

Expression, purification and characterization of zinc-finger nuclease to knockout the goat beta-lactoglobulin gene



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ABSTRACT

Engineered zinc-finger nucleases (ZFNs) have been widely used for precise genome editing. ZFNs can induce DNA double-strand breaks at specific genomic locations and drive the introduction of an insertion or deletion of base pairs at the targeted region, consequently resulting in a loss-of-function mutation. In this study, we investigated the cloning, expression and purification of ZFN fusion proteins targeting the goat beta-lactoglobulin (BLG) gene and detected the cleavage activities of these ZFN proteins in vitro and in cells, respectively. The results showed that the pET-BLG-LFN and pET-BLG-RFN prokaryotic expression plasmids can be constructed correctly and expressed efficiently in *Escherichia coli* BL21 (DE3) cells to produce the 6× His-tagged ZFN proteins that can be purified by Ni-IDA-Sefinose Column. The predetermined sequence of BLG can be recognized and excised both in vitro and in goat fibroblasts by the purified ZFN fusion proteins, which demonstrated that the purified ZFN fusion proteins can be used as gene modification tools to knock out the BLG gene. Furthermore, these results lay the foundation for eliminating allergen BLG from goat milk and improving the quality of goat milk products.

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Introduction

Goat milk is the primary source of nutrition for humans second only to cow milk. However, some infants are allergic to goat milk products in the first year of life [1]. Beta lactoglobulin (BLG),¹ which is a major whey protein of ruminant milk but absent in human milk [2], is considered an allergen for infants [3–5]. The milk allergen has been reduced through several technologies, including heat treatment, fermentation, hydrolyzed protein desensitization, glycation, and RNAi [6,7]. However, these approaches cannot solve this problem thoroughly. By contrast, Gene targeting can work as a direct and complete method to reduce and even eliminate the

allergen BLG from goat milk without any change in the other milk components. Traditional gene targeting technology is inefficient and time consuming. The appearance and development of engineered chimeric nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9, significantly improve the efficiency of gene targeting [8].

ZFNs, the efficient site-specific gene modification tools, are created by fusing both the recognition motif Cys₂His₂ zinc fingers and the cleavage domain type IIS restriction enzyme FokI [9–11]. The FokI cleavage domain must dimerize to cut DNA, so a monomer does not work at single binding site. It needs to construct two sets of Cys₂His₂ zinc fingers directed to neighboring sequences and joined each to a monomeric cleavage domain [12,13]. Only both the combined left and right ZFNs can induce site-specific double-strand breaks (DSBs) [8] and trigger cellular repair mechanisms involving either error-prone non-homologous end joining (NHEJ) [14,15] or homology-directed repair (HDR) [16]. Therefore, BLG knockout with ZFNs is an appropriate technology to solve the problem of milk allergy.

Although ZFNs have been successfully applied for gene disruption or addition in mouse [17,18], porcine [15] and human cells [19,20] by traditional transfection methods and mRNA delivery systems, they are limited by high random insertion and off-target cleavage [21]. These limitations and defects can be avoided by

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¹ Abbreviations used: DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; LB, Luria-Bertani; His, histidine; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl-sulfate–polyacrylamide gel electrophoresis; ZFNs, Zinc-finger nucleases; DSBs, DNA double-strand breaks; BLG, beta lactoglobulin; NLS, nuclear localization signal; FokI, type IIS restriction enzyme; *E. coli* BL21 (DE3), *Escherichia coli* BL21 (DE3); NHEJ, non-homologous end joining; HDR, homology-directed repair; mRNA, messenger ribonucleic acid; RNAi, RNA interference; TALEN, transcription activator-like effector nuclease; CRISPR/Cas9, clustered regulatory interspaced short palindromic repeat/Cas 9; DTT, DL-Dithiothreitol; PMSF, phenyl-methylsulfonyl-fluoride; DMEM, Dulbecco's Modified Eagle Media; SCNT, somatic cell nuclear transfer.

the method of direct delivery of ZFNs as proteins. Compared with plasmid delivery systems, direct delivery of site-specific nuclease proteins has more advantages, including no risk of insertional mutagenesis, reduced toxicity and few off-target cleavage events [22]. Previous studies showed that direct delivery of ZFNs or TALENs proteins yielded highly efficient gene knockout in various mammalian cell types [22,23].

In this research, we purified ZFN proteins targeting the goat BLG gene from *Escherichia coli* by using Ni-IDA-Sefinose column and assayed their cutting efficiency and specificity in vitro and in cells. Finally we investigated the feasibility of using direct protein delivery of ZFNs to knock out the BLG gene.

Materials and methods

Construction of the expression vector

Two prokaryotic expression constructs pET-BLG-L/RFN were generated to express a pair of ZFNs targeting goat BLG gene

Table 1
Primer sequences used in this study.

Primer name	Sequences (5'–3')
5'EcoRI-NLS	CCGGAATTC CCCAAGAAGAAGAGGAAGGT
3'FokI-XhoI	AACAGATGGCTGGCAACTAG
BLG5-F	GCATCCCACTGCTCCTG
BLG3-R	CCATCGATT TTCAGCTCCTCCACGTACACT
SNA-F	TCGTAGAGGAAGCCACCCCG
SNA-R	CACACGTGGCAACATTGGATCTT

Notes: Letters underlined means restriction sites, bolded means protective base. F means sense primer, while R means anti-sense primer.

(GenBank: Z33881). The left (L) and right (R) zinc fingers that were flanked by N-terminal nuclear localization signal (NLS) [24] and C-terminal specialized FokI cleavage domain were PCR amplified from their respective templates pZFN3 and pZFN4 with the primers 5'EcoRI-NLS and 3'FokI-XhoI listed in Table 1. The respective templates pZFN3 and pZFN4 and their sequences were engineered and customized by the CompoZr ZFN Operations Group at Sigma-Aldrich Biotechnology (Fig. 1). The ZFNs used in this study are available through Sigma-Aldrich (St. Louis, MO; lot number: 01051223MN). PCR products were digested with EcoRI and XhoI (TaKaRa, Dalian, China), then recovered from agarose gel by the DNA purification kit (Tiangen, Beijing, China) and finally cloned into the EcoRI and XhoI restriction sites of the pET-32a (+) expression vector (Novagen, Madison, USA) to generate the plasmids pET-BLG-LFN and pET-BLG-RFN. Sequencing analysis (Sangon, Shanghai, China) was conducted to verify the construction of pET-BLG-L/RFN (Fig. S1).

Expression of ZFN fusion proteins

The expression of ZFN fusion proteins was performed according to the method described by Thomas Gaj and Rodriguez [25,26] with some modifications. The ZFN prokaryotic expression plasmids pET-BLG-L/RFN were transformed into BL21 (DE3) respectively and the transformed BL21 (DE3) were incubated at 37 °C for 12–16 h to form single colonies. One of the grown single colonies was chosen to grow overnight in 5 mL LB medium containing 100 µg/mL ampicillin at 37 °C. 100 µL of the overnight inoculant cultures continuously grown in 50 mL LB media supplemented with 100 µM ZnCl₂, 100 µg/mL ampicillin, 100 µg/mL chloramphenicol and 0.2% glucose until the OD600 reached 0.8. In order to confirm optimum conditions of the ZFN proteins expression, induction

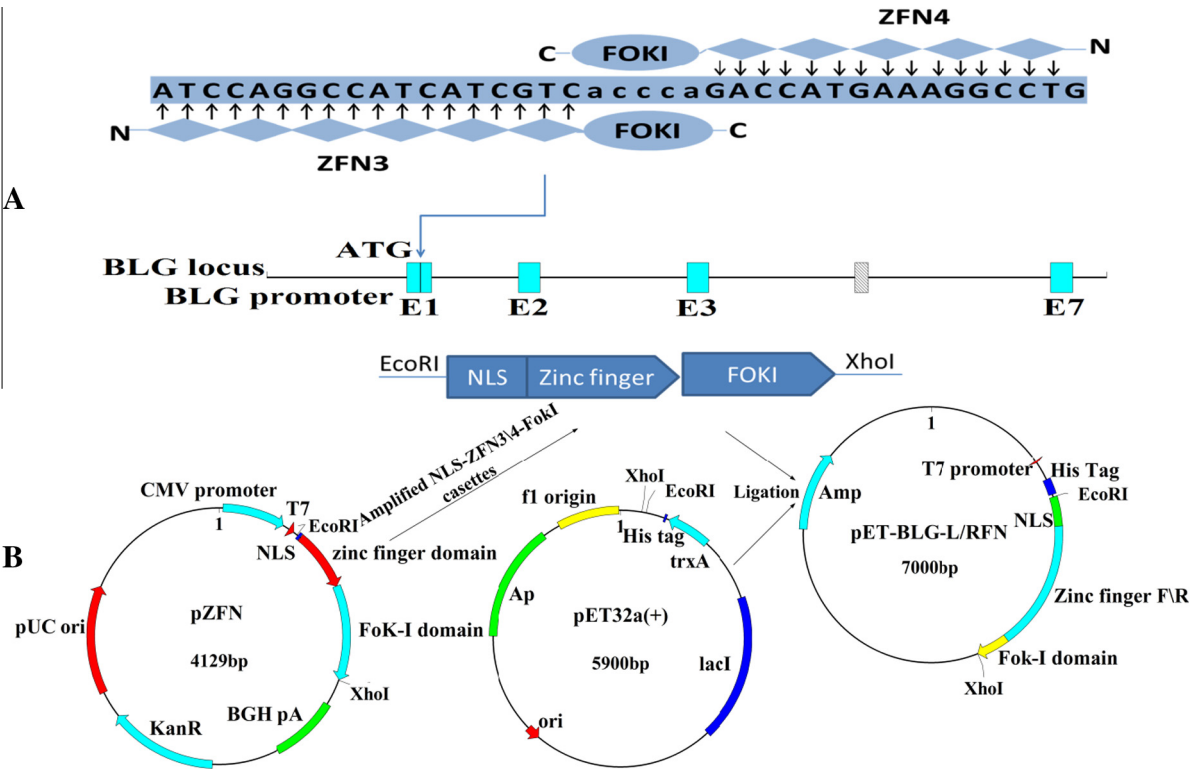


Fig. 1. Schematic overview depicting the binding sites of the left and right ZFNs and the construction of the prokaryotic expression vectors pET-BLG-L/RFN. (A) Cartoons of one pair of ZFNs binding at specific genomic sites. The binding sites of the left and right ZFNs are located in the Exon 1 of BLG gene. The DNA sequence of the primary binding site for each ZFN is uppercase; the cut sites cleaved by dimerization of the FokI nuclease domains are lowercase. (B) The flow chart of pET-BLG-L/RFN construction. Clone the NLS-ZFN3/4-FokI cassettes by PCR with their respective mammalian expression vectors as templates. Then, doubly digested the amplicon with EcoRI and XhoI and inserted it into the prokaryotic expression vector pET 32a (+) which was cut with the same endonucleases.

temperature and time were optimized. Protein synthesis was induced with 1 mM β -D-1-thiogalactopyranoside (IPTG) for 4 h at 25 °C and 30 °C respectively to optimize the induction temperature. The culture was induced with 1 mM IPTG and incubated at 25 °C for different times (4.0 h, 5.0 h, 6.0 h and 8.0 h) to optimize the expression time, respectively. The expression level of total cells was analyzed by SDS–PAGE and the weights of the fusion proteins expressed in BL21 (DE3) were compared with protein molecular weight marker (Transgen, Beijing, China). And then we compared the experimental molecular weight of two purified ZFN proteins with the theoretical value (60.4 kDa (ZFN3) and 57.6 kDa (ZFN4), respectively). Finally, the optimal induction temperature and time were obtained. After BL21 (DE3) containing pET-BLG-L/RFN was induced under the optimal condition, the cells were harvested and resuspended in lysis buffer (50 mM Tris–HCl, 500 mM NaCl, 100 μ M ZnCl₂, 1 mM PMSF, 1 mM MgCl₂, 1 mM β -mercaptoethanol, 10 mM imidazole, 0.2% Triton X-100 and 10% glycerol, pH 8.0). Each 1 g of harvested cells was resuspended in 10 mL lysis buffer, and then cells were lysed using standard sonication conditions on ice. The cellular debris was removed by centrifugation at 12,000 rpm (HFA15.50 rotor, Biofuge Stratos, Germany) for 20 min at 4 °C, while the resulting supernatants were assessed by SDS–PAGE and protein weight concentration was assessed using Bradford assay method, with BSA as standard protein. The molar concentrations of the two purified ZFN proteins can be converted from the weight concentrations through the formula (Picomoles Protein = $\frac{\mu\text{g Protein}}{\text{Protein Size (kDa)}} \times \frac{\text{kg}}{10^9 \mu\text{g}} \times \frac{10^{12} \text{ pmol}}{\text{mole}}$).

Purification of ZFN fusion proteins

1 L growths of the *E. coli* containing pET-BLG-L/RFN were cultured in Erlenmeyer flask respectively until the OD₆₀₀ of cells reached 0.8, and then protein synthesis was induced with 1 mM IPTG at 25 °C for 4 h. The expression cells BL21 (DE3)/pET-BLG-L/RFN were harvested by centrifugation at 12,000 rpm (HFA15.50 rotor, Biofuge Stratos, Germany) for 20 min at 4 °C. The pellet was resuspended in 30 mL of native lysis buffer (50 mM Tris–HCl, 500 mM NaCl, 100 μ M ZnCl₂, 1 mM PMSF, 1 mM MgCl₂, 1 mM β -mercaptoethanol, 10 mM imidazole, 0.2% Triton X-100 and 10% glycerol, pH 8.0). Cells were lysed using standard sonication conditions on ice, and the soluble fraction was isolated by centrifugation at 12,000 rpm for 30 min at 4 °C.

ZFN proteins in the soluble fraction were purified using a Ni-IDA-Sefinose Column (Sangon, Shanghai, China) and then eluted with imidazole (250 mM) in the presence of 50 mM NaH₂PO₄, 300 mM NaCl, 100 μ M ZnCl₂ and 10% glycerol at pH 8.0. The purified proteins were assessed for purity by SDS–PAGE. The weight concentration of ZFN proteins was determined by Bradford assay, with BSA as standard protein. Each protein was concentrated by ultrafiltration with Amicon Ultra-15 centrifugal filter (Millipore; USA) and dialyzed into HEPES buffer (20 mM HEPES, 90 μ M ZnCl₂, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, pH 7.4) before long-term storage at –80 °C in 50% glycerol.

Western blot assays

The purified ZFN proteins were resolved by electrophoresis by 12% SDS–PAGE gel and then electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The 6 $\times$ His-tagged ZFN proteins were detected using an anti-His Tag monoclonal antibody (Beyotime, China) as primary antibody in western blot analysis [27,28]. Finally, the proteins were detected by the enhanced chemiluminescence (ECL) method.

In vitro DNA cleavage analysis

Cleavage analysis was performed based on the method described as previously [11]. The ZFNs target site of BLG gene was amplified using the primers BLG5-F and BLG3-R (Table 1) and then cloned into pMD-19-T vector (TaKaRa, Dalian, China) forming the recombinant plasmid named pMD-19-BLG. The pMD-19-BLG plasmid was digested with ClaI restriction endonuclease (TaKaRa, Dalian, China) and recovered by the DNA purification kit (Tiangen, Beijing, China). The obtained linear segment was cut by ZFN proteins as substrate DNA in vitro. Briefly, ZFN fusion proteins (0.12 μ g (ZFN3) and 0.11 μ g (ZFN4)) were added into pre-heating ZFN reaction buffer (20 mM Tris–acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 90 μ M ZnCl₂, and 5 mM DTT, pH 7.9) containing 100 ng of substrate DNA and the final concentration of each ZFN protein was 100 nM. Cleavage reactions were conducted at 37 °C in the total volume of 20 μ L for increasing times (5 min, 10 min, 20 min, 30 min, 45 min and 60 min).

Cell culture and ZFN fusion proteins treatments

Goat fetal fibroblast cells were cultured in DMEM (Gibco, USA) medium with 10% FBS in a humidified 5% CO₂ atmosphere at 37 °C and harvested at 70–80% confluence; then seeded onto 24-well plates at a density of 2×10^5 cells per well. The treatment procedure of ZFN fusion proteins were as follows [25]. Purified ZFN3 and ZFN4 fusion proteins were diluted respectively at various concentrations in Opti-MEM (Gibco, USA) containing 90 μ M ZnCl₂ at pH 7.4; the soluble cell extract (*E. coli* containing pET-32a (+)) without ZFN as a negative control was also diluted in Opti-MEM. The fibroblast cells were washed with Opti-MEM (Gibco, USA) and then incubated with each 250 μ L of ZFN3 dilution and ZFN4 dilution or 500 μ L of negative control dilution. The molar concentrations of each ZFN protein in the different treatment groups were 1 μ M, 1.5 μ M, 2 μ M and 2.5 μ M respectively and the specific weight concentrations were 60, 91, 121, 151 μ g/mL for ZFN3 protein and 58, 86, 115, 144 μ g/mL for ZFN4 protein, respectively. Specifically, Cells were incubated in Opti-MEM containing both ZFN3 and ZFN4 proteins for 1 h at 37 °C. Then both the treated cells and negative control cells were washed and maintained at 37 °C with serum-containing medium for 24 h. This process was repeated up to 4 times over four consecutive days, and then the cells were harvested for analysis.

Surveyor nuclease assay

Genomic DNA was extracted using TIANamp Genomic DNA Kit (Tiangen, Beijing, china) from the ZFN proteins treated cells and negative control cells, the BLG target locus of ZFN proteins was amplified from the genomic DNA using the PCR primers SNA-F and SNA-R listed in Table 1. The Surveyor nuclease assay (Transgenomics, USA) was adapted for quantification of the minor deletions and insertions typically induced by NHEJ because they are susceptible to the mismatches created by error-prone repair in PCR products. In this study, the Surveyor nuclease assay was used to detect whether the minor deletions and insertions existed in the BLG target locus of ZFN proteins. Cleavage products were detected by electrophoresis and then the frequency of endogenous gene mutation generated in the genomic DNA was quantified by densitometry [29].

Results

Construction of pET-BLG-LFN and pET-BLG-RFN expression plasmids

The flow diagram of pET-BLG-L/RFN construction is schematically represented in Fig. 1B. The NLS-ZFN3/4-FoKI fusion cassettes

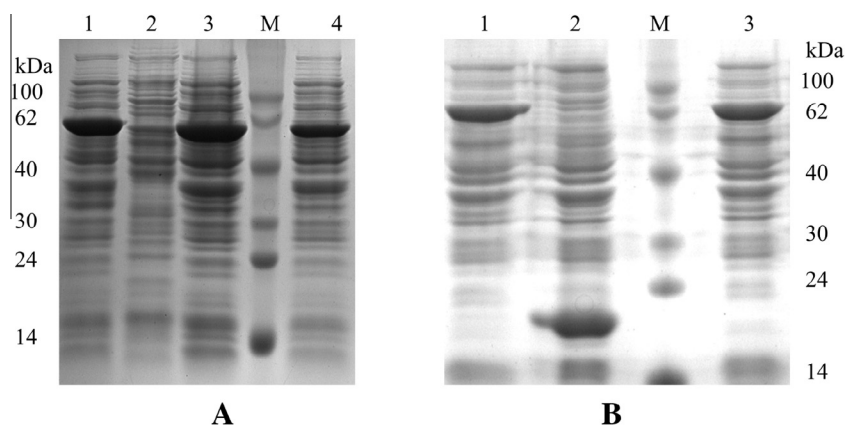


Fig. 2. Expression of ZFN proteins in BL21 (DE3). (A) The determination of optimal induced temperature. Lanes 1, 2 represented the BL21 (DE3) pET-BLG-LFN induced temperatures of 25 °C and 30 °C, respectively; Lanes 3, 4 represented the BL21 (DE3) pET-BLG-RFN induced temperatures of 25 °C and 30 °C, respectively. (B) Soluble expression of ZFN fusion proteins in *E. coli* BL21 (DE3), analyzed by SDS-PAGE. Lane 2, negative control, soluble protein of *E. coli* transformed with pET-32a (+) vector; Lane 1, soluble cell proteins of *E. coli* containing pET-BLG-RFN; Lane 3, soluble cell protein of *E. coli* containing pET-BLG-LFN; M, molecular weight marker (kDa).

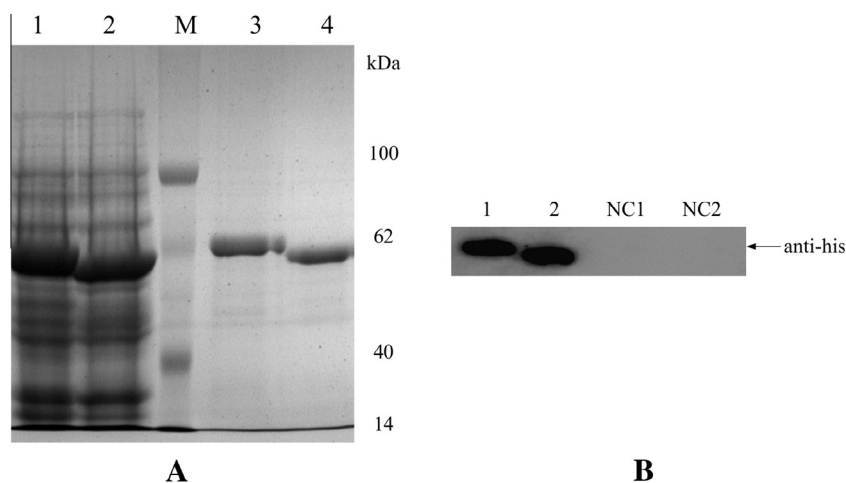


Fig. 3. Purification of ZFN proteins with elution buffer under native conditions and western blot analysis. (A) Lanes 1 and 2, soluble protein of *E. coli* containing PET-BLG-L/RFN; Lanes 3 and 4, 5 µg and 4 µg of each ZFN fusion protein purified after a His-tagged affinity chromatography; M, molecular weight marker (kDa). (B) Identification of purified ZFN3 and ZFN4 proteins by western blot analysis with an anti-His Tag monoclonal antibody. Lanes 1 and 2, purified ZFN3/ZFN4 proteins were blotted with anti-his-tag antibodies; Lanes NC1 and NC2, soluble bacterial proteins of BL21 (DE3) containing PET-BLG-L/RFN without induction with IPTG were blotted with anti-his-tag antibodies.

were amplified from their respective mammalian expression vectors pZFN3 and pZFN4 and cloned into the pET-32a (+) expression vector to generate the plasmids pET-BLG-L/RFN (Fig. 1B). The correct construction was identified by restriction enzyme digestion (data not shown) and further verified by sequence analysis (Fig. S1).

The left (L) and right (R) ZFNs mammalian expression vectors pZFN3 and pZFN4 were designed to target the Exon 1 of goat BLG gene to introduce frame-shift mutations, which can lead to the production of null alleles (Fig. 1A). The principal mechanism was that the left (L) and right (R) ZFNs can create DSBs which will be repaired through the NHEJ or HR pathway. When the exogenous repair template was absent, the overall efficiency of intrinsically error-prone NHEJ was higher than HR at all cell cycle stages, thus the repair of the DSBs tended to be low fidelity [30]. Furthermore, NHEJ in particular can produce localized mutations due to deletion and/or insertion of short sequences at the break, resulting in loss of BLG protein expression [31].

Expression and purification of ZFN fusion proteins

The expression capacity of *E. coli* containing pET-BLG-L/RFN was identified by SDS-PAGE analysis after optimizing the induction

temperature and time, respectively. As shown in Fig. 2A, the better induction temperature was 25 °C and 1 mM IPTG concentration was sufficient to obtain high yield of proteins. Considering that there was no obvious difference of the yields from 4 to 8 h after induction (data not shown), we chose 4 h as our final induction time, because it was the minimal time required in results. SDS-PAGE results showed that there were two obvious bands approximately 60 kDa matched with the theoretical molecular weights produced in soluble bacterial proteins respectively, while no corresponding specific band was detected in negative control under the optimized induction conditions (Fig. 2B).

ZFN proteins in the soluble lysates were purified using a Ni-IDA-Sefinose Column. The purified results showed that all other bands almost disappeared after purification whereas only the single bands we need became more obvious respectively and their molecular weights were approximately 60 kDa (Fig. 3A). Purified ZFN proteins were also identified by western blot analysis with an anti-His Tag monoclonal antibody. We found that the anti-His antibodies combined specifically with the 6× His-tagged ZFN proteins but not the soluble cell extract without induction with IPTG (Fig. 3B). The weight concentration of the purified ZFN proteins was measured after purification and concentration. The final

Table 2
Purification of ZFN fusion proteins from *E. coli*.

Step	ZFN3				ZFN4			
	Total protein ^b (mg)	Target protein ^c (mg)	Overall yield ^d (%)	Purity (%)	Total protein ^b (mg)	Target protein ^c (mg)	Overall yield ^d (%)	Purity (%)
Sonicated supernatant ^a	41.5	5.23	100	13	40.6	5.06	100	12
NIA-agarose	0.67	0.55	10.5	82	0.64	0.51	10.1	80
Dialyzed and concentrated	0.54	0.45	8.6	83	0.52	0.42	8.3	81

^a From 1 L of *E. coli* culture induced with 1 mM IPTG for 4 h at 25 °C.

^b Total protein content was determined by Bradford protein assay.

^c Target protein was estimated by quantifying the amount in each gel lane by densitometry.

^d The overall yield was calculated based on the amount of ZFN3/ZFN4 fusion protein.

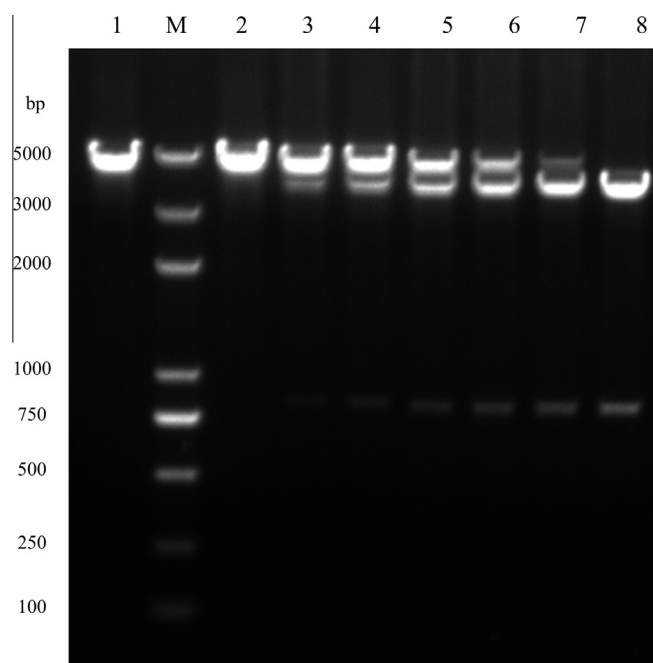


Fig. 4. Detection the activity of ZFN recombinant proteins in vitro. Lane 1, Negative control group that was added with water. Lane 2, Negative control group that was added with the soluble cell extract without ZFN. Lanes 3–8, linearized substrate fragment was digested into two parts with length of 3580 bp and 791 bp for increasing times (5 min, 10 min, 20 min, 30 min, 45 min and 60 min), respectively. M, Trans 2K Plus DNA Marker.

yields of the fusion proteins were 0.45 mg/L (ZFN3) and 0.42 mg/L (ZFN4), respectively (Table 2).

Detection the activity of recombinant proteins in vitro

During the activity analysis of recombinant proteins in vitro, the linearized substrate fragment was incubated with ZFN proteins in reaction buffer for increasing times (5 min, 10 min, 20 min, 30 min, 45 min and 60 min) at 37 °C. Furthermore, two negative controls including the linearized substrate fragment incubated with water and incubated with soluble cell extract (*E. coli* containing pET-32a (+)) without ZFN were also added.

The results demonstrated that the fragment was not even cut in the two negative controls (Fig. 4, Lanes 1 and 2), whereas the DNA fragments could be digested into two parts with the expected size by the purified ZFN proteins in various cutting time (Fig. 4, Lanes 3–8). Meanwhile, the Clal-cut plasmid was cut more completely with the increased cutting time. So we can conclude that approximately 1 h was needed for thoroughly cutting in vitro.

Surveyor nuclease assay

Following the examples of ZFN proteins used in other cells [25], the goat fetal fibroblast cells were incubated with the dilutions of both ZFN proteins containing N-terminal NLS peptides and then harvested for genome DNA extraction. The BLG target locus of ZFN proteins was amplified from the genome DNA and detected whether the disruption was existed in the target locus using the Surveyor nuclease assay. Surveyor nuclease is a mismatch-specific endonuclease that recognizes mismatches in heteroduplexed DNA and cleaves both strands on the 3' side of the mismatch, providing specific information on mutation location. Especially in our study, when the DSBs were repaired through NHEJ, the Surveyor nuclease would recognize the mutation produced at the BLG locus and cleave both strands of the PCR products. It can be seen from Fig. 5A that 754-bp PCR products can be amplified from the genome DNA of different concentrations (1 μ M, 1.5 μ M, 2 μ M and 2.5 μ M) of ZFN proteins-treated cells (Lanes 4–7), untreated cells (Lane 1) and soluble cell extract without ZFN treated cells (Lane 3) as two negative controls. The 754-bp PCR products were not even cut in the two negative controls by the Surveyor nuclease (Fig. 5A, Lanes 1 and 3); whereas the PCR products amplified from the genome DNA of different concentrations of ZFN proteins-treated cells could be digested into two parts with the expected size (Fig. 5A, Lanes 4–7). The level of Surveyor nuclease cleavage was determined by the ratio of cleaved products to the parental band which reflected the frequency of the endogenous BLG gene disruption mediated by ZFN proteins. The measuring results showed that the frequency of targeted gene cleavage (0, 1.2%, 2.0%, 2.6%, and 2.9%) was increased when the molar concentration of ZFN proteins was 0, 1.0, 1.5, 2.0, 2.5 μ M, respectively (Fig. 5B).

Discussion

The aim of this study was to establish an expression and purification protocol to produce ZFN proteins to knock out the BLG gene. The production of the ZFN proteins was to explore an alternative avenue of gene targeting method which can overcome some risks, such as safety problems in gene-based delivery method and inefficiency in mRNA delivery system [32]. Our study has shown that ZFN proteins delivery resulted in gene knockout as the transient transfection of ZFN expression vectors.

When the purified ZFN proteins were used as gene modification tools, three main abilities including penetrating cells, entering the nucleus and cleavage were needed. Nuclear localization signal (NLS) residues, the specific amino acid sequence on the signaling proteins, was fused at the N-terminus of the ZFN peptides for nuclear translocation [33,34]. Furthermore, the nonspecific cleavage domain of the FokI restriction endonuclease was linked to the modular zinc finger proteins (ZFPs) domains at the C-terminus

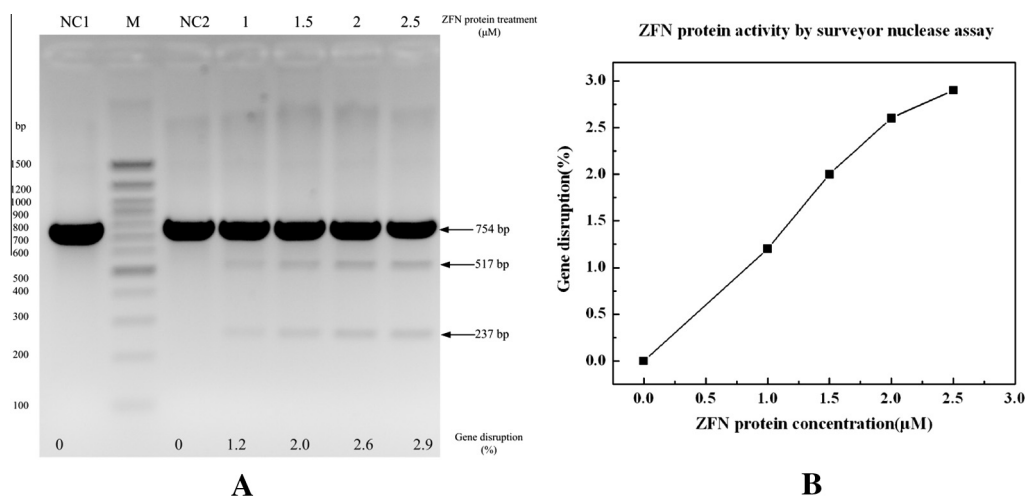


Fig. 5. Surveyor nuclease assay of the ZFN proteins activity at the goat BLG loci. The bands were quantitated by ethidium bromide staining and densitometry to determine the frequency of gene disruption. (A) The 754-bp PCR amplicon is cleaved into ~517- and ~237-bp fragments. Numbers at the bottom of each lane indicate % of the BLG gene disruption frequency. (B) The frequency of the endogenous BLG gene disruption is plotted against ZFN proteins concentration. The frequency of endogenous gene disruption which was determined by the Surveyor nuclease assay increased with ZFN proteins as the molar concentration range was between 0 and 2.5 μM.

to generate the cleavage ability. It is worth to be mentioned that cell-penetrating peptides (CPPs) have been powerful transport tools to deliver various proteins into the cell membranes previously [35,36], but ZFNs fused to protein transduction domains such as HIV-1 TAT protein or polyarginine motif were difficult to express and purify [23]. Meanwhile, standard ZFN proteins already possess intrinsic cell-penetrating capabilities because of the net positive charge of ZFP-DNA binding domains [25]. Therefore, the ZFPs were only fused to NLS at the N-terminus and FokI domain at the C-terminus without cell-penetrating peptides.

In this research, we demonstrated that purified ZFN protein can be used as a gene modification tool to knock out the BLG gene and cleavage activity existed in vitro and in cells. The ZFN fusion proteins can recognize and excise the desired sequence of BLG in vitro. The Surveyor nuclease assay of ZFN-treated cells verified targeted mutagenesis in goat fibroblast cells, revealing the presence of the anticipated ZFN-induced insertions and/or deletions at the BLG locus. The Surveyor nuclease has been widely used to detect a variety of mutations [37] in the human [38], zygotes [39] and mammal [40] genomes accurately. The function of some genes would be lost through the NHEJ pathway and the Surveyor nuclease assay can detect this kind of mutation.

The efficiency of the purified fusion protein in the Surveyor nuclease assay seems low; the possible reasons are summarized as follows. First, the cleavage needs two purified ZFN proteins active in a cell simultaneously. They must penetrate cell membranes and enter the cell nucleuses to identify the BLG gene. Meanwhile, both of them must keep activity and produce DSBs in the targeted gene, and then the BLG gene will produce mutation through NHEJ pathway. So some proteins may not reach the targeted location to cleave the targeted gene BLG and induce the mutation during this process. And the activities of some proteins decrease in this process, which also limits the cleavage frequency. Second, we have tried to optimize the experiments to increase the cutting efficiency in fibroblast cells by increasing the amount of proteins or treating time. But as the amount of purified fusion protein increased, direct application of excessive amounts of ZFN proteins generally resulted in protein precipitation and led to a lot of dead cells during the experiment. Similarly, prolonging treating time still cannot improve the cutting efficiency. The similar experimental results have been also obtained from the previous research [25], in which incubation with the human CCR5 ZFN proteins

achieved a high efficiency of gene disruption when the treatment time was 1 h and extended periods of incubation (>60 min) did not increase the frequency of genome modification. The possible reason may be that the amount of ZFN proteins entering the cells reaches its peak within 1 h and the specific mechanism need to be investigated in the future studies. Finally, we decided to choose 1 h as the treatment time.

The gene-modified goat fibroblast cells can be converted to clonal cell clusters by limiting dilution and the clonal cell clusters can be used as donor cells for somatic cell nuclear transfer (SCNT) to produce transgenic goats [15,41], which has significance for BLG free goats' production and milk products improvement.

In conclusion, induction temperature and time were optimized in the expression process of ZFN proteins and the 6× His-tagged ZFNs were purified from BL21 (DE3) by using Ni-IDA-Sefinose Column. Furthermore, cleavage activities of the purified ZFN proteins both in vitro and in cells were detected. The results demonstrated that the purified ZFN proteins can be used as gene modification tools to knock out the BLG gene in goat fibroblasts. This gene-free delivery system has a great potential for BLG free goats' production as the targeted modification cells can be used for SCNT. Our investigation lays the foundation to improve milk qualities and properties.

Competing interests

The authors have declared no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.pep.2015.04.004>.

References

- [1] A. Host, Frequency of cow's milk allergy in childhood, *Ann. Allergy Asthma Immunol.* 89 (2002) 33–37.
- [2] G. Invernizzi, E. Annoni, A. Natalello, S.M. Doglia, M. Lotti, In vivo aggregation of bovine beta-lactoglobulin is affected by Cys at position 121, *Protein Expr. Purif.* 62 (2008) 111–115.
- [3] S. Sharma, P. Kumar, C. Betzel, T.P. Singh, Structure and function of proteins involved in milk allergies, *J. Chromatogr. B Biomed. Sci. Appl.* 756 (2001) 183–187.
- [4] G.H. Kontopidis, C. Holt, L. Sawyer, Invited review: β -lactoglobulin binding properties, structure and function, *J. Dairy Sci.* 4 (2004) 12.
- [5] K. Ponniah, T.S. Loo, P.J. Edwards, S.M. Pascal, G.B. Jameson, G.E. Norris, The production of soluble and correctly folded recombinant bovine beta-lactoglobulin variants A and B in *Escherichia coli* for NMR studies, *Protein Expr. Purif.* 70 (2010) 283–289.
- [6] P. Davis, C. Smales, D. James, How can thermal processing modify the antigenicity of proteins?, *Allergy* 56 (2001) 56–60.
- [7] B.M. Ehn, T. Allmere, E. Telemo, U. Bengtsson, B. Ekstrand, Modification of IgE binding to beta-lactoglobulin by fermentation and proteolysis of cow's milk, *J. Agric. Food Chem.* 53 (2005) 3743–3748.
- [8] T. Gaj, C.A. Gersbach, C.F. Barbas 3rd, ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering, *Trends Biotechnol.* 31 (2013) 397–405.
- [9] J. Wang, G. Friedman, Y. Doyon, N.S. Wang, C.J. Li, J.C. Miller, K.L. Hua, J.J. Yan, J.E. Babiarz, P.D. Gregory, Targeted gene addition to a predetermined site in the human genome using a ZFN-based nicking enzyme, *Genome Res.* 22 (2012) 1316–1326.
- [10] E.A. Moehle, J.M. Rock, Y.L. Lee, Y. Jouvenot, R.C. DeKolver, P.D. Gregory, F.D. Urnov, M.C. Holmes, Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases, *Proc. Natl. Acad. Sci. U.S.A.* 104 (2007) 3055–3060.
- [11] J. Guo, T. Gaj, C.F. Barbas 3rd, Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases, *J. Mol. Biol.* 400 (2010) 96–107.
- [12] J.C. Miller, M.C. Holmes, J. Wang, D.Y. Guschin, Y.-L. Lee, I. Rupniewski, C.M. Beausejour, A.J. Waite, N.S. Wang, K.A. Kim, An improved zinc-finger nuclease architecture for highly specific genome editing, *Nat. Biotechnol.* 25 (2007) 778–785.
- [13] J. Bitinaite, D.A. Wah, A.K. Aggarwal, I. Schildkraut, FokI dimerization is required for DNA cleavage, *Proc. Natl. Acad. Sci.* 95 (1998) 10570–10575.
- [14] Y. Santiago, E. Chan, P.-Q. Liu, S. Orlando, L. Zhang, F.D. Urnov, M.C. Holmes, D. Guschin, A. Waite, J.C. Miller, Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases, *Proc. Natl. Acad. Sci.* 105 (2008) 5809–5814.
- [15] P. Li, J.L. Estrada, C. Burlak, A.J. Tector, Biallelic knockout of the alpha-1,3 galactosyltransferase gene in porcine liver-derived cells using zinc finger nucleases, *J. Surg. Res.* 181 (2013) e39–e45.
- [16] N. Klymiuk, P. Fezert, A. Wunsch, M. Kurome, B. Kessler, E. Wolf, Homologous recombination contributes to the repair of zinc-finger-nuclease induced double strand breaks in pig primary cells and facilitates recombination with exogenous DNA, *J. Biotechnol.* 177 (2014) 74–81.
- [17] P. Perez-Pinera, D.G. Ousterout, M.T. Brown, C.A. Gersbach, Gene targeting to the ROSA26 locus directed by engineered zinc finger nucleases, *Nucleic Acids Res.* 40 (2012) 3741–3752.
- [18] B. Shen, X. Zhang, Y. Du, J. Wang, J. Gong, P.H. Tate, H. Li, X. Huang, W. Zhang, Efficient knockin mouse generation by ssDNA oligonucleotides and zinc-finger nuclease assisted homologous recombination in zygotes, *PLoS One* 8 (2013) e77696.
- [19] R.C. DeKolver, V.M. Choi, E.A. Moehle, D.E. Paschon, D. Hockemeyer, S.H. Meijssing, Y. Sancak, X. Cui, E.J. Steine, J.C. Miller, Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome, *Genome Res.* 20 (2010) 1133–1142.
- [20] T. Mino, T. Mori, Y. Aoyama, T. Sera, Gene- and protein-delivered zinc finger-staphylococcal nuclease hybrid for inhibition of DNA replication of human papillomavirus, *PLoS One* 8 (2013) e56633.
- [21] V. Van Tendeloo, R. Willems, P. Ponsaerts, M. Lenjou, G. Nijs, M. Vanhove, P. Muylaert, P. Van Cauwelaert, C. Van Broeckhoven, D. Van Bockstaele, High-level transgene expression in primary human T lymphocytes and adult bone marrow CD34+ cells via electroporation-mediated gene delivery, *Gene Ther.* 7 (2000) 1431–1437.
- [22] J. Liu, T. Gaj, J.T. Patterson, S.J. Sirk, C.F. Barbas III, Cell-penetrating peptide-mediated delivery of TALEN proteins via bioconjugation for genome engineering, *PLoS One* 9 (2014) e85755.
- [23] R. Ru, Y. Yao, S. Yu, B. Yin, W. Xu, S. Zhao, L. Qin, X. Chen, Targeted genome engineering in human induced pluripotent stem cells by penetrating TALENs, *Cell Regen.* 2 (2013) 1–8.
- [24] H. Xiong, S. Li, Z. Yang, R.R. Burgess, W.S. Dynan, E. coli expression of a soluble, active single-chain antibody variable fragment containing a nuclear localization signal, *Protein Expr. Purif.* 66 (2009) 172–180.
- [25] T. Gaj, J. Guo, Y. Kato, S.J. Sirk, C.F. Barbas 3rd, Targeted gene knockout by direct delivery of zinc-finger nuclease proteins, *Nat. Methods* 9 (2012) 805–807.
- [26] D.C. Rodriguez, K. Camargo, T.G. Tripsianes, J. Kapp, J. Mendes, B. Schubert, B. Cordes, B. Reif, Cloning, expression and purification of the human islet amyloid polypeptide (hIAPP) from *Escherichia coli*, *Protein Expr. Purif.* 106 (2015) 49–56.
- [27] H. Jiang, Y. Sui, Y. Cui, P. Lin, W. Li, S. Xing, D. Wang, M. Hu, X. Fu, Expression, purification, and characterization of human osteoclastic protein-tyrosine phosphatase catalytic domain in *Escherichia coli*, *Protein Expr. Purif.* 107 (2015) 7–12.
- [28] L. Zhou, Z. Zhao, B. Li, Y. Cai, S. Zhang, TrxA mediating fusion expression of antimicrobial peptide CM4 from multiple joined genes in *Escherichia coli*, *Protein Expr. Purif.* 64 (2009) 225–230.
- [29] D.Y. Guschin, A.J. Waite, G.E. Katibah, J.C. Miller, M.C. Holmes, E.J. Rebar, A rapid and general assay for monitoring endogenous gene modification, *Methods Mol. Biol.* 649 (2010) 247–256.
- [30] Z. Mao, M. Bozzella, A. Seluanov, V. Gorbunova, DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells, *Cell Cycle* 7 (2008) 2902–2906.
- [31] M.R. Lieber, Y. Ma, U. Pannicke, K. Schwarz, Mechanism and regulation of human non-homologous DNA end-joining, *Nat. Rev. Mol. Cell Biol.* 4 (2003) 712–720.
- [32] Y. Cai, R.O. Bak, J.G. Mikkelsen, Targeted genome editing by lentiviral protein transduction of zinc-finger and TAL-effector nucleases, *Elife* 3 (2014) e01911.
- [33] M.A. Zanta, P. Belguise-Valladier, J.-P. Behr, Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus, *Proc. Natl. Acad. Sci.* 96 (1999) 91–96.
- [34] L.J. Brandén, A.J. Mohamed, C.E. Smith, A peptide nucleic acid–nuclear localization signal fusion that mediates nuclear transport of DNA, *Nat. Biotechnol.* 17 (1999) 784–787.
- [35] E. Koren, V.P. Torchilin, Cell-penetrating peptides: breaking through to the other side, *Trends Mol. Med.* 18 (2012) 385–393.
- [36] F.S. Hassane, A. Saleh, R. Abes, M. Gait, B. Lebleu, Cell penetrating peptides: overview and applications to the delivery of oligonucleotides, *Cell. Mol. Life Sci.* 67 (2010) 715–726.
- [37] D.Y. Guschin, A.J. Waite, G.E. Katibah, J.C. Miller, M.C. Holmes, E.J. Rebar, A Rapid and General Assay for Monitoring Endogenous Gene Modification, *Engineered Zinc Finger Proteins*, Springer, 2010. pp. 247–256.
- [38] E.E. Perez, J. Wang, J.C. Miller, Y. Jouvenot, K.A. Kim, O. Liu, N. Wang, G. Lee, V.V. Bartsevich, Y.-L. Lee, Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases, *Nat. Biotechnol.* 26 (2008) 808–816.
- [39] T. Mashimo, T. Kaneko, T. Sakuma, J. Kobayashi, Y. Kunihiro, B. Voigt, T. Yamamoto, T. Serikawa, Efficient gene targeting by TAL effector nucleases cojoined with exonucleases in zygotes, *Sci. Rep.* 3 (2013).
- [40] J.C. Miller, S. Tan, G. Qiao, K.A. Barlow, J. Wang, D.F. Xia, X. Meng, D.E. Paschon, E. Leung, S.J. Hinkley, G.P. Dulay, K.L. Hua, I. Ankoudinova, G.J. Cost, F.D. Urnov, H.S. Zhang, M.C. Holmes, L. Zhang, P.D. Gregory, E.J. Rebar, A TALE nuclease architecture for efficient genome editing, *Nat. Biotechnol.* 29 (2011) 143–148.
- [41] X. Liu, Y. Wang, Y. Tian, Y. Yu, M. Gao, G. Hu, F. Su, S. Pan, Y. Luo, Z. Guo, F. Quan, Y. Zhang, Generation of mastitis resistance in cows by targeting human lysozyme gene to beta-casein locus using zinc-finger nucleases, *Proc. Biol. Sci.* 281 (2014) 20133368.