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Original Article

A method to induce high expression of human lysozyme in the milk of transgenic mice

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Abstract: Objective: To investigate a low cost and efficient method to induce high expression of human lysozyme (hLYZ) in milk. Methods: a hybrid gene locus strategy was applied in this study, to construct a 50-kb bovine-αS1-casein-hLYZ hybrid gene locus, which was used as a mammary-gland-specific expression vector. The transgenic mice were generated. The expression of human lysozyme of the milk was measured by Western blot. The concentration of rhLYZ was determined by Elisa method. Results: the expression of hLYZ of the milk samples from transgenic lines 15-4, 23-7, 23-11 could be detected by Western blot, while there was no expression in wild type mice. In the quantitative analysis by ELISA assay, the expression level of of hLYZ in the 3 lines was at the 5.35-6.06 g/l, while line 23-7 showed the highest expression level. Conclusion: Our transgenic mice carrying the bovine-αS1-casein-hLYZ hybrid gene locus represent a model system for the cost-effe

Keywords: Human lysozyme, bovine-αS1-casein, transgenic mice, hybrid gene locus

Introduction

Human lysozyme (hLYZ), also known as muramidase, can hydrolyze the mucopolysaccharide-β (1⟶4) glycosidic bonds of bacterial cell walls. It is an important non-specific defense factor in the human body, participating in various immunological reactions [1]. Human lysozyme is present in a wide range of cells, tissues, and organs. Its concentration is about 0.5 mg/mL in human, which is 1500-4000 times higher than that in cow, sheep and goat [2]. Owing to its intrinsic antimicrobial activity, hLYZ plays a vital role in preventing intestinal diseases and boosting infant health [3, 4]. However, the resources are limited and the cost of production is high. Therefore, its large-scale production for clinical use is desirable.

Transgenic farm animals that secrete recombinant proteins into their milk, via efficient transgene expression, provide the opportunity to obtain high-quality and low-cost target protein [5]. In view of the utility of the mammary gland as a potential bioreactor, using of the mammary gland bioreactor system is not only a good new way to produce hLYZ, but also a process to avoid mastitis through the resisting against the growth of bacteria in dairy animals [6]. Maga et al. has successfully induced expression of hLYZ in the mammary gland of transgenic mice [6]. Shortly thereafter, guidance with the bovine-α-S1-casein sequence in transgenic rabbits led to the successful expression of human interleukin-2 [7]. Furthermore, human protein C was expressed in the milk of transgenic pigs and reached the level of 1 g/L [8].

In our previous study, we developed a hybrid gene locus method, in which the genomic coding sequence of a milk protein gene is completely replaced by a foreign protein from the start codon to the stop codon. We also developed a successive three-step gap-repair method to achieve the above method. A 50-kb mouse whey acidic penomic-human lactoferrin genomic (mWAP-hLF) hybrid gene locus was constructed in this way, which led to hLF being expressed in the milk of transgenic mice at an extremely high level of 29.8 g/L [9]. In addition,

a 38.4-kb mouse whey acidic penomic-human tissue plasminogen activator (mWAP-htPA) hybrid gene locus was constructed, leading to htPA being expressed in the milk of transgenic mice at a level of 3.3 g/L [10]. A 37-kb mouse whey acidic penomic-human serum albumin (mWAP-hSA) hybrid gene locus was produced, and rhSA was expressed at a high level of 11.9 g/L in milk [11]. Therefore, using this method is feasible to obtain extremely high level expression of target protein.

In the present study, regulatory elements of the bovine-αS1-casein gene locus were chosen to direct expression of the hLYZ genomic coding sequence, and then a bovine-αS1-casein-hLYZ hybrid locus was constructed. Using ELISA, we measured the expression level and calculated the single-copy locus expression.

Materials and methods

Gene clone

The BAC clone CH240-428L4 for the bovine-αS1-casein gene locus and RP11-1143G9 (GenBank No. AC020656) containing the hLYZ gene locus were purchased from the BACPAC Resources Center of American the Children's Hospital. The gap-repair method used was based on the Red recombinant system encoded by the pKD46 vector (GenBank No. AY048746.1) [12].

Construction of the pBR322-gap-repair vector

T1-T6, six 400-500-bp homologous arms, were amplified by PCR with BAC as a template, which were then individually subcloned into the pMD18-T vector. T1 was located in the region 9 kb upstream of the bovine-αS1-casein start codon. T2 was located in the region just upstream of the bovine-αS1-casein start codon. T1 and T2 were used for gap repair of the 5-kb 5'-flanking region of the bovine-αS1-casein gene locus. T5 was located in the region 9 kb upstream of the bovine-αS1-casein stop codon, while T6 was located in the region just upstream of this codon. T5 and T6 were used for gap repair of the 9-kb 5'-flanking region of the bovine-αS1-casein gene locus. T3 was located in the region just downstream of the hLYZ start codon, while T4 was located in the region just upstream of the hLYZ stop codon. T3 and T4 were two arms used for gap repair of the whole 20-kb hLYZ genomic sequence from ATG to TGA. All six homologous arms were ligated together in the following order: T1 was ligated to T2 via an *S*wa I site, T3 was ligated to T4 via a *P*me I site, while T5 was ligated to T6 via an *H*pa I site through PCR connection between T2 and T3 because between T1 and T6 homologous with each arm sequence spell non-trace enzyme sites connection, there were no suitable enzyme loci between T4 and T5 arms, only with homology themselves *E*coR I connection. All enzyme sites were native to the bovine-αS1-casein or hLYZ sequence and the six arms were seamlessly ligated, with no base pairs being introduced or deleted. After ligation, the six arms were cut out from the pMD18-T vector by PvuI enzyme digestion and then cloned into the PvuI site of pBR322.

Successive three-step gap repair

For the first step of gap repair, DH10b bacteria containing the bovine-αS1-casein BAC insert were transfected with the pKD46 plasmid. Then, the transformants carrying the bovine-αS1-casein BAC insert and pKD46 were incubated in 5-ml SOB cultures with ampicillin/chloramphenicol double-resistance LB liquid medium at 30°C and 220 r/min, after OD_{600} reached at of 0.20-0.25, 1 mol/L L-arabinose was added. This mixture was then incubated for 45-60 min until an OD_{600} reached at 0.45-0.50. After that it was centrifuged at 4000 r/min for 15 min. The bacterial sediment was collected using cold aseptic water, and then electrocompetent was made after concentrating 100-fold and washing three times with ice-cold 10% glycerol. Electroporation was performed using a Cell-Porator (Gbico) with a voltage booster and 0.15-cm chambers according to the manufacturer's instructions. This process featured 25 μl cells and 100 ng pBR322-gap-repair vector, which was linearized by *H*pa I to dissociate the homologous arms T5 and T6. The shocked cells were supplemented with 1 ml of SOC, incubated for 1 h at 37°C, and then half of the mixture was spread onto an LB plate containing 50 μg/ml tetracycline.

In the second step, the gap repair was initiated with the plasmid resulting from the first step. The 9-kb 3'region of the pBR322-gap-repair vector was linearized by *P*me I to dissociate the homologous arms T3 and T4, and then electro-

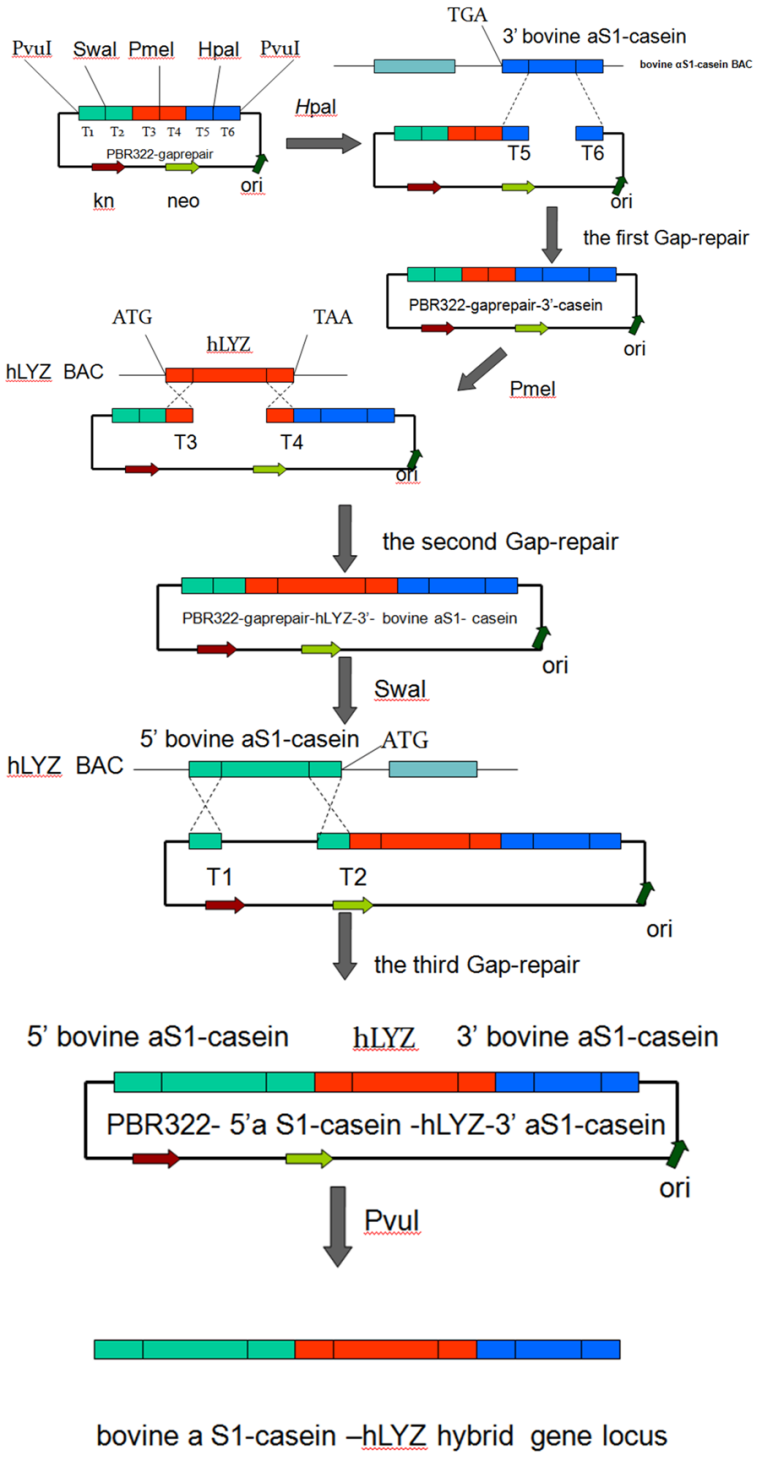

Figure 1. A sketch of the three-step gap-repair method for construction of the bovine-αS1-casein-hLYZ hybrid gene locus. First, homologous arms T5 and T6 were dissociated by *Hpa* I to catch the 9-kb 3'-flanking region of the bovine-αS1-casein. Second, homologous arms T3 and T4 were dissociated by *Pme* I to catch the 4.3-kb hLYZ genomic coding sequence from ATG to TGA. Third, homologous arms T1 and T2 were dissociated by SwaI to catch the 8.6-kb 5'-flanking region of the bovine-αS1-casein. The final bovine-αS1-casein-hLYZ hybrid gene locus was released from the pBR322 vector by *Pvu* I digestion and used for subsequent microinjection.

porated into competent DH-10β bacteria containing the hLYZ BAC insert and pKD46 plasmid.

In the third step, the gap repair was initiated with the plasmid resulting from the second step. The pBR322-gap-repair vector-9-kb 3'-5-kb hLYZ was linearized by Swa I to dissociate the homologous arms T1 and T2, and then electroporated into competent DH10β bacteria containing the bovine-αS1-casein BAC insert and pKD46 plasmid once again. Other procedures were the same as in the first step of gap repair.

Six homologous arms of T1-T6, 400-500 bp in length, which were amplified by PCR with BAC as a template, were seamlessly ligated together for cloning into the PBR322 vector to construct the successive three-step gap-repair vector. In the first step, homologous arms T5 and T6 were dissociated by *Hpa*I digestion, and the linearized vector was electroporated into competent cells containing the bovine-αS1-casein BAC clone and pKD46 plasmid. The plasmid pKD46 expressed the Red system under the control of a well-regulated promoter to avoid unwanted recombination events under non-inducing conditions. The 9-kb 3'-flanking region of bovine-αS1-casein was gap-repaired. In the second step, homologous arms T3 and T4 were dissociated by *Pme*I digestion, and then electroporated into bacteria containing the hLYZ BAC clone and pKD46 plasmid; then, the 4.3 kb of the hLYZ genomic sequence from the start codon ATG to the stop

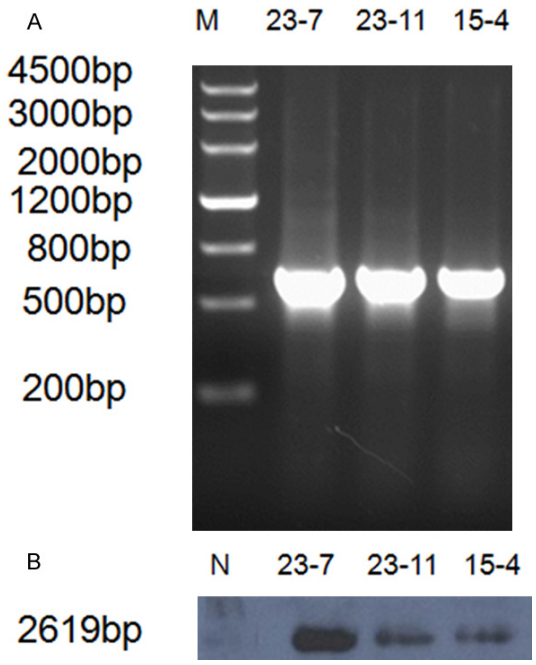

Figure 2. Identification of transgenic mice by PCR and Southern blot analysis of genomic DNA. A. PCR analysis of tail DNA was performed with the primer pair mouse-F and mouse-R corresponding. A product of 630 bp was generated by PCR after 30 cycles (94°C, 30 s, 58°C, 30 s, 72°C 1 min). M: DNA marker III, 15-4, 23-7, 23-11: samples from the three transgenic mouse lines. B. Southern blot analysis of transgenic mice. A 2619-bp band representing the region from introns 1 to 3 of the hLYZ genomic sequence was obtained by *Sca*I-HF and *Nco*I-HF digestion of genomic DNA, with a 630-bp PCR fragment as a probe located between introns 1 and 2 of the hLYZ genomic sequence. N: negative control sample from wild-type mice; 15-4, 23-7, 23-11: samples from the three transgenic mouse lines.

codon TGA was gap-repaired. In the third step, started from the second step, the arms T1 and T2 were dissociated by *Swa*I digestion, and electroporated into competent cells containing the bovine-αS1-casein BAC clone and pKD46 plasmid once again; then, the 8.6-kb 5'-flanking region of bovine-αS1-casein was gap-repaired. Lastly, the bovine-αS1-casein -9-kb 3'-f-4.3-kb hLYZ-8.6-kb 5'-f was obtained. The three fragments were then seamlessly ligated, which resulted in the successful construction of bovine-αS1-casein-hLYZ; the bovine-αS1-casein gene locus was exactly replaced by the 4.3-kb hLYZ genomic coding sequence from the start codon to the stop codon. The bovine-αS1-casein-hLYZ hybrid gene locus was proven to be accurate by enzyme digestion and sequence analysis. The three-step gap-repair process is shown in **Figure 1**.

Generation of transgenic mice

The 50-kb bovine αS1-casein-hLYZ hybrid gene locus was released from the pBR322 vector by PvuI digestion and purified by agarose gel electrophoresis. The DNA was microinjected into fertilized C57BL/6 mouse eggs, and the eggs were reimplanted into pseudopregnant females. The mice were housed at the transgenic mouse facility. Total genomic DNA was prepared from a short segment of mouse tail to check for integration of the injected DNA. PCR of tail DNA was performed with the primer pair mouse-F and mouse-R (mouse-F, 5'-CTGTGACTACAGGTGTGTACCAC-3'; mouse-R, 5'-TCATAGCAGATACATAGGCTGATG-3') corresponding to the bovine-αS1-casein and hLYZ gene. As a result, a PCR product of 630 bp was generated after the following schedule: 94°C for 4 min, and then 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. In case of integration of the bovine αS1-casein-hLYZ hybrid gene locus construct. Southern blot analysis of SacI-HF and NcoI-HF double-enzyme-digested tail DNA was performed in accordance with the standard procedure, following the protocols provided in the DIG High Prime DNA Labeling and Detection Starter Kit II. The probe used to check for integration of the construct was a 630 bp fragment located between intron 1 and intron 3 of hLYZ, which was amplified by the specific primer pair mouse-F and mouse-R (mouse-F, located in intron 1 of hLYZ; mouse-R, located in intron 3 of hLYZ), and then labeled with digoxigenin.

The bovine-αS1-casein-hLYZ construct was released from the pBR322 vector by *Pvu*I digestion and microinjected into C57BL/6 mouse eggs, resulting in three transgenic mouse lines. These three lines appeared to inherit the transgene stably over several generations, so they were used in a breeding program to follow the inheritance and expression of the transgene from generation to generation. Integration of the final hybrid gene locus was confirmed by PCR and Southern blot analysis. A 630-bp PCR product was generated from the bovine-αS1-casein-hLYZ integrated unit (**Figure 2A**). The mouse genomic DNA was digested with EcoRI and probed with an hLYZ probe, resulting in the identification of a 2619 kb band representing the fragment from intron 1 to intron 3 of the hLYZ genomic sequence (**Figure 2B**). As indicated by the homologous BLAST, there was no similarity between all introns of the hLYZ gene

Table 1. Primers used in this study

No	Primer sequences	Length of product (bp)
T1F	5'-GCCCGATCGGCCTTGTGGCTCGAATCTTCTAG-3'	
T1R	5'-GCATTTAAATGCACTGGCAATTTCTTGGTAA-3'	332
T23F	5'-GCATTTAAATATTGACGCTTCTCTATTC-3'	
T23R	5'-TGGCGATCGGTTTAAACATCTATTCACTCAAAG-3'	551
J1F	5'-TCTGAGGGACTCCACAGTTATG-3'	
J1R	5'-TAACACATACCCACTGCTCCTG-3'	680
J2F	5'-AAATATTGACGCTTCTCTATTCCTC-3'	
J2R	5'-TTCCTTTATCGGGTATCTCTGG-3'	627
J3F	5'-GGAACTCTAGGAGTCAAACGTG-3'	
J3R	5'-TGGCGATCGGTTTAAACATCTATTCACTCAAAG-3'	876
Mouse-F	5'-CTGTGACTACAGGTGTGTACCAC-3'	
Mouse-R	5'-TCATAGCAGATACATAGGCTGATG-3'	630

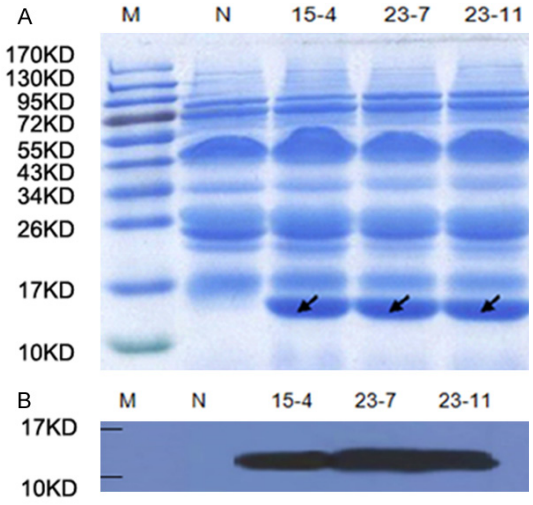

Figure 3. Expression of hLYZ in the milk of transgenic mice. A. Milk samples were applied to SDS-PAGE and then stained for total protein. *M* protein marker, *N* milk sample from wild-type mice; 15-4.23-7.23-11 lines from the three transgenic mouse lines. Numbers on the left indicate the molecular weights of the protein standards. B. Western blot analysis of hLYZ. Milk samples were diluted and applied to western blotting using a monoclonal rabbit anti-hLYZ antibody. M: protein maker; N: sample from non-transgenic mice (day 12 lactction); 15-4, 23-7, 23-11: milk samples from three transgenic mice lines (day 12 lactction).

and the mouse counterpart no similarity can be found all introns of the hLYZ gene and the mouse counterpart.

Determination of transgene copy numbers

The integration copy number for each transgenic mouse strain was determined according to our previous report [9]. The mouse-F and mouse-R primers were used to assess the Ct values of the hLYZ gene; both primers were located in intron 3 of hLYZ.

Analysis of hLYZ expression

For SDS-PAGE, milk samples were diluted (1:40) in phosphate-buffered saline at equal volumes (10 μl), which were loaded under reducing conditions onto a 10% SDS/PAGE gel. The milk of wild-type mice was used as a negative control, and hLYZ standard from transgenic rice was used as a positive control. Western blotting was performed in accordance with standard protocols. After blotting, the membrane was blocked for 1 h in Tris-buffered saline (pH 7.5) containing 0.2% Tween-20 and 5% bovine serum albumin. The membrane was then incubated for 1 h with a primary monoclonal rabbit anti-hLYZ antibody diluted at 1:500 in 0.2% Tween 20 and 1% Protifar in TBS (pH 7.5). Then, the membrane was washed with TBS containing 0.2% Tween-20 for 1 h at room temperature, after which it was incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Enhanced chemilumin-escence detection was then performed using Na-luminol and p-coumaric acid.

The concentration of rhLYZ was determined using an AssayMax Human Lysozyme

ELISA kit (milk) (Assaypro, Brooklyn, NY) was used according to the manufacturer's protocol. The activity of rhLYZ was calculated relative to the standard curve of known hLYZ concentrations.

Primers

All the primers were listed in **Table 1**. Primers J1F and J1R were used to achieve the first step of PCR identification of the 3' bovine-αS1-casein gene fragment. The primers J2F and J2R were used to achieve the second step of PCR identification of the hLYZ gene fragment. The primers J3F and J3R were used to achieve the third step of PCR identification of the 5'bovine-αS1-casein gene fragment. The primers mouse-

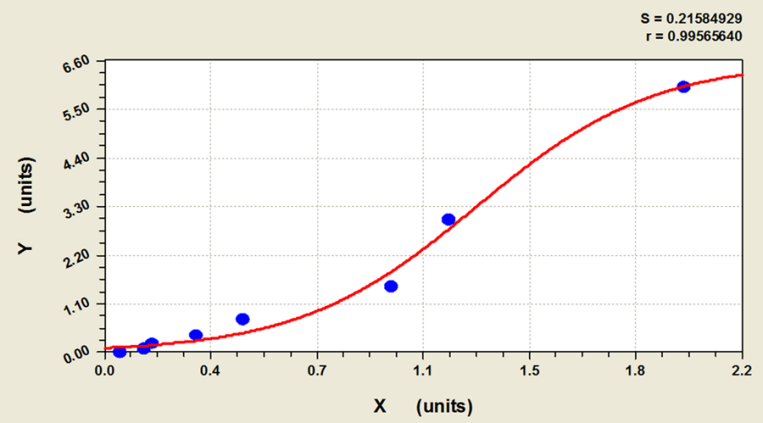

Figure 4. A standard graph for the determination of hLYZ concentration by ELISA. Human lysozyme standard dilution into six gradient. X-axis: standard density, Y-axis: absorption values reflecting luminosity.

Table 2. Expression of human lysozyme in transgenic mouse milk. Expression levels represent the values of milk pools from female mice at day 12 of lactation for each transgenic line

Rat lines	Human lysozyme expression (g/L)
15-4	5.88
23-7	6.06
23-11	4.35
N	0.00

N: negative control.

F and mouse-R were used for the identification of transgenic mice.

Results

High hLYZ expression measured by Western blot

To evaluate the expression of hLYZ, milk was collected from three female transgenic mouse lines at day 12 of lactation. The band of about 16 KD was illustrated in SDS-PAGE analysis of milk samples from transgenic lines 15-4, 23-7, 23-11, consisting of a significant amount of hLYZ (**Figure 3A**). In western blot analysis, compared with wild-type mouse milk, a unique protein band of 16 kDa was observed in the milk of three transgenic mice. There was no band of 16 kDa in the negative control sample (**Figure 3B**).

High hLYZ expression measured by ELISA

The expression level of hLYZ in milk was determined by total antigen assay ELISA. Human lysozyme standard was diluted to 6.0, 3.0, 1.5, 0.75, 0.375, 0.187, 0.093, and 0.000 μg/ml, and ELISA standard curve fitting equation was used CurveExpert software analysis, finally got the data analysis (**Figure 4**). The expression level of of hLYZ in the 3 lines was at the 4.35-6.06 g/l (**Table 2**), while line 23-7 showed the highest expression level.

Discussion

In this study, the transgenic animal mammary bioreactors model that express high levels of recombinant human lysozyme in milk was built. The mammary gland has been considered as a potential bioreactor for the expression of recombinant proteins. The results show that the high expression of recombinant proteins requires optimized constructs, featuring an optimized integration locus. In previous study, rhLZ was expressed in the milk of the transgenic animal using 23 kb of a bovine-αS1-casein regulatory element, which resulted in rhLYZ being expressed at concentrations from 0.25 to 0.07 g/L [13]. Therefore, lysozymes were chosen in this study. In addition, transgenic goats were established by standard pronuclear microinjection, which led to rhLYZ expression in milk at a level of 0.27 g/L [14]. In another study, the milk from transgenic mice could produce hLYZ at the level of 1.4 g/L [15], which was achieved by cloning the genomic sequence of hLYZ into the commercial expression vector pBC1. Moreover, transgenic cattle expressing rhLYZ in milk were produced at a level of 25.96 mg/L [16]. The hybrid construct of goat β-casein fused to rhLYZ gDNA seemed to be an optimized construct, as the rhLYZ was expressed at a desirable level in transgenic livestock. It was reported that 0.026 g/L of rhLYZ appeared in transgenic cow milk [17]. In contrast, rhLYZ was expressed at a very low level in transgenic cloned pigs, namely, 0.32 mg/L [18]. To replace the genomic sequence of human lactoferrin (hLF) in the hLF BAC clone with that of hLYZ, which is optimized for the hLYZ expression vector, five transgenic mouse lines were generated, four of which expressed rhLYZ at levels from 1.2 to 1.76 g/L [19]. Our expression cassette was set based on the hybrid gene locus strategy works, which includ-

ed both the expression level and the proportion of high productive transgenics. A 50 kb mWAP-hLF hybrid gene locus was constructed and an extremely high level expression of rhLF (16.7-29.8 g/L) was found in the milk [9]. A 38.4 kb mWAP-htPA hybrid gene locus was constructed, which gave high level expression of rhtPA attained to 3.3 g/L [10]. A chimeric mWAP-hLYZ vector was constructed and the expression of rhLYZ in transgenic mice attained to 35 g/L [20].

In our study, a bovine-αS1-casein gene was used as a promoter to drive the expression of hLYZ, while bovine-αS1-casein itself is the endogenous high expression protein in ruminants. After bovine-αS1-casein gene was introduced into the murine germline, transgene expression occurred in all transgenic mice, and was confines to the lactating mammary gland. The highest levels of expression were obtained with a transgene containing 14.2 kb of 5'flanking sequence, which was 20 mg/ml [21]. High expression of GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor) could be induced in transgenic mice by a bovine-αS1-casein gene [22]. The above research showed that the bovine-αS1-casein could not only guide the expression of exogenous gene in bovine mammary epithelial cell, but also guides expression in transgenic mice, rabbits or sheep, which may be related to the conservation of the expression regulation of milk protein gene. Moreover, the bovine-αS1-casein gene is very similar to the mouse casein gene, which is possibly originated from the same ancestor. Therefore, the bovine-αS1-casein was chosen as the regulatory element to guide the expression of hLYZ in mammary gland. Red homologous recombination system was used to replace part of the genome, which was used to build a milk target protein hybrid locus. In the current study, a bovine-αS1-casein-LYZ heterozygous locus containing a relatively intact bovine-αS1-casein gene upstream and a control sequence downstream was established.

When the established bovine-αS1-casein-hLYZ heterozygous locus were transferred by the microinjection method and integrated into fertilized egg cells in mice, the mouse mammary gland bioreactor was achieved. 631 pieces of mouse fertilized eggs were transplanted to 23 receptors in rat. Among 23 offspring mice, PCR and Southern blot identification revealed three transgenic mice, yielding a positive rate of 13.0%, which proved that the hybrid loci could be consolidated in mice, and the efficient expression of lysozyme could be obtained. Our current study established a heterozygous locus that was integrated into fertilized egg cells of mice, which need less transgenic mice. The reasons should be: (i) the rearing environment caused abnormal female fertility; (ii) the superovulation of false pregnant mothers was not good; (iii) the toxicity of the heterozygous locus; and (iv) the large size of the bovine-αS1-casein-hLYZ hybrid gene fragment would cause problems in gene integration. The amount of rhLYZ in milk was also quantified by ELISA, which showed that three transgenic mouse lines had a high level of expression. This proved that the bovine-αS1-casein BAC vector provided consistent expression, and our expression cassette based on the hybrid gene locus strategy functioned efficiently. The expression level of hLYZ reached as high as 6.06 g/L, while another cases reached at 5.88 g/L and 4.35 g/L. There was no abnormal phenomenon in transgenic mice, which meant that our hybrid loci are safe. In the process of breeding the F_1 generation of transgenic mice, 50 F_2 mice were obtained, of which 22 (44%) were genetically modified, conforming to the Mendelian law of inheritance. It proved that the stable genetic traits of transgenic mice could be obtained.

In conclusion, the results of this study show that the mammary gland expression vector with the bovine-αS1-casein-hLYZ heterozygous locus can be expressed in mice and there was no effect on normal protein expression in mouse milk. As the milk of dairy cows is easy to be obtained and continuously available, the use of mammary gland bioreactor system of dairy cows may provide a new way to produce hLYZ and transfer the benefits of human milk to cow milk.

Disclosure of conflict of interest

None.

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