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# Study on Bovine Mammary Specific Expression Vector of Expressing Human Lysozyme Gene

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**Abstract:** To study the bovine mammary gland bioreactor, the 5' and 3' flanking sequence of bovine β-casein (*CSN2*) gene were cloned and connected to the eukaryotic expression vector of pC DNA3.0 which had been modified. The human Lysozyme gene (*hLYZ*) was inserted into the site between 5' and 3' flanking sequence after the vector named pC was identified and formed the vector named as pC-hLYZ subsequently. To determine whether the constructed vector could drive the hLYZ expression, the pC-hLYZ and positive control hLYZ plasmid were wrapped by the PEI and injected into lactation rabbits through mammary gland centre duct. Milk was collected and the expression status was detected after 72 h. The plate inhibition assay showed that pC-hLYZ eukaryotic expression vector could be a good driver for *hLYZ* gene expression in rabbit mammary gland. The results demonstrated that the bovine mammary gland specific expression vector of hLYZ had been constructed successfully. The establishment of the vector laid the foundation for further study of bovine mammary gland expression technology.

**Key words:** CSN2, hLYZ, Clone, Mammary gland specific expression, bovine, rabbit

## INTRODUCTION

Lysozyme (LYZ) was a kind of non-specificity defenses factor which existed in human and animal's body fluids or tissues widely (Fokunang *et al*., 2005; Idenyi *et al*., 2006; Sharifi-Yyazdi *et al*., 2007; Anand *et al*., 2010; Han *et al*., 2011). The LYZ was usually used in clinical and food industries result from the significant pharmacological effects such as anti-bacterial, eases pain, anti-virus, anti-tumor and enhance immune system (Rong and Ling, 1999; Yu *et al*., 2009; Maga and Murray, 1995). LYZ was main obtained from chicken egg white which own high levels of LYZ (3-4 mg $mL^{-1}$). However, the activity was only 1/3 of hLYZ and may even elicit immune response like allergic reaction when used in human being (Zhu and Sun, 2006).

The natural LYZ can be collected from human milk, placental extract or saliva but very limited (Sun *et al*., 2004). Therefore, LYZ was considered as one of the main nutritional differences between human and animal milk and it was also the main object about to research animal milk more like human milk (Maga and Murray, 1995). Many studies showed that animal breast tissue was the most ideal place for the expression of transgenic exogenous medical protein although many expression systems had been constructed (Yang *et al*., 2008). It produced medical protein with the ability of high output and low cost, the protein was processed and modified after translation and also easy to be separated (Shi *et al*., 2008). At present, the animal mammary gland bioreactor possess the fastest research progress and the best development perspective. The basic principle is that the target gene with important development value will be fused with the regulatory sequence of lactoprotein gene to construct the mammary gland specific expression genetic component.

Subsequently, it is injected into the animal embryos to obtain the transgenic offspring. When the animals lactating, the exogenous gene will express under the control of lactotropin and the regulatory sequence of lactoprotein gene, the expression level can meet or exceed the normal content of lactoprotein (Archibald *et al*., 1990). Therefore, these transgenic animal breasts are like natural factories of producing activity protein and we can collect the valuable bioactive protein constantly as long as the animals are fed. In this study, we constructed the bovine mammary gland expressing vector of hLYZ and detected the foundation to expressed hLYZ for exploring the feasibility of bovine mammary gland expressing hLYZ.

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Table 1: The three pairs of primer sequences for PCR amplification

| Primers | Primer sequences (5'-3') | Product size/kb | Annealing temp. (°C) |
|---|---|---|---|
| CSN2-5' | F: AGTCGCGAATTGCTGGCTTTTAAATTTTCCCT | 1.8 | 59 |
| | R: ATGGTACCACGCGTAAGGAGGAGCTGAATGGATAATGA | | |
| CSN2-3' | F: CTGGTACCTAAGCTTTAGGAGATTAGAGGC | 1.1 | 55 |
| | R: ATCTCGAGATGCCTAAGGGTTAATTTATTG | | |
| hLYZ | F: ATACGCGTATGAAGGCTCTCATTGTTCTGGGGCT | 1.5 | 57 |
| | R: CTGGTACCGTGTTGCAAAGCCTTTAATTAGAATG | | |

NruI, TCGCGA; KpnI, GGTACC; MluI, ACGCGT; XhoI, CTCGAG

## MATERIALS AND METHODS

**Plasmid, host cell and animals**: Eukaryotic expression vector pC DNA3.0, vector pMD19-T and DH5α were purchased from TaKaRa (Dalian, China). The cDNA of hLYZ was synthesized by Heng Yin Biotech (Shanghai). The experimental rabbits (20 individuals) were reared in the same management system in XinMin rabbit company located in Guizhou, Guiyang, P.R. China.

**Primers design and PCR amplification:** According to the sequence of *CSN2* gene (GenBank No.: AY352050) and part of *hLYZ* gene (GenBank No.: NC_000012.11), three pairs primers to make Restriction Endonuclease Analysis (REA) were designed using the primer 5.0 Software (Table 1).

The 25 μL volume of PCR amplification contained: 100 ng genomic DNA, 0.5 μM each primer, 1×buffer (including 1.5 mM $MgCl_2$), 200 μM dNTPs and 1 units of Taq DNA polymerase (MBI, Fermentas). The PCR cycling conditions consisted of an initial single cycle for 10 min at 95°C followed by 35 cycles of cycling consisting of 94°C for 60 sec, annealing for 60 sec and 72°C for 90 sec with a final extension at 72°C for 10 min.

The PCR fragments of three pairs primers were cloned into the pMD19-T vector (Takara, China) and then sequenced for confirmation in both directions, respectively. All the sequences were compared with the published sequence in GenBank by using the Blast search program at the NCBI website.

**Expression vector construction:** Restriction enzyme PvuI was used to treat the pC DNA3.0 vector and transformed DH5á competent cells, then the plasmids were extracted and named as pA. pA and CSN2 gene 5'-pMD19-T vector were cutting by using restriction enzyme Nru I+Kpn I to obtain cohesive end pA and CSN2-5' flanking, respectively and connected their with T4 ligase and transformed DH5α competent cells and then extracted the plasmids and named it as pB. In order to get cohesive end CSN2-3' flanking and pB, Xho I+Kpn I were used to cut *CSN2* gene 5'-pMD19-T vector and pB, then linked their with T4 ligase and names as pC. pC was digested by Mlu I and sticky end fill flat using Klenow fragment to obtain pC-hLYZ. All productions were identified by enzyme digestion and PCR amplification.

**hLYZ transient expression in rabbits mammary gland:** The healthy adult rabbits which obtained one day ago were utilized as laboratory animals. The pC-hLYZ vector and the positive vector were wrapped by PEI and 5% glucose solution, respectively.

Experimental animals were allotted to experimental groups and control group randomly, the experimental groups were injected with recombinant plasmid of different doses.

The doses were 100 μL (50 μg plasmid), 200 μL (100 μg plasmid), 400 μL (200 μg plasmid), 500 μL (250 μg plasmid) and 600 μL (300 μg plasmid), respectively. The control group was injected with blank vector, repeated injection after 24 h. The milk were collected after 48 h and tested the effect of hLYZ.

**Statistical analysis:** All data were analysed by Excel 2007 (Microsoft, USA).

## RESULTS AND DISCUSSION

**PCR amplification:** After the 30 cycles PCR amplification for the 5' and 3' flanking sequence of *CSN2* gene and the sequence of *hLYZ* gene, researchers obtained 2 μL production of the PCR extension reaction to run agarose gel electrophoresis and stain by EB then the sizes were 1.8, 1.1 and 1.5 kb, respectively which matched the initial anticipation (Fig. 1 and 2).

**Identification of the *CSN2* gene and hLYZ-T vector:** The CSN2 5'UTR-T vector was cut by using the enzymes NruI and KpnI, the sequences were about 1.8 and 2.7 kb (Fig. 3). The CSN2 3'UTR-T vector was cut by XhoI and KpnI then the sequences were 2.7 and 1.1 kb (Fig. 4). Furthermore, the hLYZ-T was cut by KpnI and MluI and the sequences were 1.5 and 2.7 kb. It showed that the sizes were same as the expected ones.

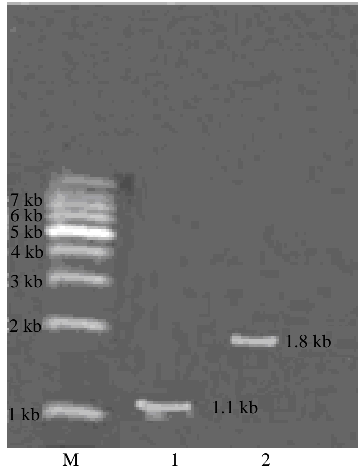


Fig. 1: PCR production of *CSN2* gene 5' and 3' lanking sequence. Lane M: 1 kb DNA landder (TIANGEN), Lane 1: PCR production of CSN2 3'UTR; Lane 2: PCR production of CSN2 5'UTR

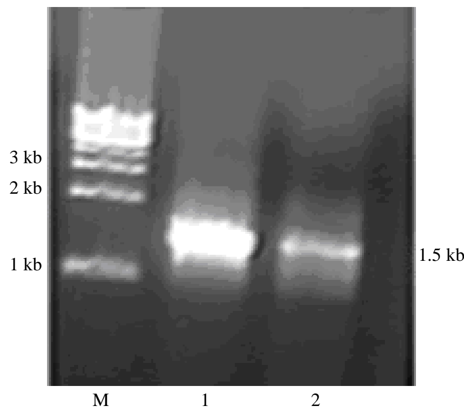


Fig. 2: PCR production of hLYZ. Lane M: 1 kb DNA landder (TIANGEN); Lane 1, 2: PCR production of hLYZ

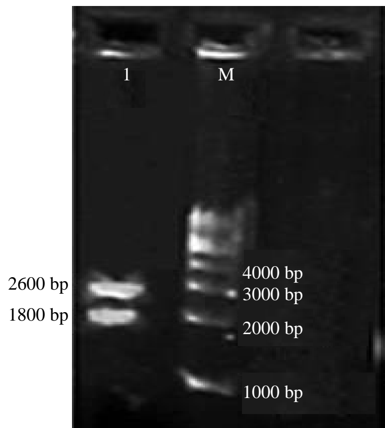


Fig. 3: Identification of CSN2 5'-portion-T vector Lane M: 1 kb DNA landder (TIANGEN), Lane 1: NruI+KpnI cutting of CSN2 5'UTR-T vector

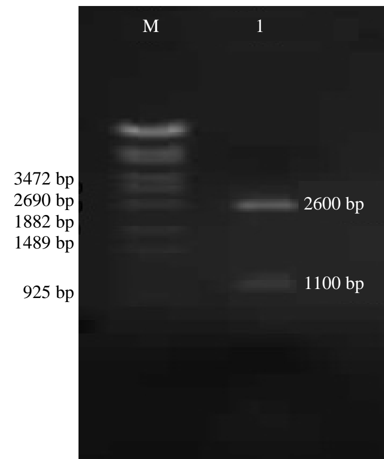


Fig. 4: Identification of CSN2 3'-portion-T vector. Lane M: λ-EcoT14digest DNA Marke (TIANGEN), Lane 1: XhoI+KpnI cutting of PSP-II 3'UTR-T vector

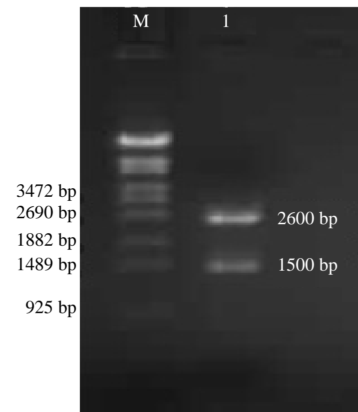


Fig. 5: Identification of hLYZ-T vector. Lane M: λ-EcoT14digest DNA Marke (TIANGEN), Lane 1: XhoI+KpnI cutting of hLYZ-T vector

**Construction of bovine mammary specific expression vector of expressing human lysozyme gene:** The 5' and 3' flanking sequence of *CSN2* gene and *hLYZ* gene were cloned into the pC DNA3.0 vector which have been reformed then identified by enzyme digestion and PCR. The results showed that pB was 4.4 kb, pC was 5.5 kb and pC-hLYZ was 6.9 kb and they also agreed with the outcome of anticipation (Fig. 4-8).

**Transient expression in rabbit mammary gland:** The pC-hLYZ vectors with different doses were injected into the rabbits' mammary gland which had been wrapped by PEI. The milk were collected after 48 h of injection and the *in vitro* drug sensitive test was used to investigate the effect, at last we discovered the bacteriostasis. There were mild bacteriostasis to *Staphylococcus aureus* with 50 μg

Fig. 6: Identification of pB vector. Lane M. λ-EcoT14digest DNA Marker (TIANGEN), Lane 1: NruI+KpnI cutting of pB vector

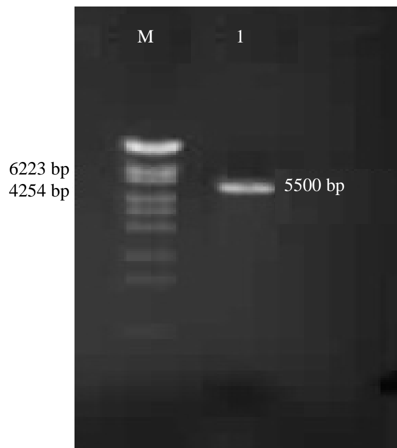


Fig. 7: Identification of pB vector. Lane M: λ-EcoT14digest DNA Marker (TIANGEN), Lane 1: NruI+KpnI cutting of pC vector

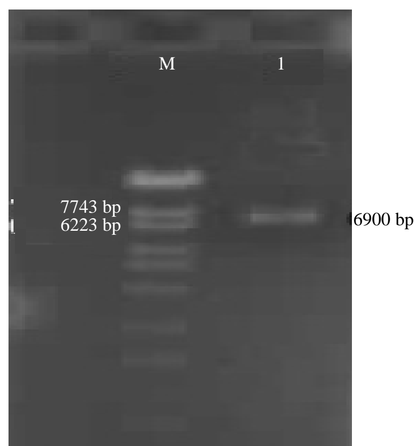


Fig. 8: Identification of pC-hLYZ vector. Lane 1: MluI cutting of pC VectorfpC-hLYZ vector, Lane M: λ-EcoT14digest DNA Marker (TIANGEN)

Fig. 9: hLYZ inhibition of *Staphylococcus aureus* test results; 1) Injection pC-hLYZ of 50 ug; 2) Injection pC-hLYZ of 100 ug; 3) Injection pC-hLYZ of 200 ug; 4) Injection pC-hLYZ of 300 ug; 5) Penicillin group 500 IU

Fig. 10: hLYZ inhibition of *Streptococcus agalactial* test results; 1) Injection pC-hLYZ of 50 ug; 2) Injection pC-hLYZ of 100 ug; 3) Injection pC-hLYZ of 200 ug; 4) Injection pC-hLYZ of 300 ug; 5) Penicillin group 500 IU

dose of vector and the bacteriostasis was better when the dose was 200 μg. However, the inhibition zone had no emarkable increase when injected with 300 μg recombinant plasmid compared with the dose of 200 μg. the mammary gland showed necrosis and milk could not be collected when the injection reached 400 μg time$^{-1}$.

Moreover, the same results obtained when studied the bacteriostasis to *Streptococcus agalactiae*. The blank control didn't show bacteriostasis to the two bacterium (Fig. 9-11).

**Content of transient expression hlyz in rabbit milk:** Bacteriostasis significance was represented by the width of inhibition zone (W) (Zhang *et al*., 2004):

Fig. 11: Antibacterial test results of the control group; 1) Control group; 2) Injection pC-hLYZ of 50 ug; 3) Penicillin group 500 IU

Table 2: Human lysozyme against *Staphylococcus aureus* and *Streptococcus agalactiae* of antibacterial cativity

| | *Staphylococcus aureus* $2.28\times10^7$ CFU $mL^{-1}$) | | | *Streptococcu agalactiae* ($5.32\times10^6$ CFU $mL^{-1}$) | | |
|---|---|---|---|---|---|---|
| Dose ($\mu$g $time^{-1}$) | D (mm) | W (mm) | Bacter-iostasis | D (mm) | W (mm) | Bacter-iostasis |
| 50 | 7.50 | 0.75 | - | 13.25 | 3.63 | +++ |
| 100 | 9.50 | 1.75 | + | 15.50 | 4.75 | +++ |
| 200 | 10.25 | 2.13 | + | 17.25 | 5.63 | +++ |
| 300 | 10.35 | 2.18 | + | 17.75 | 5.88 | +++ |
| 400 | -- | -- | -- | -- | -- | -- |
| 500 IU penicillin (control) | 8.25 | 1.13 | + | 14.25 | 4.13 | +++ |

--: Did not collect the milk; +++: W>3 mm, the inhibition effect was strong; ++: 2.5<W<3.0 mm, the inhibition effect was middle; +: 1 mm<W<2.5 mm, inhibition; -: W<0.9 mm, weak or no inhibition

$$W = \frac{D\text{-}d}{2}$$

Where:

W = Width of inhibition zone
D = Diameter of inhibition zone
d = Diameter of the paper (6 mm) (Table 2)

The mammary specific expression vector of expressing *hLYZ* gene was constructed by using *CSN2* gene regulatory sequences which contained the 5' (1.8 kb) and 3' (1.1 kb) flanking sequence (it contained part of the last intron and the last untranslated extron), the 3' flanking sequence contained polyA and G/T group, these sequences played an important part in mRNA transcription and the stability of cytoplasm translation regulation. More importantly, they possess the obvious interreaction chronically result in the endogenous transcription of CSN2 under the induction of reasonable factor (hormone). Researchers cloned the pC DNA3.0 vector which contained the 5' and 3' flanking sequence of *CSN2* gene and the cDNA of *hLYZ* gene then the mammary specific expression vector pC-hLYZ was constructed. In this specific expression vector the structural gene of *CSN2* was replaced by the cDNA of hLYZ and based on the endogenous expression regulation style. If the expression vector's integration site is suitable, it will drive the expression of exogenous target gene in mammary gland epithelial cells and tissue.

The successful construction of pC-hLYZ vector laid the foundation for the regulation mechanism and regulation characteristic of the 5' and 3' flanking sequence of *CSN2* gene. However, the expression quantity of pC-hLYZ vector, the mechanism and characteristic of the 5' and 3' flanking sequence of *CSN2* gene even whether there is hormonal dependency element are all need further study (Liu *et al*., 2004).

pC-hLYZ were wrapped by PEI and injected into rabbit's mammary gland and the dose were 100 μL (50 μg plasmid), 200 μL (100 μg plasmid), 400 μL (200 μg plasmid), 500 μL (300 μg plasmid) and 600 μL (400 μg plasmid), respectively. This experiment demonstrated that the expression effect was closely related to the volume of plasmid. When the dose was 100 μg, there was an acceptable effect but the inhibition zone was no longer larger as the dose increased when the dose was 300 μg/ time. When the injection reached 400 μg $time^{-1}$ the mammary gland showed necrosis.

## CONCLUSION

Calcium phosphate precipitation, DEAE-glucan method, electricity wear method and liposomes transfection method are common transient transfection methods (Sambrook and Russell, 2002). Liposomes transfection method is reliable and has good repeatability but it is expensive and need large dosage (Thierry *et al*., 1997). PEI was found as an effective gene transfection reagent and applied in transgenic study of cellls and mammals widely (Zhang *et al*., 2000). The plasmids with hLYZ cDNA were wrapped by PEI transfection to the mammary glands of rabbit and the expression effect was perfect.

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