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Exogenous ß-galactosidase decreased lactose in caprine mammary epithelial cells

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ABSTRACT

This study was conducted to express exogenous ß-galactosidase and make lactose hydrolyzed in mammary epithelial cells which could provide a valuable way for obviate lactose intolerance.

PCR was used to get the 5' flanking regulatory region of bovine αS1 casein gene (1.2kb) from the genomic DNA and construct the vector. β-galactosidase was expressed under the control of 5' flanking regulatory regions of bovine αS1 casein gene (1.2kb) in caprine mammary epithelial cells (CMECs). *LacZ* gene expression specificity was detected by 5-bromo-4-chloro–3-indolyl-β-D-galactoside (X-gal) staining of the β-galactosidase activity. In the cell culture supernatant and cell extracts, β-galactosidase was detected within 24 to 144 h after transfection. β-galactosidase of the cell extracts had a gradually decrease tendency in 24 to 72 h. In contrast, β-galactosidase of cell culture supernatant had a gradual increase tendency. In the cell extracts, β-galactosidase could be detected in 1 to 3 generation. HPLC showed that the lactose concentration of CMECs was reduced by β-galactosidase and the effects held significantly in 24 to 144 h post-transfection. β-galactosidase could express in CMECs and significantly reduced the lactose concentration of CMECs, these findings would provide an available way for obviate lactose intolerance.

Key words: β-galactosidase, Lactose, Lactose intolerance, Mammary epithelial cells

Adult-type hypolactasia (also known as primary lactose malabsorption or lactase non-persistence) is the world's most common enzyme deficiency. It is due to the decline of the activity of the small intestine brush border lactasephlorizin hydrolase (LCT), the disaccharidase involved in the hydrolysis of milk's main carbohydrate lactose. As a result of this autosomal-recessive trait, lactose, which is normally digested in proximal to mid-jejunum, reaches the colon where it is fermented by bacteria to fatty acids and gas, causing bloating and diarrhoea (Terjung and Lammert 2007). In a minority of individuals, the same symptoms are due to a secondary lactase deficiency which is caused by distinct pathologies of the small intestine (e.g., giardiasis, celiac disease or Crohn's disease). Several authors have suggested that consuming small amounts of milk does not exert any effects in lactose non-persistent individuals, yet this condition is apparently the major reason for avoiding milk products worldwide (Savaiano *et al*. 2006, Lember *et al*. 2006, Krawczyk *et al*. 2008). Rutter *et al.* (1953) found that the inclusion of 20% lactose in poultry diets impaired growth and caused diarrhoea. Furthermore, they found lactose, but not galactose, in the serum of experimental chicken indicating the absence of the enzymes necessary to hydrolyze lactose into glucose and galactose. In addition, lactase activity has been previously shown to be at low levels in the enterocytes isolated from the small intestine of broiler chickens. Mozdziak *et al*. (2003) generated lines of transgenic chickens carrying the *Escherichia coli* lacZ gene and expressing bacterial β-galactosidase. Bacterial β-galactosidase has the ability to hydrolyze lactose, which cannot be utilized as a source of energy by birds, to glucose and galactose that participate in glycolysis to generate ATP. β-galactosidase catalyzing the hydrolysis of lactose into its constituent monosaccharides, glucose and galactose, attracted attention of researchers and dairy industry because of nutritional (lactose intolerance), technological (crystallization), and environmental (pollution) problems associated with this major milk carbohydrate. So, a kind of effective, convenient and green method for hydrolysis of lactose is needed.

αS1-casein is the most abundantly expressed milk protein in the cow. It was found that short rat β-casein promoter constructs (<3kb) express the transgene only at a low level in the mammary gland (Lee *et al.*1988), while high level and lactationally stimulated expression was observed using 3.8kb (but not 1.7kb) of the bovine β-casein promoter or 21kb of the bovine *αS1*-casein promoter. The necessity to use extended casein promoter segments for strong, mammary gland and lactation-restricted expression of transgenes had been confirmed ever since (Brophy *et al*.2003). In this study,

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lacZ gene was expressed in CMECs under the control of 5' flanking regulatory regions of bovine *αS1* casein gene (1.2kb) and determined the concentration of lactose in transformant. This built a biologic model for the further study on the hydrolysis of lactose in dairy animal milk.

MATERIALS AND METHODS

Vector construction: The 5' flanking sequence of bovine *αS1* casein gene was amplified from the genomic DNA of bovine with 2 synthetic primers (primer 1: 5'-GGGTACCAAAGTGGACAGCCTGAA-32 ; primer 2: 5' -ACCGGTTGCAGACTTTTGCTT CC-32), which contained *Acc65I* and *AgeI* site respectively. The PCR conditions used in amplifying the 5' flanking sequence of bovine *α S1* casein gene were 95°C (45s), 57.4°C (45s) and 72°C (2 min) for 35 cycles. The amplified DNA was ligated into pGEM-T vector to create plasmid *αs1p*-T. The constructed plasmid was confirmed by nucleotide sequencing and restriction mapping. The plasmid *αs1p*-T and was digested with *Acc65I* and *AgeI* and ligated into the same enzyme digested pSV-galactosidase control vector, resulting in the recombinant vector *gαs1p-psv*. The recombinant vector was confirmed by restriction mapping.

Culture and identification of CMECs: The CMECs established by our team were used in all experiments. The cell lines were originally derived from a biopsy specimen of a mammary gland from a lactating (35-days postparturition) Guanzhong goat. The established cultures showed no evidence of a transformed phenotype and maintained anchorage dependence and contact inhibition. Cytokeratin–18 is a specific marker of mammary epithelial cells. The fluorescence of CMECs was visualized with confocal laser scanning microscope.

Transfection and X-gal staining of CMECs: CMECs were seeded at 2×10^4 cells cm^{-2} in 24-well plates. Until cells accreting 80%, the *gαs1p*-psv plasmids were introduced into CMECs by Tfx-20. After transfection 48 h cells were stained to detect X-gal. Cells were fixed for 5 min at room temperature with 3% formaldehyde and incubated for 16h at 37°C with X-gal reaction buffer (40 mmol L^{-1} citric acid/ sodium phosphate buffer, of 1 g L^{-1} X-gal, 150 mmol L^{-1} NaCl, 2 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} potassium ferricyanide and 5 mmol L^{-1} potassium ferrocyanide).The untransfected CMECs acted as negative control. They were both observed by inverted microscope.

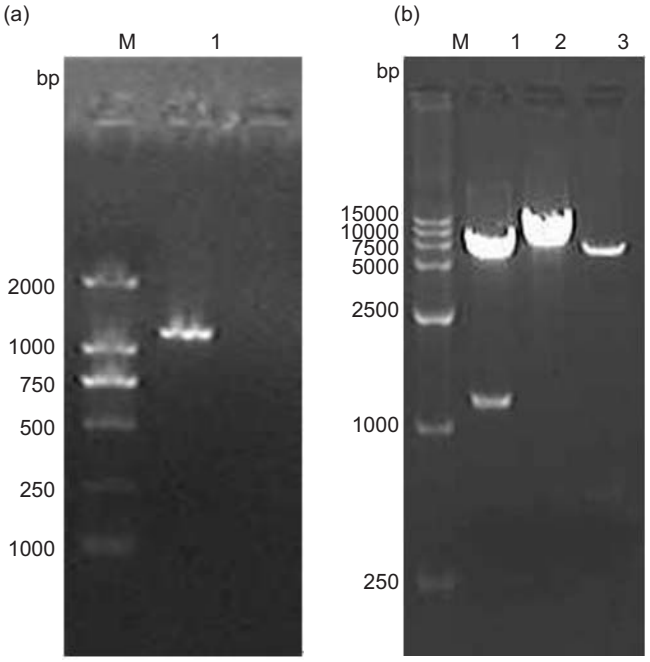
ß-galactosidase activity assay: The cell culture supernatant and cell extracts were collected at 24, 48, 72, 96, 120 and 144 h post-transfection. The detection reagent was prepared according to the manufacturer's instructions. The mixtures were assayed by chemiluminescence detector and got relative light unit (RLU). Negative controls were consisted of untransfected CMECs in the same period correspondingly. In addition at 48 h post-transfection, CMECs were detached with 0.05% trypsin-0.04% EDTA, washed 3 times with modified Hanks balanced salt solution, and transferred culture at a 2-fold of dilution. After passage 48 h, culture supernatant were assayed by the same way upon until would not yield expression product (ß-galactosidase). Negative controls were consisted of untreated CMECs in the same generation correspondingly.

Lactose assay HPLC: Initially, lactose standard curve was constructed with lactose standard substance by HPLC, concentration of lactose and peak area were used to set up abscissa and ordinate. The column temperature was set at 25°C and the UV detector was set at 195 nm. The mobile phase was acetonitrile (5%). The flow rate was 0.6 ml min^{-1}. The cell extracts were collected at 24, 48, 72, 96, 120 and 144 h after transfection, and determined the concentration of lactose by HPLC.

Statistical analysis: All data were expressed as mean ±SD. from at least 3 separate experiments. Statistical significance was calculated with SPSS windows version 16.0 by Student's t-test or one-way ANOVA. Differences at $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Construction of the eukaryotic expression vector: Using specific primers, fragments that cover the whole 5' flanking



Figs A–B. **A.** Identification of 5' flanking sequence of bovine *αS1* casein gene. Lane M, DNA marker: D2000; lane 1, PCR product of 5' flanking sequence of bovine *αS1* casein gene, the size of fragment is about 1.2 kb. **B.** Identification of recombinant of gαs1p-psv. Lane M, DNA marker D15000; lane 1, product of *gαs1p*-psv digested with *Acc65I* and *AgeI*; 2 fragments of psv (about 6.2kb) and *αS1* (about 1.2kb); lane 2, product of *gαs1p*-psv digested with *AgeI*, the size of fragment is about 7.4kb; lane 3, product of psv plasmid digested with *Acc65I* and *AgeI*, the sizes of fragments are 520bp and 6.2kb.

sequence of bovine *αS1* casein gene were amplified by PCR. The cloned sequence of 5' flanking sequence of bovine *αS1* casein gene contained 1,195 bp, which was consistent with the Genbank (Fig.1A and Supp1).

By restriction enzyme digestion and conjunction with PSV plasmid, we constructed the recombinants vector *gαs1p*-psv. After transformation positive cloning was screened in LB broth with 100 lg/mL ampicillin. The plasmid of *gαs1p*-psv was purified and confirmed by digested with *Acc65I* and *AgeI* restriction enzyme. The product of enzyme cutting was analysed on 1.0% agarose gel electrophoresis (Fig.1B).

Culture and identification of CMECs: Caprine mammary epithelial cell colonies, when plated on plastic, attached within 2 to 3 days as clumps from which the cells spread out onto the plastic as round single cell sheets. We further investigated homogeneity at the fourth passage. The presence of cytokeratin-18 in CMECs was demonstrated by double stain immunofluorescence using FITC labelled cytokeratin-18 (green) and PI labelled nuclei (red) (Fig.2A). Negative control consisted of the fibroblast of goat mammary, which could not be stained by cytokeratin-18 antibody (Fig.2B).

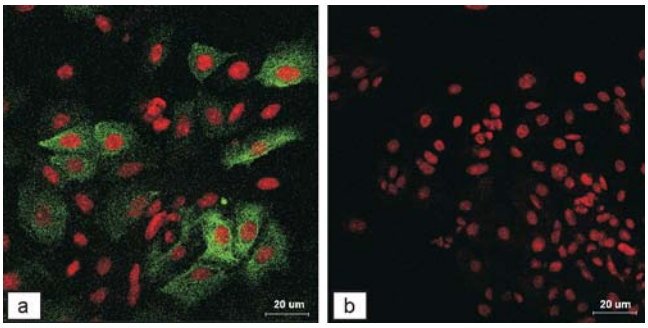


Fig.2. Expression of cytokeratin-18 in CMECs. **A**. Double stain immunofluorescence using FITC labelled cytokeratin-18 (green) and PI labelled nuclei (red). **B.** Negative control. PI labelled nuclei of goat fibroblast (red).

X-gal staining of CMECs: CMECs after transfected with *gαs1p*-psv 48h, were stained with X-gal reaction buffer to detect the expression of *ß*-galactosidase. Then the cells were observed by inverted microscope. The bright blue staining-indicative of *ß*-galactosidase expressed in the transfected

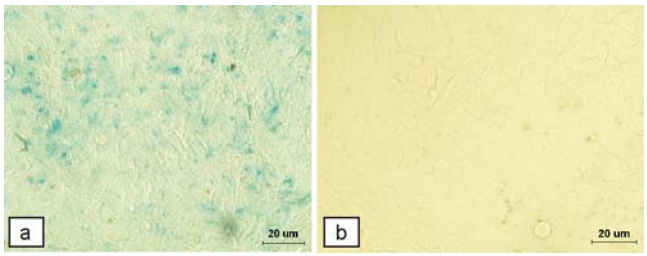


Fig.3. X-gal staining of CMECs. **A**. In transfection of CMECs with *gαs1p*-psv, *ß*-galactosidase could catalyze the disassociation of X-gal and showed blue color. **B**. Negative control consisted of untransfected CMECs.

CMECs, which showed the expression of *ß*-galactosidase (Fig.3A). Nearly no staining was observed in untransfected CMECs (Fig.3B).

ß-galactosidase activity assay: CMECs were seeded at 2×10^4 cells cm^{-2} in 24-well plates, treated with transfection *gαs1p*-psv and incubated at 37°C in a 5% CO_2 incubator. We assayed *ß*-galactosidase of transfected cells at 24, 48, 72, 96, 120 and 144 h post-transfection by chemiluminescence detector. In the cell extracts, the quantity of *ß*-galactosidase had a gradual decrease tendency in 24 to 72 h, gradually decreased after 96 h, but significantly higher than the negative control group (Fig. 4A). In contrast, *ß*-galactosidase of cell culture supernatant had a gradual increase tendency, and obviously higher than the negative control group (P<0.01) (Fig. 4B).

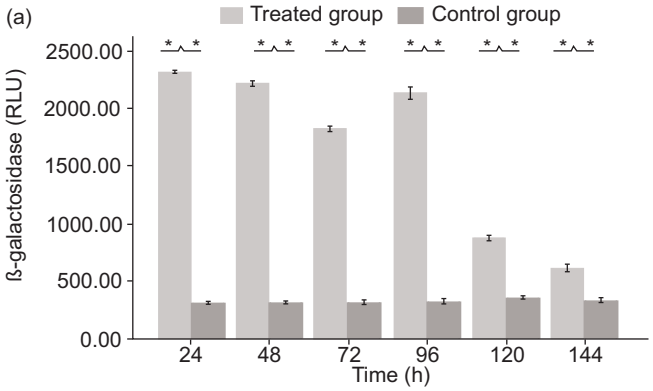


Fig. 4A. Expression of *ß*-galactosidase in the cell extracts. *ß*-galactosidase levels of cell extracts at the 24-144h time point post-transfection are obviously higher than the negative control. Values are means±SD (n=3);** means P<0.01.

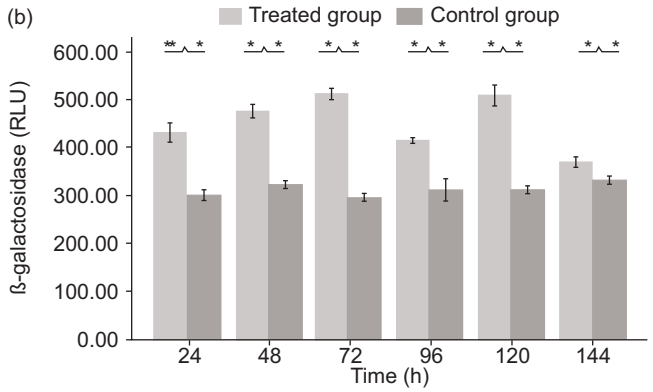


Fig.4B. Expression of *ß*-galactosidase in the cell culture supernatant. *ß*-galactosidase levels of cell culture supernatant at the 24–144h time point post-transfection are higher than the negative control. Values are means±SD (n=3), ** means P<0.01.

To evaluate the expressions of ß-galactosidase, we also assayed *ß*-galactosidase of transfected cells at 1 to 3 generation by chemiluminescence detector. In the cell supernatant, the quantity of *ß*-galactosidase had gradually

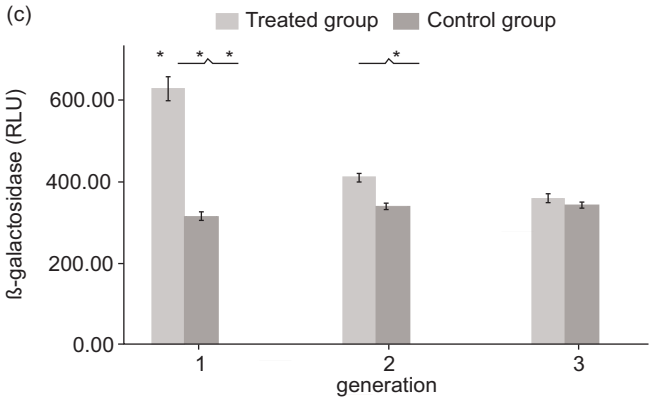


Fig. 4C. Expression of ß-galactosidase in passages. ß-galactosidase levels of cell culture supernatant at different generations are shown.Values are means ± SD (n=3); * means P<0.05, ** means P<0.01.

decrease tendency in 1 to 3 generation (Fig.4C). In first generation, the difference of *ß*-galactosidase expression was obviously significant between transfection cells and negative control (P<0.01). The difference in second generation was significant (P<0.05), but till the third generation the difference was not significant (P>0.05).

Lactose assay by HPLC: The cell extracts were collected at 24, 48, 72, 96, 120 and 144 h after transfection, and determined the concentration of lactose by HPLC. Lactose concentration of transfected cells was significantly lower than non-transfected control at 24, 48, 72, 96, 120 and 144 h (P<0.01) (Fig.5).The results suggested that exogenous *ß*-galactosidase may influence lactose content secreted by caprine mammary epithelial cells.

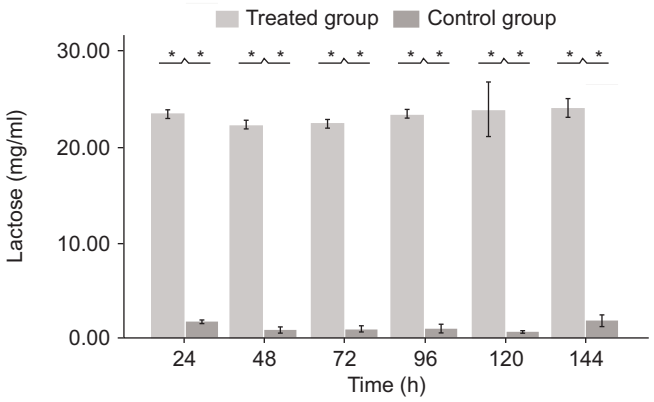


Fig.5. Concentration of lactose at different time. Concentration of lactose in cells at the 24h–144h post-transfection was significantly lower than non-transfected control. Values are means±SD (n=3). ** means P<0.01.

Lactose intolerance is a recognized common problem in many children and most adults throughout the world. Although rarely life-threatening, the symptoms of lactose intolerance can lead to significant discomfort, disrupted quality of life (Heyman 2003). Enzyme-replacement therapy with microbial exogenous lactase (obtained from yeasts or fungi) represents a possible strategy for primary lactase deficiency. Enzymes could be added in a liquid form to milk before its consumption or administered in a solid form (capsules or tablets) together with milk and dairy products. This strategy is effective in reducing both H_2 breath excretion and subjective manifestations of discomfort after milk ingestion (Montalto *et al.* 2006). Therapy compliance with ß-galactosidase is assured by good palatability (Montalto *et al.* 2005), though there are some reported taste alterations (Suarez *et al.* 1995). But this method is also expensive and inconvenience for many people. In this study, we described the cloning and expression of ß-galactosidase in dairy animal mammary epithelial cells, and aimed to find out a kind of new way to solve the problem of decrease the quantity of lactose in milk and release lactose intolerance.

The bovine *αS1* casein gene extends over 17,508 bp with 1,138 bp of exon and 16,370 bp of intron DNA. Thus, the size ratio of exon vs intron DNA is 1:14.4. The gene is split into 19 exons, ranging in size from 24 to 385 bp, and 18 introns from 90 bp to 1 967 bp. There are 2 potentially functional 'TATA' boxes in *αS1* promoter region, with the sequence TTTAAAT at -29 being linked to the major transcription initiation site, part of this sequence belongs to an 11 bp direct repeat motive AAATAGCTTGG, which is also located at –572.

The caseins are encoded by a cluster of single copy genes localized on chromosome 6 in ruminants (Rijnkels *et al.* 1997) and the promoter regions controlling milk gene expression are documented (Rosen *et al.*1999). Using the bovine *αS1*-casein promoter, it was found in mice that the –14 kb promoter construct gave the highest level of expression in the mammary gland and was developmentally controlled according to the bovine rather than the mouse scheme. The core identified here at –10 kb in the bovine *αS1*-casein promoter conceivably specifies one of the distal, highly relevant control elements contributing proper and strong lactation-dependent expression of the bovine *αS1*-casein gene (Vanselow *et al.* 2006).

In this study, we cloned –2 to –1 197 5' flanking regulatory regions of bovine *αS1* casein gene, and this 1 195 bp fragment including some important upstream regulatory elements of bovine *αS1* casein gene, which could direct *LacZ* expression specifically in CMECs. In the cell culture supernatant and cell extracts, *ß*-galactosidase was detected within 24–144 h post-transfection. In the cell extracts, the expression of *ß*-galactosidase had a gradually decrease tendency in 24–72 h. In contrast, the quantity of *ß*-galactosidase in the cell culture supernatant had a gradually increase tendency. It suggested that *ß*-galactosidase could express and secrete into the extracellular environment normally. In the cell extracts, the quantity of *ß*-galactosidase had a gradual decrease tendency in generation 1 to 3. HPLC showed that *ß*-galactosidase reduced the lactose concentration of CMECs and the effects

held significantly in 24–144 h post-transfection. In conclusion, all these data demonstrated that exogenous *β*-galactosidase was able to express in eukaryotic cells. Its expression products have significant activity and could significantly decrease lactose in CMECs. Most importantly, this findings would lead a valuable and original way for obviate lactose intolerance.

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