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Expression of recombinant myostatin propeptide pPIC9K-Msp plasmid in *Pichia pastoris*

W. Du[1,2], J. Xia[2], Y. Zhang[2], M.J. Liu[1,2], H.B. Li[2], X.M. Yan[2], J.S. Zhang[2], N. Li[2], Z.Y. Zhou[2] and W.Z. Xie[2]

[1]College of Animal Science, Shihezi University, Shihezi, China
[2]Xinjiang Academy of Animal Science, Urumqi, China

Corresponding authors: M.J. Liu / Y. Zhang
E-mail: liumingjm2@163.com / zhangyangzy2@126.com

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ABSTRACT. Myostatin propeptide can inhibit the biological activity of myostatin protein and promote muscle growth. To express myostatin propeptide *in vitro* with a higher biological activity, we performed codon optimization on the sheep myostatin propeptide gene sequence, and mutated aspartic acid-76 to alanine based on the codon usage bias of *Pichia pastoris* and the enhanced biological activity of myostatin propeptide mutant. Modified myostatin propeptide gene was cloned into the pPIC9K plasmid to form the recombinant plasmid pPIC9K-Msp. Recombinant plasmid pPIC9K-Msp was transformed into *Pichia pastoris* GS115 by electrotransformation. Transformed cells were screened, and methanol was used to induce expression. SDS-PAGE and western blotting were used to verify the successful expression of myostatin propeptide with biological activity in *Pichia pastoris*, providing the basis for characterization of this protein.

Key words: Myostatin propeptide; Recombinant plasmid; Biological activity

INTRODUCTION

McPherron et al. (1997) reported that a novel transforming growth factor called myostatin (MSTN), cloned from mouse skeletal muscle cDNA library, inhibited skeletal muscle growth (McPherron et al., 1997). Increased level of MSTN propeptide inhibits or affects the biological activity of MSTN (Thies et al., 2001; Hill et al., 2002; Zimmers et al., 2002; Lee, 2004). Yang et al. (2001) reported that transgenic mice with myostatin precursor protein overexpression showed a 20-110% increase in body weight, but bred normally without other features Lee et al. (2011) recombined rockfish myostatin propeptide with maltose binding protein expressed in *Escherichia coli* to obtain soluble rockfish myostatin propeptide. Wolfman et al. (2003) found that Asp-76 on the mouse myostatin peptide was the specific cleavage site of metalloproteinase BMP-1/TLD. After cleavage by metalloproteinase, MSTN precursors can release the c-terminal dimer, which binds to its specific receptor, and activates downstream signaling. After Asp is mutated to Ala, the propeptide can still bind to the C-terminal dimer in the form of potential polymer, but cannot be hydrolyzed by the BMP-1/TCD protein. Injecting the mutated propeptide into mice resulted in a muscle mass increase of 25-30% (Wolfman et al., 2003).

Pichia pastoris is an expression host that has been rapidly developed in recent years (Hamilton et al., 2006; Li et al., 2014). *P. pastoris* has many advantages over the *E. coli* system in RNA cleavage, protein folding, and glycosylation. Furthermore, it also has efficient expression, high yields, and easy purification compared with insect expression systems. In light these advantages, this study aims to express the myostatin propeptide protein in *P. pastoris*. The myostatin propeptide protein was cloned into the pPIC9K vector following codon optimization and mutation, and was expressed in the cell strain GS115. Our findings serveas a basis for further studies on the biological functions of myostatin propeptide.

MATERIAL AND METHODS

Bacterial strains and plasmids

The pPIC9K plasmid and the *P. pastoris* yeast strain GS115 were purchased from Invitrogen (Waltham, MA, USA). *E. coli* DH5α was used for genetic engineering and CMCC44102 was used as the indicator bacteria.

Enzymes and biochemistry reagents

*Eco*RI, *Not*I, *Sac*I, and T4 DNA ligase and lysis buffer were purchased from Bao Bioengineering, Co., Ltd. (Dalian, China). Taq DNA polymerase and ampicillin were products of Shanghai Hi-tech Bioengineering Technology, Co., Ltd. (China). Tryptone yeast extract was purchased from Oxoid (Nepean, Canada). The centrifugal filter units with molecular weight cut-offs of 3 and 10 ku were purchased from Millipore. Yeast nitrogen base (YNB) was purchased from Sigma (St. Louis, MO, USA).

Media

YPD, MD, MM, BMGY, and BMMY media were all prepared according to the recipe in the Invitrogen *Pichia pastoris* expression instruction.

Expression of myostatin propeptide in *Pichia pastoris*

Optimization and mutation of myostatin propeptide codon

We designed the optimized myostatin propeptide genetic codon using the primer 5.0 software, according to the sheep myostatin cDNA coding sequence (Gal-2 Accession No. NM-001009428) reported in NCBI as well as the, and mutated Asp-76 into Ala. Appropriate restriction sites for the enzymes *Eco*RI and *Not*I as well as protecting bases were added at both ends of the gene. Gene synthesis and T vector cloning were performed by Shanghai Bioengineering Co., Ltd.

Construction of pPIC9K-Msp recombinant plasmid

The pPIC9K and pMD18-T-Msp vectorswere double digested with *Eco*RI and *Not*I, respectively. Ligation products were transformed into competent DH5α cells. Transformed cells were amplified by colony PCR using the primers 5'AOX1 (5'-GACTGGTTCCAATTGACAAGC-3') and 3'AOX1 (5'-GCAAATGGCATTCTGACATCC-3'). Positive clones were screened and plasmids possessing correct PCR products were sent to Shanghai Bioengineering Technology Co., Ltd. for sequencing. The recombinant plasmid was named pPIC9K-Msp.

Electrotransformation of Pichia pastoris GS115

P. pastoris competent cells GS115 was prepared following the manufacturer's guidelines (Invitrogen). The linear recombinant expression plasmid was cut by *Sal*I, and was transformed electronically into GS115. Transformation solution (200 µL) was applied on a MD plate without histidine, and cultured for 3 days at 30°C to screen positive colonies. Single colonies were picked up using sterile pipet tips from the MD plate and placed into YPD media. PCR was performed for colony identification using 1µL lysed supernatant as template. Primers for 5'AOX1 and 3'AOX1 were used to screen for successfully transformed cells. Sterile water was used as blank control.

Induction and expression of recombinant yeast transformant

Positive yeast transformants, which were successfully transformed with pPIC9K-Msp and the empty vector pPIC9K, were inoculated into a 250 mL bottle containing 50 mL BMGY growth media. Yeast was cultured at 30°C and 250 r/min. They were then collected and induced at 28°C for 4 days in 100 mL BMMY media. During induction, samples were collected every 24 h and methanol was added to maintain the anhydrous methanol level at 5 mL/L. Samples were centrifuged at 12,000 *g* for 2 min. Supernatants were concentrated 10-folds and gene expression was verified by Tricine-SDS-PAGE and western blotting as described previously (Zhang et al. 2014).

RESULTS

Detection of myostatin pro-peptide gene sequence

Shanghai Bioengineering Co., Ltd. performed the sequencing analysis, and confirmed the length to be 762 bp, which was identical to the sequence as expected.

Restriction enzymedigestion and identification of recombinant plasmids

The successfully transformed pPIC9K-Msp recombinant plasmid was digested by *Sal*I. The plasmid fragment was amplified, and PCR products were visualized on a 1% agarose gel. A 10,000-bp band was seen, which was in line with our expectations, as the vector was 9267 bp in length and the target gene was 762 bp in length. The results show that the construction of the recombinant pPICK9K-Msp plasmid was successful (Figure 1).

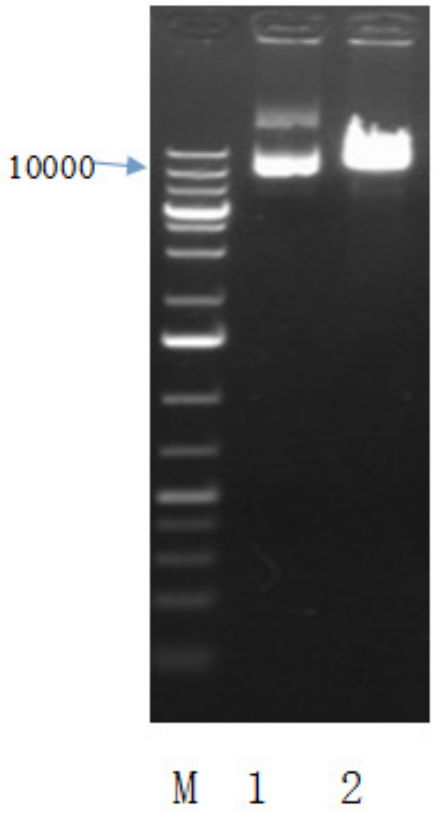

Figure 1. PCR products following Sal I digestion *Lane M*: DNA ladder; *lane 1*: non-linear plasmid; *lane 2*: 10,029 bp linear fragment after *Sal*I digestion (vector 9267 bp + gene of interest 762 bp).

PCR detection of recombinant plasmid pPIC9K-Msp

Primers targeting the yeast *AOX1* gene were used during PCR amplification. Two bands were amplified because most of the transformants were the Mut^+ type, and lacked the *AOX1* gene to replace that from the wild type GS115 gene. The transformed plasmid contained one wild type GS115 *AOX1* gene, about 2200 bp, and a copy of the recombinant integrated target gene, about 1160 bp. PCR results further demonstrated that the transformants were methanol dependent wild type Mut^+(Figure 2).

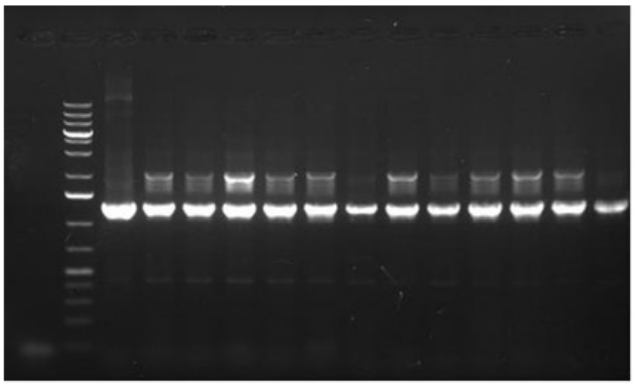

Figure 2. PCR detection of recombinant plasmid pPIC9K-Msp using the AOX primer. Recombinant plasmids were amplified by standard PCR amplification system, with reaction volumes of 15 µL. *Lane M*: DNA ladder; Positive: pPIC9K-MSP plasmid was used as template; Negative: sterile water was used as control; *lanes M1-M13*: GS115/pPIC9K-MSP transformants.

Target protein quantification by SDS-PAGE and western blot

After 48 and 72 h induction, samples were analyzed by SDS-PAGE electrophoresis, and specific bands were visualized at approximately 28 kDa. This is the theoretical size of the target protein, MSP. Following glycosylation, the protein would become larger than its theoretical size. This correlates with the two clear bands shown in the SDS-PAGE results in Figure 3. As indicated in the western blot, two significant hybridization bands were present, while in control samples the hybridization bands were negative (Figure 4). These results indicate that the MSTN propeptide was successfully expressed in the yeast culture supernatants.

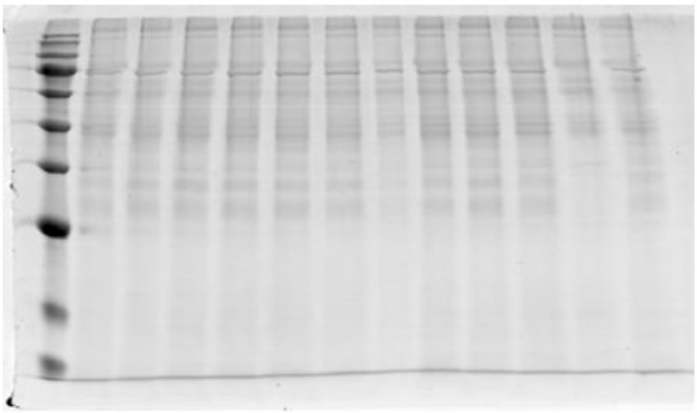

Figure 3. SDS-PAGE analysis of pPIC9K-MSP expression in recombinant *Pichia pastoris. Lane M*: ladder; *lanes M1-M13*: GS115/pPIC9K-MSP transformants.

Figure 4. Western blotting analysis of pPIC9K-MSP expression in recombinant *Pichia pastoris*.

DISCUSSION

In this study, a *P. pastoris* system was used to express MSTN propeptide due to its many advantages. This eukaryotic expression system has been broadly used for exogenous expression of various proteins, and overcomes many of the short comings of the prokaryotic expression system. It has a lot of advantages in expression yield, biological activity and genetic stability: 1) the strong alcohol oxidase (AOX) promotor can induce abundant and stable expression of the exogenous protein; 2) target protein can be secreted into the extracellular space and processed by translational modification, such as glycosylation, formation of disulfide bonds, etc.; 3) protein expression is flexible, can be either intracellular or extracellular, with high density growth; 4) the exogenous gene can be integrated into the yeast chromosome by homologous recombination, resulting in genetic stability; 5) *P. pastoris* has low levels of protein secretion, thus allowing the exogenous protein to become the dominant protein in the growth media; 6) *Pichia pastoris* has low levels of glycosylation compared with *Saccharomyces cerevisiae*. In *Pichia pastoris*, the sugar chain contains 8-14 mannose residues in each side chain, which is much shorter than the 50-150 mannose residues on each side chain in *Saccharomyces cerevisiae* (Han 2006; Liu et al., 2013; He et al., 2014).

Yeast expression systems exhibit codon usage bias. Rare codons, especially in concentrated areas of rare codons, rate of protein translation is restricted, and interferes with protein expression. This study performed codon optimization and modification on the myostatin propeptide gene, which was cloned into the pPIC9K vector, and successfully expressed in the GS115 strain. The successful construction of the pPIC9K-Msp recombinant plasmid was successfully expressed in GS115. However, expression yield of the target protein was relatively low. This may be due to several factors. First, characteristics of exogenous protein are the primary factor for determining the success or failure of protein expression. Secondly, physical and chemical characteristics of the exogenous protein, transformant phenotype, package expression or secretion induced expression, as well as the induction conditions can all affect the expression efficiency of target protein. Taken together, the factors influencing exogenous gene expression in *P. pastoris* are complex and diverse. At present, we successfully expressed pPIC9K-MSP in *P. pastoris* GS115, and the next step is to optimize protein expression to ensure that pPIC9K-Msp can be expressed in *P. pastoris* with high efficiency. This will provide a theoretical basis for the basic research and practical use of myostatin in treating muscle atrophy related disease as well as in animal husbandry.

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