



## Cloning and Prokaryotic Expression of the Mature Fragment of the Chinese Yellow Bovine Myostatin Gene

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**ABSTRACT :** Myostatin is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) super-family. It acts as a negative regulator for skeletal muscle growth. Myostatin mutations are characterized by a visible, generalized increase in muscle mass in double muscled cattle breeds. To understand the biochemistry and physiology of the Chinese Yellow bovine myostatin gene, we report here for the first time expression of the gene in *Escherichia coli* (*E. coli*). Primers of the myostatin gene of Chinese Yellow Cattle were designed on the basis of the reported bovine myostatin mRNA sequence (Gen-Bank Accession No. NM005259) and optimized for *E. coli* codon usage. *XhoI* and *EcoRI* restriction enzyme sites were incorporated in the primers, and then cloning vector and expression vector were constructed in a different host bacterium. The expressed protein had a molecule mass of about 16 kDa as determined by SDS-PAGE under reducing conditions. The expressed protein reacted specifically with myostatin monoclonal antibody on immunoblots. Our studies should lead to the investigation of the differences in myostatin genes of various cattle and could benefit human health and food animal agriculture. (**Key Words :** Prokaryotic Expression, Chinese Yellow Bovine, Myostatin Gene)

### INTRODUCTION

Myostatin is a member of the TGF- $\beta$  super-family. It is expressed almost exclusively in skeletal muscle and acts as a negative regulator for skeletal muscle growth. Like many other TGF- $\beta$  super-family member proteins, myostatin appears to be produced as a precursor protein composed of a signal sequence, a N-terminal pro-peptide domain (pro-domain), and a C-terminal mature domain (Zimmers et al., 2002). Based on the cDNA sequence of its gene, the mature form of myostatin consisting of 109 amino acids, appears to be formed upon removal of the N-terminal prodomain by proteolytic cleavage at a tetrabasic (Arg-Lys-Arg-Arg) site (Lee and McPherron, 2001). The amino acid sequence of the mature form of myostatin is identical among human, murine, rat, porcine, chicken, and turkey specie, and only a few amino acid differences exist in other mammalian species. This remarkable conservation through evolution suggests a common and highly conserved function (McPherron and Lee, 1997). Various studies indicate that

the mature form of myostatin forms a disulfide bond linked dimer similar to many other members of the TGF- $\beta$  super-family (McPherron et al., 1997; Lee and McPherron, 2001; Thies et al., 2001).

Myostatin is detected very early in the myotome of developing mouse (Zimmers, 2002) and cattle embryos (Lee and Mcpherron, 1997) and its expression continues in the muscle through the adulthood. It is located in the cytoplasm of muscle fibers and is absent from connective tissue (Thomas et al., 2000). In rats, myostatin protein levels are higher in the slow-fiber dominated muscles than in the fast-fiber dominated muscles (Taylor et al., 2001). In contrast, myostatin mRNA and protein level are higher in fast-fiber-dominated muscles than in slow-fiber-dominated muscles of mice (Gray, 1990) and rats (Israel et al., 1992). There appears to be no such difference in humans (Eder and Fersht, 1995).

Presently, intensive selection over many generations has resulted in a number of highly muscled cattle (McPherron and Lee, 1997), sheep (Cockett et al., 1994), and pigs (Brenig et al., 1992). In two breeds of double-muscled cattle, Belgian Blue and Piedmontese, mutations on the myostatin gene result in either the elimination of the complete protein or the substitution of a cysteine for a tyrosine residue (Grobet et al., 1997; Kambadu et al., 1997; Carole et al.,

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2002). This suggests that myostatin may act as a regulator of skeletal growth, bovine, because of extensive selection for large muscle mass, unlike mice (McPherron et al., 1997), may have associated genetic changes that allow for increase in muscling in a heterozygous animal. The Chinese Yellow Cattle is a famous traditional cattle breed and have also become major meat cattle in China. Most Chinese Yellow Cattle also have a double-muscle phenotype though the genetic basis to this phenotype has not been reported.

Although myostatin is predicted to be a negative regulator of skeletal muscle development, its role in fully developed skeletal muscle is puzzling. Myostatin knockout mice exhibit a 2-3 fold increase in skeletal muscle mass without any impact on the size of other organs as compared to wild-type mice (McPherron et al., 1997). Dysfunctional mutations in the myostatin gene are observed in double muscled cattle (Kambadur et al., 1997; McPherron and Lee, 1997; Grobet et al., 1998). In addition, systemic administration of myostatin induces significant muscle wasting in mice (Zimmers et al., 2002). Conversely, administration of antimyostatin antibody to adult mice significantly increases skeletal muscle mass (Whittemore et al., 2003). It has been suggested that the mutations in the gene might be utilized as markers for the double-muscled phenotype (Bellinge et al., 2005). Because no difference was found between double muscled and normally muscled cattle embryos or their adult muscles in terms of timing or level of expression myostatin (Grobert et al., 1997; Kambadur et al., 1997), mutations at the protein level were likely responsible for the phenotype in these breeds. Therefore, it is suggested that myostatin in muscle may be one of the long sought inhibitors that specifically control the growth of individual tissues or organs. Although the sheep (Rong et al., 2005) and swine (Ma et al., 2005) myostatin promoters cloned and sequenced, also recombinant myostatin of Piedmontese has expressed in *E. coli* (Thomas et al., 2000; Sasha et al., 2002), but expression protein of the Chinese Yellow Bovine myostatin gene *in vitro* had not reported yet.

## MATERIALS AND METHODS

### Amplification of myostatin cDNA

Total RNA was extracted from a Chinese Yellow bovine muscle using a commercial extraction kit (Trizol, Gibco-BRL, Rockville, MD) and mRNA was purified from the total RNA using an oligo (dT) column. Messenger RNA (100 ng) was used for oligo-dT primed reverse transcription with 200U SuperScript II RNase H reverse transcriptase (Gibco-BRL, Rockville, MD). In a 50  $\mu$ l reaction mixture, 5ul of the above reverse transcription reaction mixture and 0.2  $\mu$ M primers were mixed with 2 IU Taq polymerase mixtures (PCR Supermix High Fidelity, Gibco-BRL,

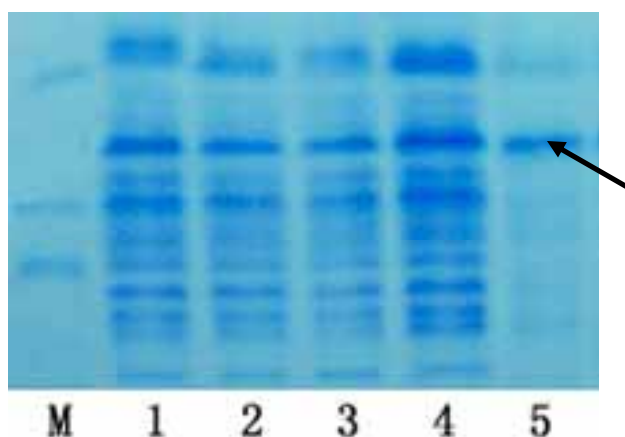
Rockville, MD) to amplify 369 bp of cDNA coding for the mature fragments of bovine myostatin. Thermal cycling parameters consisted of pre-amplification denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 2 min, and then a 5 min final extension at 72°C. Primers were designed on basis of the reported bovine myostatin mRNA sequence (Gen-Bank Accession No. NM005259) and optimized for *E. coli* codon usage *Xho*I and *Eco*RI restriction enzymes site were incorporated in the primers. The forward primer was 5'-CCGGGTACCGTAACAGACACCAAAA -3' and the reverse primer 5'-GAATTCATGAACACCCACAGCGATCTACT -3' corresponding to bases 760-1,125 bp of bovine myostatin mRNA sequences.

### Construction of myostatin expression vector

Amplified myostatin cDNAs were purified from PCR mixtures using a commercially available PCR purification kit (Amersham-Pharmacia, Piscataway, NJ) and subsequently inserted into a cloning vector (pCR T7/NT-TOPO, Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Recombinant DNAs were introduced by heat shock into competent *E. coli* for characterization of the construct and its propagation, and maintenance. Clones with correct insertions were identified using PCR and restriction analysis of plasmids carrying the recombinant cDNAs. After the initial screening, plasmids of selective clones were subjected to DNA sequence analysis for the final selection of an expression plasmid carrying myostatin sequence that was 100% homologous to the bovine sequence reported in the literature.

### Expression and Purification of recombinant myostatin

The plasmid carrying the myostatin sequence was isolated and introduced by heat shock into expression competent *E. coli* harboring the lambda DE3 (BL21) lysogen which carries the T7 RNA polymerase under the control of the lac UV5 promoter. The transformation mixture (250  $\mu$ l) was added to LB medium (10 ml) containing 100  $\mu$ g/ml ampicillin and 34  $\mu$ g/ml chloramphenicol for overnight growth at 37°C with vigorous shaking. This culture was used to inoculate 200 ml of prewarmed LB medium containing 100  $\mu$ g/ml ampicillin and 34  $\mu$ g/ml chloramphenicol. The expression of the recombinant myostatins was induced when the culture reached by adding 1 mmol/L isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) until the final concentration was 0.2 mmol/L (lane 4), 0.4 mmol/L (Figure 1, lane 3), 0.6 mmol/L (Figure 1, lane 2), 0.8 mmol/L (Figure 1, lane 1), respectively, an OD600 of 0.6-0.7. The culture was then incubated for 5 h at 37°C. The expressed mature myostatin protein was purified from *E. coli* using affinity



**Figure 1.** The expression and purification of the expressed bovine mature myostatin protein. The plasmid was constructed by carrying the 100% homologous myostatin sequence and introduced by heat shock into expression competent *E. coli* harboring the lambda *DE3* (*BL21*) lysogen that carries the T7 RNA polymerase under the control of the lac UV5 promoter. The expression of the recombinant myostatins was induced when the culture reached by adding 1 mmol/L isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) until the final concentration was 0.2 mmol/L (lane 4), 0.4 mmol/L (lane 3), 0.6 mmol/L (lane 2), 0.8 mmol/L (lane 1), respectively, an OD600 of 0.6-0.7. The maximum expression amount condition was when IPTG final concentration was 0.2 mmol/L, inducing times was 5 h, inducing temperature is 37°C (lane 4) in this study. The expressed mature myostatin protein from *E. coli* was purified using affinity chromatography in nickel columns according to standard procedures (lane 5) (Svend et al., 1989).

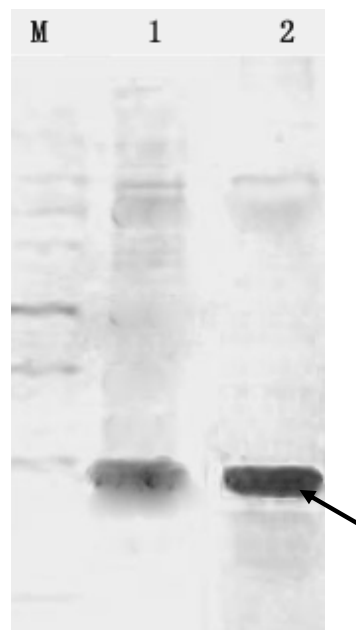
chromatography on nickel columns according to standard procedures (Svend et al., 1989).

#### SDS-PAGE

SDS-PAGE was performed as the method of Laemmli (Laemmli, 1970). Samples were mixed with loading buffer in the presence or absence of 1.5%  $\beta$ -mercaptoethanol and boiled for 5 min before loading. After electrophoresis, bands were visualized by Coomassie brilliant blue staining.

#### Western blotting

Myostatins fractionated by 12% SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane. After blocking with TBST (100 mM Tris-HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20), the membrane was incubated with a monoclonal anti-myostatin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200 for 1 h at room temperature. After washing with TBST, the membrane was incubated with a 1:5,000 dilution of rabbit anti-goat IgG antibody (Sigma-Aldrich) conjugated with alkaline phosphatase for 1 h at room temperature, followed by color development with the BCIP/NBT substrate (Sigma,



**Figure 2.** Western blotting assay. Myostatins fractionated by SDS-PAGE were transferred to a nitrocellulose membrane electrophoretically. After blocking, the membrane was incubated with monoclonal anti-myostatin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) raised against a peptide region of the mature form of myostatin and then subsequently incubated with secondary antibodies conjugated with alkaline phosphatase, followed by color development with the BCIP/NBT substrate. Lane 1 showed the un-purified myostatin protein and lane 2 showed the purified myostatin protein. Arrowhead indicates the mature fragment of myostatin protein.

St. Louis, MO).

## RESULTS

#### Expression and purification of recombinant mature bovine myostatin

The expression of mature myostatins is shown in Figure 1. Typically, 5 hours incubation after IPTG addition was enough to induce maximum expression. The expressed recombinant myostatins were designed to consist of 369 bp mature myostatin sequence, thus the estimated molecular weights of the mature myostatins was around 15.6 kDa. On SDS-PAGE under induced conditions, the recombinant myostatins migrated as a band at about 16 kDa (Figure 1, lane 4). The expressed mature myostatin protein from *E. coli* was purified using affinity chromatography in nickel columns according to standard procedures (Figure 1, lane 5). We also optimized expression conditions such as IPTG concentration, inducing time, inducing temperature, medium and vector. We got the maximum expression amount when the *BL21* (*DE3*) was host bacterium, the concentration of IPTG was 0.2 mM/L, inducing time was 5 hours, inducing temperature was 37°C (Figure 1, lane 4).

### Western blotting analysis

The expressed myostatin protein was analysed and immunoblotted using a commercially available monoclonal antiserum directly against the furin-digested products of the mature fragment of bovine myostatin (Figure 2). The 16 kDa of the expressed protein was recognized by the antiserum. Lane 1 of the Figure 2 shows the un-purified myostatin protein and lane 2 of the Figure 2 shows the purified myostatin protein. The arrowhead indicates the mature fragment of myostatin protein.

### DISCUSSION

Myostatin gene was discovered by studying transforming growth factor- $\beta$  (McPherron et al., 1997) which can control muscle increase and is a negative regulator. Although the phenotype of myostatin deficient animals hypothesis, formulated in the 1,960s that myostatin may be one of the specific growth inhibitors of muscle development, several questions remain exact mechanism of myostatin function (Kocamis and Killefer, 2002). First, does myostatin circulate in the blood? If so, do binding proteins in the TGF- family such as follistatin and Noggin regulate its activity in the circulation or does it have its own specific binding proteins? Second, can myostatin inhibit the growth of skeletal muscle in adult animals? If so, is it dose-dependent? Third, is myostatin involved in highly muscled callipyge sheep (Cockett, 1994), Pietrain pigs (Brenig and Brem, 1992; Li et al., 2002; Sun et al., 2002) or chicken (Moon, 2005)? Fourth, how does myostatin interact with the growth factors that have been well documented to stimulate skeletal muscle growth? A complete understanding of the biochemistry and physiology of myostatin could be beneficial to human health and food animal agriculture (Nara et al., 2001).

Presently, scientists have used gene cloning, expression techniques, gene knock out and trans-gene technique to decrease or deactivate the role of myostatin *in vitro*, thereby encouraging skeletal muscle growth and elevating the animal productivity (Grobet et al., 1998; Hamrick et al., 2003; Gregory, 2004).

Some studies indicate that careful validation of binding characteristics of anti-myostatin antibodies is essential in using anti-myostatin antibodies to determine the expression protein of myostatin *in vivo* (Lee and McPherron, 2001). Other studies demonstrate that highly efficient refolding *in vitro* is possible for the unprocessed porcine myostatin, but not for the mature form of myostatin expressed in *E. coli* as inclusion bodies, and that the myostatin prodomain facilitates the folding of mature myostatin (Hyung-Joo Jin, et al., 2004).

In this study, we firstly cloned cDNA coding for the mature fragments of the Chinese Yellow bovine myostatin

in an expression vector. We then optimized expression conditions such as IPTG concentration, inducing time, inducing temperature, medium and vector. A 16 kDa of inducing expression protein could show by using SDS-PAGE (Figure 1). It was also confirmed by western blotting that the expressed protein is the mature fragment of bovine myostatin (Figure 2).

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