SOLUBLE EXPRESSION OF PORCINE MYOSTATIN PROPEPTIDE IN AN *ESCHERICHIA COLI* EXPRESSION SYSTEM USING MALTOSE BINDING PROTEIN AS A FUSION PROTEIN

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ABSTRACT

Myostatin (MSTN) is a potent negative regulator of skeletal muscle mass. The activity of MSTN is inhibited by MSTN propeptide, the N-terminal fragment of unprocessed MSTN cleaved off during post-translational MSTN processing, indicating the potential of MSTN propeptide to enhance skeletal muscle growth in livestock. The objective of this project was to produce bioactive wild-type porcine MSTN propeptide (pMSTNProW) and its mutated form at the cleavage site (pMSTNProM) in *E. coli*. The *pMSTNProW* and *pMSTNProM* genes were separately cloned into pMAL-c5X vector downstream of the *maltose-binding protein* (*MBP*) gene, and were transformed and expressed in *E. coli* Top10F' strain. The MBP-pMSTNPro proteins were purified by the amylose-resin affinity chromatography, followed by the anion exchange chromatography. For each mL of cell culture, about 38.7 ug of soluble MBP-pMSTNProW protein and 37.0 ug of soluble MBP-pMSTNProM protein were purified by amylose-resin affinity chromatography. By using anion exchange chromatography, about 10.4 ug of MBP-pMSTNProW protein and 9.3 ug of MBP-pMSTNProM protein per mL cell culture were recovered and further purified. MBP was removed from the pMSTNPro proteins by Factor Xa digestion. Approximately 4.2 ug of pMSTNProW and pMSTNProM proteins were purified per mL of cell culture. Western blot analysis further confirmed the soluble expression of pMSTNPro proteins. Bioactivity tests verified the MSTN-suppressing capacity of six different forms of pMSTNPro proteins *in vitro*. All the pMSTNPro proteins demonstrated a similar tendency on inhibiting MSTN activity, indicating they were bioactive and their inhibitory effects on MSTN activity were dosage-dependent. Further studies are needed to examine the potency and binding characteristics of different

forms of the pMSTNPro proteins and the interaction between metalloprotease and MBP-pMSTNPro proteins. The results of this study demonstrate that a large production of pMSTNPro is possible using an *E. coli* system to study the potential of pMSTNPro to improve muscle growth in pigs.

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LITERATURE REVIEW

1.1 Transforming growth factor β superfamily

The transforming growth factor β (TGF-β) superfamily is a group of secreted growth and differentiation factors (GDF) (McPherron and Lee, 1997). The members of this superfamily are involved in a variety of biological functions including development, differentiation, reproduction, immune responses, bone growth, apoptosis, tissue homeostasis and regeneration (Kolodziejczyk and Hall, 1996; McPherron et al., 1997; Hoodless and Wrana, 1998; Schulz et al., 2008).

1.2 Myostatin (MSTN)

Myostatin (MSTN), also known as GDF-8, is a member of the TGF-β superfamily which plays an important role in regulating of skeletal muscle growth and development (Kambadur et al., 1997; McPherron and Lee, 1997). Hypermuscularity caused by natural mutations on the *MSTN* gene has been identified in several mammalian species, including cattle (Kambadur et al., 1997; McPherron and Lee, 1997), dogs (Shelton and Engvall, 2007), sheep (Hadjipavlou et al., 2008), and human (Schuelke et al., 2004). The amino acid sequence of the carboxy-terminal of MSTN is highly conserved across species like mouse, human, cattle, pig, dog, chicken, fish, and mollusc, which suggests a potentially common and conserved function of MSTN on inhibiting skeletal muscle growth (McPherron et al., 1997; Hu et al., 2010b).

MSTN is expressed during embryonic, fetal, and postnatal stages of skeletal muscle development, suggesting its crucial role in myogenesis (Kambadur et al., 1997;

McPherron et al., 1997). A deletion of the C-terminal region of the *MSTN* gene in mice resulted in significant increases in skeletal muscle mass throughout the body due to both hyperplasia and hypertrophy (McPherron et al., 1997). Additionally, loss of functional MSTN increased muscle regeneration by enhancing satellite cell activation and self-renewal, thus promoting muscle growth and repair during postnatal stage (McCroskery et al., 2003; McCroskery et al., 2005). Conversely, overexpression of MSTN in skeletal muscle resulted in a decreased muscle mass and fiber size and increased fat content (Reisz-Porszasz et al., 2003). Increased expression of MSTN has also been associated with atrophic conditions like sarcopenia (Leger et al., 2008; Gonzalez-Freire et al., 2010). Hence, MSTN plays important roles in regulating muscle hypertrophy, regeneration and atrophy.

1.3 Genetics of MSTN

MSTN gene has been highly conserved across species through evolution (McPherron et al., 1997). *MSTN* gene of cattle was mapped to chromosome 2q11, which contains the *mh* locus for muscular hypertrophy (Smith et al., 1997) and is syntenic to the region of human *MSTN* gene at chromosome 2q32.2 (McPherron and Lee, 1997). *MSTN* gene was also mapped to chromosome 7p11 in chicken (Sazanov et al., 1999), chromosome 37q11 in dog (Grzes et al., 2009), and chromosome 15q2.3 in porcine (Sonstegard et al., 1998).

Naturally occurring muscular hypertrophy in animals is associated with various forms of *MSTN* gene mutations including missense and nonsense mutations, deletions in the coding region, splice site mutations, or mutation in the 3'-untranslated region (UTR) of the gene, thereby supporting the important role of the *MSTN* gene in skeletal muscle growth and development (Thomis et al., 2004; Bellinge et al., 2005; Clop et al., 2006; Mosher et al., 2007; Boman et al., 2009). However, since no functional MSTN mutation has been identified in pigs, the role of *MSTN* gene underlying muscular differences in pigs is still unclear (Jiang et al., 2002a; Stinckens et al., 2005).

A complete porcine *MSTN* gene has a size of 7626 bp (Stinckens et al., 2009), consisting of three exons with sizes of 373, 374 and 381 bp, and two introns with sizes of 1808 and 1977 bp (Stratil and Kopecny, 1999). The porcine *MSTN* gene was expressed as two forms of mRNA (i.e. 1.5 and 0.8 kb) in skeletal muscle while the highest expression levels were found during prenatal stage and were associated with low birth weight (Ji et al., 1998). A unique expression pattern was also found in the skeletal and extraocular muscle in porcine during prenatal and postnatal growth (Patruno et al., 2008). For example, a higher mRNA level of MSTN was shown in skeletal muscles during the prenatal stage than the postnatal stage, whereas extraocular muscles maintain higher levels of MSTN mRNA than skeletal muscles even during the adult stage (Patruno et al., 2008).

Several polymorphisms have been detected in the porcine *MSTN* gene, including three in the promoter region, two in exons 2 and 3, five in intron 1, and seven in intron 2 (Jiang et al., 2002b; Li et al., 2002; Stinckens et al., 2005; Stinckens et al., 2009). Particularly, polymorphisms in the promoter region of the *MSTN* gene were associated with certain production traits in pigs, along with their effects on transcription factor binding and MSTN mRNA expression level (Jiang et al., 2002a; Liu et al., 2010). For example, two single-nucleotide polymorphisms (SNPs), g.435G>A and g.447A>G, were related to early growth and meat quality traits in pigs (Jiang et al., 2002b; Yu et al., 2007).

Meanwhile, the SNP at site 447 of the porcine *MSTN* promoter also affected the expression levels of the *MSTN* gene in the longissimus dorsi muscle in pigs at 4 weeks of age (Stinckens et al., 2009). However, the differences in MSTN expression levels do not cause the changes in muscle traits probably due to the presence of other genetic and nutritional factors (Stinckens et al., 2009; Liu et al., 2011).

1.4 Expression of MSTN

At the early stages of embryogenesis, MSTN is only expressed in the myotome compartment of developing somites (McPherron et al., 1997). During the later stages of embryogenesis and adult stages, MSTN is expressed in different muscles throughout the body (McPherron et al., 1997). While MSTN mRNA is mostly expressed in developing skeletal muscle of embryo and adult animals, it has also been reported to be expressed in other tissues including myoblast and fibroblast in regenerating skeletal muscle (Yamanouchi et al., 2000), cardiomyocytes and purkinje fibers of heart (Sharma et al., 1999; Shyu et al., 2005), spleen (Lyons et al., 2010), extraocular muscle (Toniolo et al., 2005; Patruno et al., 2008), mammary glands (Ji et al., 1998; Hosoyama et al., 2006) and, to a lesser extent, in adipose tissue (McPherron et al., 1997). In contrast with mammals, fish expresses MSTN mRNA in muscles, eyes, gill filaments, spleen, ovaries, gut, brain and, at a lower level, in testes (Maccatrozzo et al., 2001; Rodgers and Weber, 2001).

Apart from the types of tissues, muscle fiber type is also associated with different expression levels of MSTN mRNA. Muscle fiber types are classified based on their metabolic and contractile properties (Schiaffino and Reggiani, 1996). For example, the fast contracting fiber has higher concentration of glycogen, produces energy via glycolytic metabolism, but it has low resistance to fatigue; whereas the slow contracting fiber has higher concentration of mitochondria, produces energy via oxidative metabolism, and fatigue slower (Schiaffino and Reggiani, 1996). A majority of MSTN mRNA was expressed in fast muscle fibers in mice (Carlson et al., 1999). In fish, MSTN mRNA was mainly expressed in red muscles in species like brook trout (Roberts and Goetz, 2003) whereas the expression was high in white muscles in little tunny (*Euthynnus alletteratus)* (Roberts and Goetz, 2001). The expression level of MSTN mRNA is also related to the percentage of various myosin heavy chain (MHC) isoforms that is usually used as a marker of muscle fiber type (Carlson et al., 1999). For example, the MSTN mRNA expression level is associated with the expression of MHC isoform IIB in muscle (Carlson et al., 1999). MHC-IIB was absent in limb and trunk muscles, but was present in specialized eye muscles (rectus lateralis and retractor bulbi) (Maccatrozzo et al., 2004). Due to the presence of MSTN in latent form, the actual level of MSTN signaling might not be correctly reflected by the expression level of MSTN mRNA and protein under different physiological conditions or environmental stimuli (Lee, 2004).

1.5 Biosynthesis and biochemical characteristics of MSTN

MSTN is synthesized as a precursor protein of about 52 kDa with 375 amino acids (376 amino acids in rodents) encoded from a single open reading frame (McPherron et al., 1997). Like other members of the TGF-β superfamily, MSTN precursor protein contains an N-terminal signal sequence for secretion, a dibasic proteolytic processing site, and a carboxy-terminal region with nine cysteine residues, one of which contributes to the formation of a disulphide-linked MSTN dimer with a size of 25 kDa soon after translation (McPherron et al., 1997). It is assumed that the N-terminal propeptide aids in proper folding of the C-terminal region of MSTN into cysteine knot structure (Thomas et al., 2000; Jin et al., 2004). Like other members of the TGF-β superfamily, the MSTN homodimer is regarded as the biologically active form and possesses receptor-binding activity as shown *in vitro* studies (Lee and McPherron, 2001; Thies et al., 2001; Rebbapragada et al., 2003). However, due to the similarity with other members of the TGF-β superfamily, we cannot rule out the possibility that MSTN might form heterodimers with the other family members as well.

MSTN precursor protein is post-translationally processed twice by proteinases to form the biologically active molecule. First, the 24-amino acid N-terminal signal peptide is removed from the precursor protein by furin family enzymes at the Golgi apparatus (Lee, 2004; Rodino-Klapac et al., 2009; Huang et al., 2011). Second, a proteolytic cleavage at an RSRR (Arg-Ser-Arg-Arg) site positioned at amino acids 240-243 results in an N-terminal propeptide and a C-terminal mature MSTN with nonglycosylated molecular masses of 27,680 and 12,400, respectively. Due to the dibasic nature of the amino acids at the cleavage site, it is speculated that the responsible proteinase might be a member of the substilisin-like serine proteinases like furin (Wise et al., 1990). Indeed, a soluble form of furin was able to cleave the MSTN precursor protein completely at the RSRR site *in vitro* (Lee and McPherron, 2001; Thies et al., 2001; Jin et al., 2004). However, the true identity of the proteinase responsible for the second cleavage in vivo remains unknown.

After the two proteolytic cleavages, propeptide remains bound to the mature MSTN homodimer non-covalently in a latent complex, which is then secreted into the extracellular matrix of muscle (McPherron et al., 1997; Lee and McPherron, 2001; Thies et al., 2001). The propeptide keeps the C-terminal dimer inactive and prevents it from binding to the corresponding receptors of the responsive cells (Lee and McPherron, 2001; Thies et al., 2001). Particularly, native MSTN circulates in serum as a latent complex, in which over 70% of the latent form of MSTN is bound to its propeptide (Hill et al., 2002).

In order to activate the MSTN dimer, it is necessary to dissociate the propeptide from the latent complex of MSTN. This latent complex can be artificially activated by heat or acid treatment *in vitro* (Zimmers et al., 2002; Wolfman et al., 2003). Alternatively, members of the bone morphogenetic protein-1/tolloid (BMP-1/TLD) family of metalloproteinases are able to cleave the propeptide immediately N-terminal to aspartate 75, thereby activating the MSTN dimer (Wolfman et al., 2003). BMP-1/TLD family of metalloproteinases are expressed in different tissues, including central nervous system (Takahara et al., 1994), osteogenic extracts of bone (Wozney et al., 1988), fibrillar collagen, cartilage, tendon, fibroblast, placenta, gastrula and extraembryonic tissues, and developing skeletal muscle (Scott et al., 1999). Indeed, all four members of the BMP-1/TLD family are capable of cleaving both the purified propeptide and the propeptide bound to the C-terminal dimer (Wolfman et al., 2003). A mutant form of propeptide (i.e. aspartate 76 was replaced by alanine) resistant to proteolysis by BMP-1/TLD metalloproteinases was found to significantly increase muscle mass when injected in mice (Wolfman et al., 2003), thus further demonstrating the importance of BMP-1/TLD metalloproteinases in regulating the latency of MSTN.

1.6 MSTN signaling pathway

After proteolytic cleavage of propeptide from the latent complex in the extracellular matrix (ECM), the mature MSTN dimer binds to either activin receptor type II A (ActRIIA) or ActRIIB, a family of serine/threonine kinase transmembrane receptors expressed in bone marrow-derived mesenchymal stem cells (Hamrick et al., 2007) and located on the surface of the target cells (Rebbapragada et al., 2003). An *in vitro* study reported that MSTN dimer has a higher affinity to ActRIIB (Lee and McPherron, 2001). The importance of ActRII receptors in regulating MSTN signaling *in vivo* was demonstrated in transgenic mice, which expressed a dominant-negative form of ActRIIB without the kinase domain specifically in muscle using a myosin light chain promoter and enhancer (Lee and McPherron, 2001). These transgenic mice showed significant increases in muscle mass through both hypertrophy and hyperplasia, which were comparable to the effect of MSTN knockout in mice (Lee and McPherron, 2001).

Upon binding of the ligand to the ActRIIB receptor, the activin type I receptor is phosphorylated and activated, which then initiates the intracellular signaling cascade through phosphorylation of the Smad2 and Smad3 proteins (Lee and McPherron, 2001). After oligomerization with a Co-Smad protein (Smad4), the Smad complex translocates from the cytoplasm into the nucleus, where it regulates the transcription of target genes like MyoD (Langley et al., 2002; Shi and Massague, 2003).

MSTN dimer can also bind to either ALK-5 or ALK-4 type I receptors (Rebbapragada et al., 2003). ALK-4 is capable to interact with ActRII receptors whereas ALK-5 has only shown interaction with TGF-βRII receptors (Massague, 1998). This MSTN signaling pathway via ALK type 1 receptors continues by activating the cascade of Smad proteins and translocating into the nucleus to control gene transcription (Liu et al., 2001)

1.6.1 Regulation of MSTN signaling

MSTN signaling is regulated by the I-Smads proteins, Smad6 and Smad7, or an ubiquitin-mediated proteasomal degradation pathway controlled by the Smad-ubiquitin regulatory factors (SMURFS) (Attisano et al., 2001). Particularly, Smad7 inhibits both TGF-β and MSTN signalings by a negative feedback mechanism (Zhu et al., 2004). Smad7 also enhances MyoD transcriptional activity, thereby promoting the differentiation and growth of muscle (Kollias et al., 2006). Smad7 transcription can be induced by MSTN, TGF-β and other growth factors to potentially function as a cross-talk mediator of the TGF-β signaling pathway with the other signaling pathways (Yan and Chen, 2011).

As a transcriptional co-repressor in TGF-β signaling, a nuclear protein c-ski can regulate the MSTN signaling *in vivo* by stabilizing the inactive Smad 2/3/4 complex on Smad/ski binding elements (Akiyoshi et al., 1999; Lee, 2004). Also, c-ski inhibits the activities of Samd2 and Samd3 proteins by directly blocking the histone deacetylase activity and their interaction with a transcriptional coactivator (Kobayashi et al., 2007). Since c-ski promotes myogenin transactivation via interacting with MyoD/MEF2 heterodimers directly, nuclear localization of c-ski is needed for the differentiation of myoblast (Kobayashi et al., 2007). Mice with *c-ski* gene knockout have shown dramatic reduction in muscle mass (Berk et al., 1997), whereas transgenic mice overexpressing c-ski specifically in muscle resulted in dramatic muscle hypertrophy (Sutrave et al., 1990). Thus, c-ski is a positive-regulator of muscle growth via blocking activities of Smad proteins in the MSTN signaling pathway (Lee, 2004). Both Smad7 and c-ski function as the intracellular negative feedback mechanisms for MSTN and potentially other TGF-β superfamily members through Smads proteins.

1.6.2 Cross-talk between MSTN and IGF-I signalings

IGF-1 is a strong mitogen for various cell types and stimulates myoblast proliferation, differentiation and cell cycle arrest (Tollefsen et al., 1989). Particularly, myoblast differentiation induced by IGF-I is mediated by increasing the activity of p21 (Lawlor and Rotwein, 2000). MSTN also activates p21 and stimulates cell cycle withdrawal of myoblast; however, unlike IGF-1, MSTN inhibits myoblast differentiation (Rios et al., 2002; McCroskery et al., 2003). In addition, MSTN enhances the activities of Akt (Morissette et al., 2009), MAPKs extracellular signal-regulated kinase (Erk)-1/2 (Yang et al., 2006), and c-Jun N-terminal kinase (JNK) (Huang et al., 2007). Inactivating Erk-1/2 and JNK prohibits MSTN from inhibiting myoblast differentiation (Yang et al., 2006). Both MSTN and IGF-I are myoblast survival factors, implying that MSTN activation of these components of a mitogenic pathway might be associated with its ability to keep cellular quiescence in growth-arrested cells (Rodgers and Garikipati, 2008). However, it remains unclear how MSTN activates these pathways and whether the cross-talk between MSTN and IGF signaling is important to muscle growth.

1.7 Physiological roles of MSTN

1.7.1 Roles in controlling myoblast proliferation, differentiation, and quiescence

MSTN expression is detected during embryonic, fetal, and postnatal stages of development, suggesting that it plays a crucial role in myogenesis (Kambadur et al., 1997; McPherron et al., 1997). During myogenesis, myoblasts proliferate and differentiate upon termination of the cell cycle at G1 phase to form myotubes (Olson, 1992). Myogenic regulatory factors (MRFs), probably stimulated by environmental signals, initiate the expression of muscle specific genes to control the growth and differentiation of myoblasts (Thomas et al., 2000). For example, MyoD and Myf-5 are required for the formation of myoblasts (Rudnicki et al., 1992); myogenin is needed for the differentiation of myoblasts (Hasty et al., 1993); MRF4 plays a role in the maturation of myotubes (Rawls et al., 1998). Myoblast cell cycle withdrawal and differentiation are regulated by cyclin-dependent kinases (Cdks) which is a family of enzymes essential for cell cycle transitions (Thomas et al., 2000). For example, G1 cyclin/Cdk complexes, cyclin-D/Cdk4 and cyclin-E/Cdk2 control the cell cycle transition from G1 to S by phosphorylation and inactivation of the retinoblastoma (Rb) protein (Guo and Walsh, 1997). The kinases' activities of the Cdks are regulated by the p16 and p21 families of cyclin-dependent kinase inhibitors (CKIs) (Thomas et al., 2000). Particularly, Cdk4 and Cdk6 are controlled by the p16 family, while all Cdks involved in G1/S transition are regulated by the p21 family (Sherr and Roberts, 1999).

MSTN inhibits myoblast growth, MRF activities and myogenic differentiation (Langley et al., 2004). Particularly, MSTN affects the expression of Myf5 and MRF4 expression in fast-type muscle fibers, as well as the expression of MyoD expression in slower-type muscle fibers (Muroya et al., 2009). Hence, MSTN has a muscle type-specific effect in skeletal muscle growth and maintenance (Muroya et al., 2009). In the presence of functional MSTN, the p21 level is increased while the Cdk2 protein level and activity are decreased, thus inactivating the Cyclin-E/Cdk2 complex (Thomas et al., 2000). The phosphorylation of Rb protein is suppressed and the cell cycle of myoblasts is arrested at the G1 phase (Thomas et al., 2000).

MSTN, as a myoblast survival factor, inhibits myoblast hyperplasia via promoting cell cycle withdrawal and delaying differentiation by cellular quiescence (Rios et al., 2001; McCroskery et al., 2003). MSTN induces reversible cellular quiescence and prevent apoptosis of the cells by down-regulating the expression of myogenic regulatory factors, including MyoD, myogenin, Pax-3 and Myf-5 (Langley et al., 2002; Rios et al., 2002; Amthor et al., 2006). Besides, MSTN inhibits protein synthesis in differentiated C2C12 myotubes (Taylor et al., 2001). The increased number of myofibers seen in animals with heavy muscling might be the result of enhanced proliferation and delayed differetiation of myoblast due to the absence of functional MSTN (Muroya et al., 2009). MSTN limits the size of muscle by suppressing the hyperplastic growth of developing myoblasts and limiting the number of cells that are differentiated into mature myofibers (Rodgers and Garikipati, 2008). Also, MSTN inhibits the hypertrophic growth of mature muscle by decreasing protein synthesis in myofiber and prevents satellite cell renewal (Rodgers and Garikipati, 2008). Hence, MSTN plays important roles in myogenesis through controlling myoblast proliferation, differentiation and cell cycle progression.

1.7.2 Roles in controlling the prenatal and postnatal growth of skeletal muscle

MSTN is a negative regulator of skeletal muscle growth and development (McPherron and Lee, 1997). A deletion of the *MSTN* gene in mice resulted in a dramatic increase in skeletal muscle mass via hyperplasia and hypertrophy (McPherron et al., 1997). Individual muscles in MSTN null mice weigh 2- to 3-fold more than those of wild-type mice, primarily due to an increase in muscle mass without a corresponding increase in the amount of fat (McPherron and Lee, 1997). *MSTN* gene deletion in adult mice had increased the mass of individual muscle by about 50% or more than the increase in muscle mass caused by the administration of MSTN inhibitors (Whittemore et al., 2003; Welle et al., 2007). Besides, MSTN null mice showed different proportions of fiber types compared with wild type mice. Like double muscling cattle, MSTN null mice have shown less slow oxidative fibers and a higher number of fast glycolytic fibers than wild type mice (Girgenrath et al., 2005). Thus, muscle hyperplasia, hypertrophy and a shift towards fast glycolytic type fibers in MSTN null mice might be resulted from the prenatal and or perinatal loss of MSTN functions (Zhu et al., 2000; McPherron, 2010).

MSTN not only regulates the types, sizes and numbers of muscle fibers during development, but it also regulates the postnatal growth of muscle fibers. Experimental protocols used to interfere the stages of development at which MSTN signaling is modulated appears to influence the development of muscular hyperplasia and/or hypertrophy (Lee, 2004). For example, transgenic mice overexpressing MSTN inhibitors, including propeptide, follistatin, or dominant-negative form of ActRIIB, under the control of a myosin light chain promoter/enhancer have shown both muscle hyperplasia and hypertrophy as seen in MSTN knockout mice (Lee and McPherron, 2001). However, the expression of a mutant form of MSTN in mice, in which a cysteine residue in the C-terminal portion was replaced by tyrosine under the control of a chicken β-actin promoter and a CMV enhancer, resulted in muscle hyperplasia without hypertrophy (Nishi et al., 2002). By contrast, muscle hypertrophy without hyperplasia can be seen in mice with MSTN inhibition during or after the neonatal stage (Whittemore et al., 2003; Girgenrath et al., 2005). All muscle fiber types, especially for the fast glycolytic type IIB fibers, are subjected to the hypertrophic effect due to MSTN inhibition (Foster et al., 2009; Matsakas et al., 2009). However, the shifts of fiber type as found in MSTN null mice do not happen if the MSTN is suppressed in normal adults (Morine et al., 2010). Transgenic mice expressing a dominant negative MSTN with a modified furin cleavage site, controlled by a muscle creatine kinase promoter, has shown hypertrophy without hyperplasia as seen in normal adult mice administered with MSTN antibodies (Zhu et al., 2000; Whittemore et al., 2003). Hence, the phenotypic effects of MSTN on muscle fiber type, size and number vary depending on the time at which MSTN signaling is blocked during the prenatal or postnatal stage (Lee, 2004).

1.7.3 Roles in suppressing satellite cell activation, proliferation and regeneration

In addition to myoblast, satellite cell is also a target for MSTN to regulate muscle fiber size in adult animals. Satellite cells are mononucleated muscle stem cells found between the sarcolemma and the basement membrane of muscle fiber (Bischoff, 1990). The negative effects of MSTN on muscle hypertrophy are partially due to the inhibition of satellite cell activation, proliferation and renewal since the postnatal muscle growth largely depends on the fusion of these satellite cells with the existing myofibers (Bischoff, 1990).

The decrease of fiber sizes by MSTN in adult animals might suggest an important role of MSTN on suppressing the proliferation and differentiation of satellite cell. Studies using isolated satellite cells have shown that MSTN increases p21 level, a cell cycle inhibitor, and decreases CdK2 level, a molecule involved in cell cycle progression, as well as inhibits satellite cell proliferation (McCroskery et al., 2003). After inducing muscle injury by notexin, the regenerated muscles of MSTN knockout mice have shown a larger number of satellite cells per unit length, a higher proportion of activated satellite cells, elevated decorin mRNA expression, decreased fibrosis and enhanced repairing compared with those of wild type mice (McCroskery et al., 2003; McCroskery et al., 2005). The satellite cells isolated from MSTN knockout mice also showed a higher proliferation rate than those from wild type mice (McCroskery et al., 2003). Compared with the untreated littermates, muscular dystrophy (*mdx*) mice expressing a mutant uncleavable MSTN propeptide via an adeno-associated virus-8 mediated intravenous gene transfer system showed an increase in the percent of fast glycolytic type fibers (Morine et al., 2010). These results indicate MSTN normally functions to keep the satellite cells in a quiescent state (Bischoff, 1990). Hence, loss of MSTN activity, by either genetic inactivation or functional antagonism, increases muscle regeneration via enhancing satellite cell activation and self-renewal, thus promoting postnatal muscle growth and repair (McCroskery et al., 2003; McCroskery et al., 2005).

Pax7, a sequence-specific DNA-binding transcription factor, plays pivotal roles in the development of central nervous system, neural crest, dermomyotome, and myotome, satellite cells and adult muscle regeneration (White and Ziman, 2008). Overexpression of Pax7 in C2C12 myogenic cells promotes satellite cell self-renewal by slowing both myogenic proliferation and differentiation (McFarlane et al., 2008). MSTN inhibits Pax7 expression by ERK1/2 signaling (McFarlane et al., 2008). Hence, MSTN negatively regulates satellite cell activation and self-renewal through a Pax7-dependent mechanism (McCroskery et al., 2003; McFarlane et al., 2008). However, another study indicated that the activation of satellite cells is irrelevant to the muscle hypertrophy by MSTN inhibition (Amthor et al., 2009).

1.7.4 Roles in adipose tissue metabolism and insulin sensitivity

In addition to muscles, MSTN appears to plays a metabolic role in regulating adipose tissue mass and insulin sensitivity under normal and obesity-inducing conditions. MSTN null mice have shown lower absolute fat mass than wild type mice, and the difference in adipose tissue deposition between the two genotypes increases with aging (Lin et al., 2002; McPherron and Lee, 2002). Transgenic mice expressing a dominant negative ActRIIB in skeletal muscle resulted in an increase of muscle mass and a reduction of body fat (Guo et al., 2009). MSTN null mice have shown a lower level of circulating leptin, a hormone derived from adipose tissue, than wild type mice, suggesting a reduction in adiposity (Lin et al., 2002; McPherron and Lee, 2002). Mice overexpressing MSTN propeptide specifically in skeletal muscle do not show as much increase in muscle mass as MSTN null mice, and have less decrease in adiposity (Zhao et al., 2005). Hence, reducing adiposity by inhibiting MSTN might, to some extent, depend on the degree of muscle hypertrophy.

Skeletal muscle normally shows a greater response to insulin to takes up more glucose than any other tissue, so enhancing insulin sensitivity of muscle is potentially useful to increase insulin sensitivity of the whole body and to prevent the onset of diabetes (DeFronzo and Tripathy, 2009). With normal levels of blood glucose and insulin, MSTN null mice have shown higher insulin sensitivity than wild type mice under the same standard diet (McPherron and Lee, 2002; Guo et al., 2009). MSTN null mice also have better control of blood glucose level during the tolerance tests of glucose and insulin (Guo et al., 2009). MSTN null mice needs more infused glucose in response to increased insulin level, suggesting that their muscles have higher response to insulin than those of wild type mice (Guo et al., 2009). After injected with insulin, higher activities of serine-threonine kinase Akt (a mediator of insulin signaling), IGF-I and other growth factors are detected in skeletal muscle and white/brown adipose tissue of MSTN null mice compared with wild type mice, suggesting an enhanced insulin signaling (Guo et al., 2009). MSTN prevents IGF-1 from activating Akt in skeletal muscle cells (Morissette et al., 2009). However, the function of MSTN in inhibiting insulin-induced activation of Akt remains unclear.

Besides of decreased adiposity and enhanced insulin sensitivity, MSTN null mice also improve the blood lipid profile, such as lowering serum cholesterol and triglyceride levels (McPherron and Lee, 2002; Guo et al., 2009). High levels of hepatic and intramyocellular lipid cause insulin resistance (Savage et al., 2007). The hepatic triglyceride level in MSTN null mice is lower than wild type mice, indicating an increase in insulin sensitivity by the loss of MSTN (Guo et al., 2009). Like MSTN null mice, transgenic mice overexpressing MSTN propeptide or a dominant negative ActRIIB specifically in skeletal muscle result in skeletal muscle mass increase and are resistant to weight gain on high-fat diet compared to wild type (Hamrick et al., 2006; Guo et al., 2009). Other metabolic effects of high-fat diet, including serum insulin, leptin, cholesterol, and triglycerides in MSTN null mice are ameliorated compared to wild type mice (Wilkes et al., 2009). Thus, the metabolic effects of high-fat diet are opposed by the deletion of the *MSTN* gene.

1.8 Inhibition of MSTN

There are several proteins found to bind to the mature MSTN dimer or the unprocessed MSTN dimer. For example, the MSTN propeptide keeps the MSTN dimer in an inactive complex in circulation (Lee and McPherron, 2001; Thies et al., 2001). Besides, latent TGF-β-binding protein-3 keeps the unprocessed MSTN in an inactive state in the extracellular matrix (Anderson et al., 2008). Follistatin, a secreted glycoprotein, inhibits MSTN and its interaction with ActRIIB (Patel and Amthor, 2005). Other proteins like growth and differentiation factor-associated serum protein 1 (GASP1) and follistatin-like related gene (FLRG) are also bound to MSTN in serum and prevents MSTN from binding to its receptors (Hill et al., 2002). Mice overexpressing the follistatin gene or the FLRG gene specifically in skeletal muscle have increased skeletal muscle mass (Lee and McPherron, 2001). However, it remain unclear which region of MSTN is bound to each of these inhibitors and whether MSTN is bound to multiple inhibitors as a single inactive complex.

1.8.1 MSTN propeptide - a natural inhibitor of MSTN

During biosynthesis of MSTN, the N-terminal propeptide plays a crucial role in the proper folding of the C-terminal region into a cysteine knot structure (Jin et al., 2004; Funkenstein and Rebhan, 2007). Jiang et al. (2004) have found that there is no intermolecular disulfide bond formation between two monomers of MSTN propeptide. Furthermore, the inhibitory activity of MSTN propeptide does not require disulfide bonding or N-linked glycosylation (Jiang et al., 2004). Both in vitro and in vivo studies have demonstrated the regulatory role of propeptide on the activity of MSTN C-terminal dimer. For example, the activity of MSTN C-terminal dimer was inhibited by propeptide in both receptor binding and reporter gene assays (Lee and McPherron, 2001; Thies et al., 2001). In addition, overexpression of MSTN propeptide increased muscle mass in transgenic mice model, which was similar to the phenotype found in MSTN knockout mice (Lee and McPherron, 2001; Yang et al., 2001). Particularly, the increased muscle mass seen in the propeptide transgenic mice was primarily due to myofiber hypertrophy, with a 60% increase of the size of fast-twitch glycolytic muscle fiber at 9 weeks of age compared with wild type littermates (Yang et al., 2001). An *in vivo* study using A204 rhabdomyosarcoma cells transfected with a $(CAGA)_{12}$ reporter construct shows propeptide inhibits the biological activity of MSTN (Thies et al., 2001). MSTN propeptide also inhibits specific MSTN binding to L6 myoblast cells (Thies et al., 2001). These results suggest MSTN propeptide acts as an inhibitor of MSTN biological activity (Thies et al., 2001).

The MSTN-inhibitory role of propeptide was demonstrated *in vivo* studies. For example, transgenic overexpression of the MSTN propeptide enhanced muscle mass in mice, which was similar to the phenotype found in MSTN knockout mice (Lee and McPherron, 2001; Yang et al., 2001). Administration of MSTN propeptide significantly increased muscle mass (Wolfman et al., 2003). Delivery of MSTN propeptide genes by an adeno-associated viral vector system or a plasmid-mediated system enhanced muscle growth in mice and dogs (Bartoli et al., 2007; Qiao et al., 2008a; Qiao et al., 2008b; Hu et al., 2010a), indicating the potential of MSTNPro as an useful pharmacological agent to enhance skeletal muscle growth in meat-producing animals.

1.9 The use of gene fusion during recombinant protein expression

Gene fusions have been broadly used to facilitate the expression and purification of foreign protein synthesized from recombinant DNA. Most commonly, the target protein is fused to a specific binding protein during expression, such that the fusion protein can be purified by affinity chromatography using substances that bind the fusion protein as an affinity matrix (Bassford et al., 1979). For instance, a target protein fused to the C-terminus of maltose binding protein can be theoretically purified in a one step procedure by amylose-resin affinity chromatography (di Guan et al., 1988).

Mammalian proteins can be expressed at high levels in prokaryotic cells like *E. coli*. However, the expressed protein is often biologically inactive due to misfolding and accumulating as inclusion bodies (Fahnert et al., 2004). Modification of the production process and protein engineering are possible ways to enhance the solubility of protein (Dalken et al., 2010). For example, some purified inclusion body proteins can be solubilized by chemical denaturation followed by refolding (Remmert et al., 2000; Jin et al., 2004; Funkenstein and Rebhan, 2007). This method, however, does not apply to all protein species and has a large variation between the yields of different soluble proteins (Dalken et al., 2010). On the other hand, the expression of target protein fused with a heterologous protein domain as a solubilizing agent is used to directly enhance the solubility of recombinant proteins (Uhlen et al., 1992). Several examples of the commonly used protein domains include DsbA (Collins-Racie et al., 1995), thioredoxin (LaVallie et al., 1993), ubiquitin (Power et al., 1990), glutathione-S-transferase (GST) from *Schistosoma japonicum* (Tudyka and Skerra, 1997), domain 1 of the translation initiation factor IF2 (Sorensen et al., 2003), protein A (Samuelsson et al., 1994), and the maltose binding protein (MBP) of *E. coli* (di Guan et al., 1988).

1.9.1 Maltose binding protein

Maltose binding protein (MBP), a product of the *malE* gene, is a well-known monomeric and periplasmic protein that involves in maltose transport in *E. coli* to bind maltose and maltodextrin (Bassford et al., 1979; Blondel and Bedouelle, 1990). MBP is synthesized from the ribosomes and is attached to the cytoplasmic side of the cytoplasmic membrane (Randall and Hardy, 1977). A majority of periplasmic MBP can be released from *E. coli* cells cultured in a maltose-minimal medium by cold osmotic shock method (Neu and Heppel, 1965). On the other hand, high yields of MBP can also be expressed in the cytoplasm (Ehrmann et al., 1998).

A plasmid vector was constructed to synthesize a high level (about 2% of total cellular protein) of MBP fusion protein in *E. coli* (Maina et al., 1988). A recognition sequence (Ile-Glu-Gly-Arg) for the blood coagulation factor Xa protease was inserted between the target protein and MBP (Maina et al., 1988). Factor Xa has a molecular weight of 42 kDa, consisted of two disulfide-linked units of 26.7 kDa and 15.7 kDa (Rodriguez and Carrasco, 1995). After the cleavage by factor Xa, the target protein can then be purified from the MBP domain by another affinity chromatography step (Maina et al., 1988).

di Guan et al. (1988) have suggested some advantages of using MBP as the fusion protein to express and purify foreign proteins: first, the purification step can be done under physiological conditions; second, the eluted protein from the amylose resin is not prone to be denatured; third, the fusion protein can be led into various cellular components by changing the leader sequence of MBP; forth, MBP does not carry any cysteine residues to interfere with the formation of disulfide bond within the target protein. Furthermore, MBP acts as a chaperone to promote proper folding of the fusion partner by temporarily preventing its fusion partner from forming aggregation prone folding intermediates and self association, thereby increasing the yield, solubility and stability of the target protein (Pryor and Leiting, 1997; Sachdev and Chirgwin, 1998; Lee et al., 2006; Sun et al., 2011). Hence, a soluble expression of MSTN propeptide protein may be promoted by fusion with MBP.

CHAPTER 2
OBJECTIVES OF THE STUDY

Previous studies have used eukaryotic culture systems for the production of bioactive MSTN propeptide (Thies et al., 2001; Wolfman et al., 2003; Jiang et al., 2004; Li et al., 2010). Because of the cost involved in the use of eukaryotic systems and potential infection by mammalian virus (Bakhshandeh and Shahrzad, 2009), the eukaryotic expression is not suitable to produce large amount of MSTN propeptide to examine its potential to improve muscle growth in meat-producing animals. While the use of an *E. coli* system would be an economic alternative, an earlier study reported an insoluble expression of MSTN propeptide in an *E. coli* system (Rebhan and Funkenstein, 2008). However, recent studies have shown that soluble expression of bioactive fish MSTN propeptide is possible in an *E. coli* system using maltose binding protein (MBP) as a fusion partner (Lee et al., 2010b; Lee et al., 2011). Therefore, the objectives of this study were (1) to express wild-type porcine MSTN propeptide (pMSTNProW) and its mutated form at the cleavage site (pMSTNProM) in *E. coli* using MBP as the fusion protein, and (2) to examine their MSTN-suppressing capacity *in vitro*.

CHAPTER 3
MATERIALS AND METHODS

3.1 Production of cDNAs of wild type porcine MSTN propeptide (pMSTNProW) and its mutant form (pMSTNProM)

The pMSTNPro-W cDNA sequence was amplified by 'touch down' PCR using unprocessed porcine MSTN plasmid constructs as a template. The production of unprocessed porcine MSTN plasmid constructs was reported previously (Jin et al., 2004). The forward and reverse primers used were 5'-AATGAGAACAGCGAGCAAAAGGA-3' and 5'- TTGATATC**TTA**TCTCCTGGATCTTTTTGGTGTGT -3', respectively. The reverse primer contained a stop codon (bold), followed by an *EcoRV* restriction site (underlined). A metalloproteinase-resistant mutant pMSTNProM (D75A) gene was generated by PCR-mediated mutagenesis using the Muta-Direct^{TM} Site-Directed Mutagenesis Kit (iNtRON Biotechnology, Seoul, Korea).

3.2 Construction and transformation of expression vectors

The amplified pMSTNProW and pMSTNProM fragments were digested separately with *EcoRV* and ligated into the *Xmn1* and *EcoRV* sites of pMAL-c5x vector included in the pMAL^{TM} Protein Fusion & Purification System (New England BioLabs, MA, USA). DNA sequence analysis was performed for correct insertion before transformation. The two expression plasmid constructs, MBP-pMSTNProW and MBP-pMSTNProM, were then introduced into competent *E. coli* (TOP 10F' strain) by electroporation. After initial screening by restriction digestion, plasmids of selective clones were subjected to DNA sequence analysis for the final selection of an expression plasmid. The above procedures were performed by Dr. Y. J. Choi's lab at the Seoul National University (Korea) while Dr. Y.S. Kim was visiting Dr. Choi's lab in the summer of 2009 for his collaborative work.

3.3 Expression of MBP-pMSTNPro fusion proteins in E. coli

The transformants were spread on Luria-Bertani (LB) (1.0% tryptone, 0.5% yeast extract and 0.5% NaCl) agar plates containing X-gal and 100 µg/ml ampicillin. Selected colonies were used to inoculate 5 mL LB medium containing 0.2% glucose and 100 µg/ml ampicillin for overnight growth at 37°C with vigorous shaking. The 5 mL cultures were used to inoculate 25 mL LB medium containing 0.2% glucose and 100 µg/ml ampicillin, and cells were grown to an OD_{600} of 0.7–0.8. To induce the soluble expressions of MBP-pMSTNPro fusion proteins, isopropyl-β-D-thiogalactoside (IPTG) was added to the cultures to a final concentration of 0.2 mM, and then the cultures were grown at 37°C for 4 hrs under vigorous shaking. SDS-PAGE analysis of the cell cultures was performed to confirm expression of the MBP-pMSTNPro fusion proteins. The samples with the highest quantity of the fusion proteins sized at 75 kDa were selected for large scale expressions (250 ml).

3.4 Affinity-purification of soluble MBP-pMSTNPro proteins

After induction, the cell pellets were harvested by centrifugation at 4,000 g for 20 mins at 4°C. Each gram (wet weight) of cell pellets was resuspended in 5 ml of column buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.5) added with the Complete Mini Protease Inhibitor Cocktail Tablet (Roche, Mannheim, Germany). The resuspended cell solution was frozen at -20 °C overnight. After thawing in cold water, the cells were lysed by sonication in short pulses of 15 seconds for 15 min in ice-water bath. The cell lysates were then centrifuged at 20,000 g for 20 mins at 4°C. For each sample, the soluble fraction was collected and the same volume of column buffer was used to resuspend the insoluble fraction. SDS-PAGE analysis of the cell lysates, soluble, and insoluble fractions was performed to verify the soluble expressions of the MBP-pMSTNPro fusion proteins. The soluble fractions were subjected to amylose resin (New England BioLabs, MA, USA) affinity chromatography following the procedure described previously (Lee et al., 2010b). Fractions were analyzed by SDS-PAGE and Western blot to examine and characterize the purified proteins.

3.5 Anion exchange chromatography-purification of MBP-pMSTNPro proteins

The elution fractions of each fusion protein were pooled and dialysed against low gradient buffer (20 mM Tris, 25 mM NaCl, 1 mM PMSF, pH 8.0) at 4 °C overnight with 2 changes of 100 volumes. The dialysed samples was filtered through a 0.22 μm syringe filter and applied to a Mono Q HR 5/5 column (GE Healthcare Life Science, NJ, USA) monitored by a BioLogic DuoFlow System (BioRad, CA, USA). After washing the column with 30 ml of low gradient buffer, a salt gradient of 25 mM NaCl to 500 mM NaCl in 20 mM Tris and 1 mM PMSF (pH 8.0) was used to elute the proteins collected in fractions of 8 ml and the absorbance was detected at 280 nm. Based on the anion exchange chromatogram, the fractions of the peaks were examined by SDS-PAGE analysis. The fractions containing the soluble MBP-pMSTNPro fusion proteins were pooled and concentrated by ultrafiltration (Millipore, MA, USA).

3.6 Separation and purification of the pMSTNPro proteins from the MBP fusion protein

The concentrated elution of each fusion protein was dialysed against Factor Xa buffer (20 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, pH 7.5) at 4 °C overnight with 2 changes of 100 volumes. After dialysis, the MBP-pMSTNPro proteins were cleaved by Factor Xa protease (New England BioLabs, MA, USA), for 24 hrs at 4 °C to release the pMSTNPro proteins from MBP. Every 1 ng of Factor Xa was used to digest 50 ng of the MBP fusion protein. The factor Xa digested proteins were then passed through the equilibrated amylose resin column to remove any MBP containing proteins from the samples. The pass-through was subjected to SDS-PAGE analysis to examine the removal of MBP fusion partner from the MBP-pMSTNPro proteins.

3.7 Protein Assay

Protein concentration was determined by the method of Bradford (Bradford, 1976) using a Bio-Rad Protein Assay Dye Reagent with bovine serum albumin as a standard.

3.8 SDS-PAGE

SDS-PAGE was performed with 12.5% polyacrylamide gels according to Laemmli’s method (Laemmli, 1970). Samples were mixed with loading buffer containing 1.0 % β-mercaptoethanol and boiled for 5 min before loading. Protein bands were visualized by Coomassie brilliant blue staining after electrophoresis.

3.9 Western blot

Western blotting was performed to characterize/identify the purified pMSTNPro proteins. Protein bands on the SDS-PAGE gels were electrophoretically transferred to

PVDF membranes. After blocking with TBST (100mM Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20) with 3% BSA, the membranes were incubated with polyclonal anti-MSTNPro (Kim et al., 2007) or monoclonal anti-MBP antibodies at a 1:10,000 dilution for 1 hr at room temperature. After washing with TBST, the membranes were incubated with a 1:20,000 dilution of goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody conjugated with alkaline phosphatase for 1 hr at room temperature, followed by color development with the BCIP/NBT substrate (Sigma, St. Louis, MO).

3.10 Bioactivity test for the pMSTNPro proteins using the CAGA-Luciferase reporter assay

A204 rhabdomyosarcoma cells (ATCC, HTB-82) were seeded in a 96 well plate at 20,000 cells/100 μL/well in DMEM media with 10% fetal bovine serum and penicillin-streptomycin plus fungizone for 24 hrs at 37 °C with 5% CO_2. For each well, cells were quickly transfected with 0.1 μg of pGL3-$(CAGA)_{12}$-luciferase plasmid using FuGENE6 Transfection Reagent (Roche, Mannheim, Germany), as well as 0.05 μg of pRL-TK plasmid (Promega, Madison, WI) containing a renilla luciferase gene which is under the control of TK promoter. pRL-TK was used as a control plasmid to calibrate the transfection efficiency of the cells. After 24 hrs, medium was replaced with serum-free medium and incubated for 9 hrs. In each well, 3 ng/mL of mouse MSTN (R&D Systems, Minneapolis, MN) and various concentrations of pMSTNProW or pMSTNProM were added and incubated for 6 hrs. The activities of both firefly and renilla luciferases were measured by a Veritas microplate luminometer (Turner Biosystems Inc., CA, USA) using the Dual Luciferase Assay System (Promega, Madison, WI) following the manufacturer's suggested procedures. For each protein subjected to bioactivity test, the firefly/renilla luciferase ratios was calculated and a corresponding bar chart with mean and standard error of mean (SEM) were plotted using the GraphPad Prism software (Prism 5, CA, USA).

CHAPTER 4

RESULTS AND DISCUSSION

Rapid expression of proteins of interest with high quality and quantity is very important in biomedical and biological research. Mostly, *E. coli* is an attractive host for fast cloning of target genes and expression of recombinant proteins because of its advantages, such as simple handling, low cost and high yields of recombinant proteins (Cabrita and Bottomley, 2004). Additionally, cytoplasmic expression generally yields more proteins than the periplasmic expression (Sorensen and Mortensen, 2005). Nonetheless, *E. coli* is unable to process many post-translational modifications, and sometimes it expresses recombinant proteins as inclusion bodies (Cabrita and Bottomley, 2004). Indeed, *E. coli* cytoplasm is a reducing environment that hinders the formation of disulfide bonds of protein, leading to protein misfolding and formation of inclusion bodies. However, disulfide bond formation or N-linked glycosylation is not necessary for the inhibitory ability of the MSTN propeptide on MSTN (Jiang et al., 2004). Hence, cytoplasmic expression of MSTN propeptide is feasible in *E. coli*.

MSTN propeptide was previously overexpressed as inclusion bodies in cytoplasm of *E. coli* (Funkenstein and Rebhan, 2007). Since correct folding and solubility determine the bioactivity of a protein, a series of time-consuming refolding procedures were performed to obtain soluble MSTN propeptide (Funkenstein and Rebhan, 2007). In order to avoid the impediments of refolding inclusion bodies, gene fusion is an advanced technique for soluble expression of protein. Especially, MBP is an effective fusion protein to enhance the solubility of many target proteins. For example, Lee et al. (2010)

have demonstrated that MSTN propeptide of fish fused with MBP can be expressed in a soluble form in *E. coli*. In this study, a wild-type porcine MSTN propeptide and its mutated form were expressed in soluble forms in the cytoplasm of *E. coli* with the aid of MBP fusion protein.

4.1 Expression of MBP-pMSTNPro proteins

Figure 1 shows the results of SDS-PAGE analysis of the expression of the two constructs in *E. coli* (TOP 10F' strain) cells. The constructs were expected to express a 75 kDa protein consisting of an N-terminal 42.5 kDa fusion protein (MBP) plus the 29 kDa MSTN propeptides (MBP-pMSTNProW and MBP-pMSTNProM). The 75 kDa MBP-pMSTNPro proteins were detectable after induction with IPTG. SDS-PAGE analysis of cell lysates showed that the most of the induced proteins were in supernatant fractions, indicating that the MBP-pMSTNPro proteins were mainly expressed in soluble forms (Fig. 1A, lane 5; Fig. 1B, lane 5). About 99.0 ug/ml proteins were expressed from pMSTNProW construct, of which about 85.0% was in soluble form, and about 96.0 ug/ml proteins were expressed from pMSTNProM construct, of which about 84.9% was soluble proteins (Table 1). This result is in consistent with previous reports in which fish MSTN-1 prodomains were expressed in soluble forms using MBP as a fusion partner (Lee et al., 2010b; Lee et al., 2011), suggesting that MBP is an effective fusion partner in soluble expression of MSTNPro proteins of various animal species in *E. coli*.

4.2 Purification of MBP-pMSTNPro proteins by Affinity Chromatography

For purification of the MBP-pMSTNPro proteins, soluble fractions of cell lysates were applied to an amylose resin column, and eluted by the column buffer containing 10 mM maltose. When analyzed by SDS-PAGE, the elution fractions were composed of mostly the recombinant MBP-pMSTNPro proteins at 75 kDa along with minor amounts of proteins with various sizes (Fig.2 A&B). About 39 ug/ml of MBP-pMSTNProW protein and 37 ug/ml of MBP-pMSTNProM protein were purified by affinity chromatography, contributing to around 40% recovery from the total proteins (Table 1).

To further characterize the affinity-purified proteins, the elution fractions of each MBP-pMSTNPro protein were pooled and were subjected to Western blot analysis. Alongside with the supernatant of cell lysate and the pooled elution fractions, MBP and commercially available glycoslyated MSTN propeptide were applied as controls (Fig. 3). Both anti-MSTN propeptide antibody and anti-MBP antibody showed strong affinities to the 75 kDa recombinant proteins of MBP-pMSTNProW (Fig. 3A i-iii) and MBP-pMSTNProM (Fig. 3B i-iii), confirming that the 75 kDa proteins contain MBP and respective propeptides. Some bands present in the supernatant of cell lysate and affinity purified elutions were also tagged by anti-MSTN propeptide antibody and/or anti-MBP antibody, indicating that those minor proteins are probably degradation products of the MBP-MSTNPro proteins. In addition, most of the proteins in the original supernatant tagged by anti-MSTN propeptide antibody were also found in the affinity elutions, suggesting that there might be some continuous degradations of MBP-pMSNTPro proteins occurred before and after the affinity purification (Fig. 3A iii and 3b iii).

Interestingly, some protein bands in the original supernatant, tagged by both anti-MSTNPro and anti-MBP antibodies, were disappeared from the pooled affinity elutions (Fig. 3A ii-iii; Fig. 3B ii-iii). This might imply that the MBP portion of the fusion proteins was truncated at the maltose binding site such that MBP failed to bind to amylose resin properly during purification and ended up in the pass-through. Nonetheless, comparing with the extent of pMSTNPro protein degradation, MBP is still a relatively stable molecular chaperone to enhance the solubility of the pMSTNPro protein that is fused to it (Bach et al., 2001), as well as a selective fusion protein for affinity purification purpose.

In fact, upon cell disruption, proteases are released to degrade proteins during the extraction and purification steps, thereby greatly decreasing the yield of target proteins. MSTN propeptide is especially prone to enzymatic degradation, so protease inhibitors are needed to prevent protein degradation during the purification process (Jiang et al., 2004). Although the Complete Mini Protease Inhibitor Cocktail Tablets (Roche, Mannheim, Germany) was applied to the cell lysate and pooled affinity elutions, some proteases might preserve their activities such that protein degradation of the recombinant protein persisted. On the other hand, phenylmethylsulfonyl fluoride (PMSF, 1 mM), a serine-protease inhibitor, has shown an excellent ability on inhibiting proteases such that the MBP-pMSTNPro proteins were well-preserved over time. However, as a serine protease, Factor Xa is strongly inhibited by PMSF, thereby losing its ability to cleave the MBP fusion protein from the pMSTNPro proteins. Thus, PMSF had to be removed from the samples by dialysis against PBS before Factor Xa digestion and tissue culture testing. Alternatively, Jiang et al. (2004) has used the protease inhibitors PIC1 (1 mg/ml leupeptin, 2 mg/ml antipain, and 10 mg/ml benzamidine, dissolved in 10,000 U/ml aprotinin) and PIC2 (1 mg/ml chymostatin and 1 mg/ml pepstatin dissolved in dimethyl sulfoxide) to prevent the glycosylated MSTN propeptide from degradation. Yet, it remains unclear whether PIC1 and PIC2 are effective on inhibiting degradation of MBP-pMSTNPro or nonglycosylated pMSTNPro proteins.

4.3 Purification of MBP-pMSTNPro proteins by Anion Exchange Chromatography

To further purify the MBP-pMSTNPro proteins, the combined elution fractions from the affinity chromatography were applied to anion-exchange chromatography using the FPLC system with Mono Q HR 5/5 column. As shown in figures 4 and 5, a majority of proteins below 50 kDa were removed from the affinity-purified elutions during the isocratic step with low gradient buffer (6.12 mS/cm, pH 8.0) and the washing step with 40% of high gradient buffer (33.58 mS/cm, pH 8.0). The MBP-pMSTNProW protein was then eluted during a salt gradient from 34.00 mS/cm to 69.90 mS/cm, of which the peak elution was occurred at 47.00 mS/cm (Fig. 4). An average of 10.4 ug/ml of MBP-pMSTNPro-W protein (10.5% of the total proteins) was recovered by anion exchange chromatography (Table 1).

Likewise, as shown in figure 5, proteins below 50 kDa were successfully removed from the affinity-purified MBP-pMSTNProM elutions during the isocratic step with low gradient buffer (6.10 mS/cm, pH 8.0) and the washing step with 40% higher gradient buffer (33.56 mS/cm, pH 8.0). The MBP-pMSTNProM protein was also eluted during a salt gradient from 34 mS/cm to 69.85 mS/cm with a peak of elution at 46.47 mS/cm. About 9.3 ± 0.8 ug/ml of MBP-pMSTNProM (9.8% of the total proteins) were purified by anion exchange chromatography (Table 1).

However, the SDS-PAGE analysis of the eluted fractions of both MBP-pMSTNPro proteins showed a presence of about 55 kDa protein in addition to the 75 kDa MBP-pMSTNPro proteins (Fig. 4 and 5), indicating a need for further purification steps for a complete purification of the MBP-pMSTNPro proteins. The 55 kDa protein showed affinity to both the anti-MSTN propeptide and anti-MBP antibodies, suggesting that the

55 kDa is a degradation product of 75 kDa MBP-pMSTNPro proteins (Fig.6 B & C). Overall, anion exchange chromatography appears to be an effective way to purify the intact MBP-pMSTNPro proteins from their degradation products.

4.4 Separation and purification of the pMSTNPro proteins from the MBP fusion proteins

To separate the MBP fusion protein from the pMSTNPro proteins, anion exchange-purified pMSTNPro proteins were incubated with Factor Xa that cleave at the IEGR site between MBP and the pMSTNPro proteins. In figure 6A, the anion exchange chromatography-purified MBP-pMSTNProW and MBP-pMSTNProM were shown in lanes 2 and 5, respectively. The SDS-PAGE analysis of the Factor Xa digestion showed a complete disappearance of the 75 kDa MBP-pMSTNPro proteins after 24 hr incubation along with the appearance of two major bands of around 43 kDa and 29 kDa (Fig. 6A), indicating the separation of the MBP (43 kDa) from pMSTNPro proteins (29 kDa). Western blot analysis showed that the 43 kDa protein had affinity only to anti-MBP antibody, and the 29 kDa protein had affinity only to anti-MSTN propeptide antibody, confirming the identity of each protein (Fig. 6 B & C).

The pMSTNPro proteins were purified by amylose resin affinity chromatography to remove MBP or MBP-containing proteins in the samples. The pass-through containing the pMSTNPro proteins were collected from the affinity purification. About 4.2 ug/ml of pMSTNProW protein and 4.24 ± 0.27 ug/ml of pMSTNProM protein were purified from the MBP fusion protein, yielding about 4.3% and 4.4% of recovery from the total soluble proteins, respectively (Table 1). SDS-PAGE and Western blot analysis of the purified pMSTNPro proteins showed that there was a majority of intact pMSTNPro proteins at 29 kDa (indicated by the arrows), along with its degradated products at various sizes (Fig. 6). The pMSTNPro degradation products were tagged by anti-MSTNPro antibody but not anti-MBP antibody, suggesting that MBP was completely removed from the pMSTNPro proteins by the affinity purification.

4.5 Bioactivity test for the pMSTNPro proteins using the CAGA-Luciferase reporter assay

Six different forms of the purified pMSTNPro protein at various concentrations were examined in A204 cells for their capacity of suppressing MSTN activity using the pGL3-$(CAGA)_{12}$-dual luciferase reporter assay system. The commercially available mouse MSTN propeptide was used as a positive control for this system and inhibited half of the MSTN activity by a concentration of about 4 ng/ml (R&D Systems, Minneapolis, MN) (Fig. 8). In consistent with the previous study (Lee et al., 2010a), MBP has shown no inhibitory effect on MSTN activity in vitro (Fig. 7). Hence, with reference to the average firefly/renilla luciferase ratio of MBP at 1000 ng/ml (i.e. 2.50), the firefly/renilla luciferase ratio for each pMSTNPro protein form was standardized accordingly for the purposes of comparison and calculation of the corresponding concentration or dosage for a half inhibition of MSTN activity. Unfortunately, due to the variation persisted in this experiment, the half inhibition concentration for each protein was solely based on rough estimation and was not statistically compared. Hence, at this point, no precisive conclusion could be made regarding to the potency of the protein on inhibiting MSTN activity. However, all six forms of pMSTNPro proteins demonstrated a similar tendency as the commercially available mouse MSTN propeptide on inhibiting MSTN activity, of which the higher the protein concentration, the lower the MSTN activity was detected *in* *vivo*. Hence, the results from the bioactivity test indicated those six forms of pMSTNPro proteins were bioactive and their inhibitory effects on MSTN activity were dosage-dependent.

The affinity purified MBP-pMSTNProW and MBP-pMSTNProM proteins had shown the estimated concentrations at about 4 ng/ml and 56 ng/ml, respectively, for introducing half inhibition of MSTN activity (Figures 9 and 10). These results underlined a possibility that the truncated fragments of MBP-pMSTNPro proteins, resulted from protein degradation, might have conserved their inhibitory capacity on MSTN activity. Similar effect was found in a previous study, indicating the truncated MSTNPro proteins preserves full inhibitory activity as long as the protein sequence contains amino acid residues from 42 to 115 at the N-terminus (Jiang et al., 2004). Besides, the stable nature of the C-terminal region of MSTNPro protein might contribute to the stability of the whole molecule (Jiang et al., 2004), suggesting the protein degradation of the MBP-pMSTNPro proteins might occur at the C-terminus rather than the N-terminus of the full length of the pMSTNPro protein. Although the affinity purified MBP-pMSTNPro proteins demonstrated their bioactivities on suppressing MSTN activity in our assay, it remains unknown whether it is due to the presence of the N-terminal amino acid residues from 42-115, or the MBP fusion protein might play a role in promoting the inhibitory capacity of the pMSTNPro proteins.

By adding a purification step of anion exchange chromatography, the MBP-pMSTNProW protein inhibited half of the MSTN activity at the concentration of about 6 ng/ml, whereas the half inhibition concentration of the MBP-pMSTNProM protein was estimated at 40 ng/ml approximately (Fig. 11 and 12). The anion exchange chromatography purification resulted in about 26% loss of the amount of proteins previously purified from the affinity chromatography (Table 1). It is speculated that the anion exchange chromatography removed some of the truncated MBP-pMSTNPro proteins which still possessed their inhibitory capacity on MSTN activity. Thus, under this assumption, the anion exchange chromatography might not be necessary and the affinity chromatography alone might probably be effective and efficient in recovering of the bioactive MBP-pMSTNPro proteins.

After removing the MBP fusion protein and purified by affinity chromatography, the estimated concentration of the pMSTNProW protein for half inhibition of MSTN activity was at around 264 ng/ml, whereas that of the pMSTNProM protein was roughly about 60 ng/ml (Fig. 13 and 14). The pMSTNPro proteins seemed to show decreasing trends in inhibitory effects on MSTN activity after the removal of MBP fusion protein, speculating a potential role of MBP on stabilizing the pMSTNProW protein. A previous study also proposed that MBP acts as a molecular chaperone and its attachment promotes the solubility and stability of the fusion partner (Bach et al., 2001). Bach et al. (2001) also speculated that during protein expression in *E. coli*, MBP is translated and folds before the fusion partner at the C-terminus, thereby providing an opportunity for MBP to stabilize the target protein by blocking its interaction with other cellular molecules that cause aggregation. Likewise, the relatively large molecular size of MBP might potentially hinder the interaction between metalloproteinase and the pMSTNPro proteins, thus keeping the MSTN homodimer in a latent form with the MBP-pMSTNPro proteins. Nonetheless, no evidence has yet showed that MBP poses any effect to the pMSTNPro proteins on inhibiting MSTN activity. On the other hand, it was assumed that the mutation at the cleavage site of the pMSTNPro protein would be effective on extending its inhibitory effect on MSTN activity by becoming inaccessible to the metalloprotease and staying attached to the MSTN homodimer to prevent it from binding to the receptors. However, it is not clear if MBP plays a role in enhancing the stability and bioactivity of the pMSTNPro proteins, or whether metalloprotease is produced in A204 cells. Besides, the difference between the relative half inhibition concentration of the wild type and the mutant forms of pMSTNPro proteins on MSTN activity could simply be due to the variations in binding kinetics, which were not evaluated in this study.

CHAPTER 5

CONCLUSION

In this study, the wild-type of pMSTNPro protein and its mutant form at the cleavage site were successfully expressed as soluble forms in *E. coli* using MBP as the fusion protein. The MBP-pMSTNPro proteins were also purified by the amylose-resin affintiy chromatography and anion exchange chromatography. Moreover, the bioactivity tests verified the MSTN-suppressing capacity of six different forms of pMSTNPro proteins *in vitro*. All the pMSTNPro proteins demonstrated a similar tendency on inhibiting MSTN activity, of which the higher the protein concentration, the lower the MSTN activity was detected. Hence, the results from the bioactivity test indicated those six forms of pMSTNPro proteins were bioactive and their inhibitory effects on MSTN activity were dosage-dependent. MBP might be a relatively stable molecular chaperone to enhance the solubility of the pMSTNPro proteins, as well as a selective fusion protein for affinity purification purpose. Further studies are needed to examine the binding characteristics of different forms of the pMSTNPro proteins and the interaction between metalloprotease and MBP-pMSTNPro proteins. Overall, the *E. coli* expression system had demonstrated as a potentially simple and cost effective way to produce large amount of pMSTNPro proteins to examine their effects on improving muscle growth in meat-producing animals.

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Table 1. Yields of pMSTNPro proteins recovered from each purification step.

	pMSTNProW		pMSTNProM	
	Average recovery of protein per ml culture (ug/ml)*	Yield (%)	Average recovery of protein per ml culture (ug/ml)*	Yield (%)
Total proteins	98.96 ± 4.89	100.0	95.53 ± 2.05	100.0
Soluble proteins	84.58 ± 3.07	85.8	81.06 ± 2.20	84.9
Affinity chromatography	38.70 ± 7.85	39.1	36.98 ± 1.46	38.7
Anion exchange chromatography	10.43 ± 0.374	10.5	9.33 ± 0.81	9.8
pMSTNPro protein after MBP removal	4.23 ± 0.31	4.3	4.24 ± 0.27	4.4

*Protein concentration was measured by Bradford method using BSA as a standard. The protein recovery was calculated as the mean ± SEM from the purfications of a triplicate of 250 mL culture of each type of pMSTNPro protein.

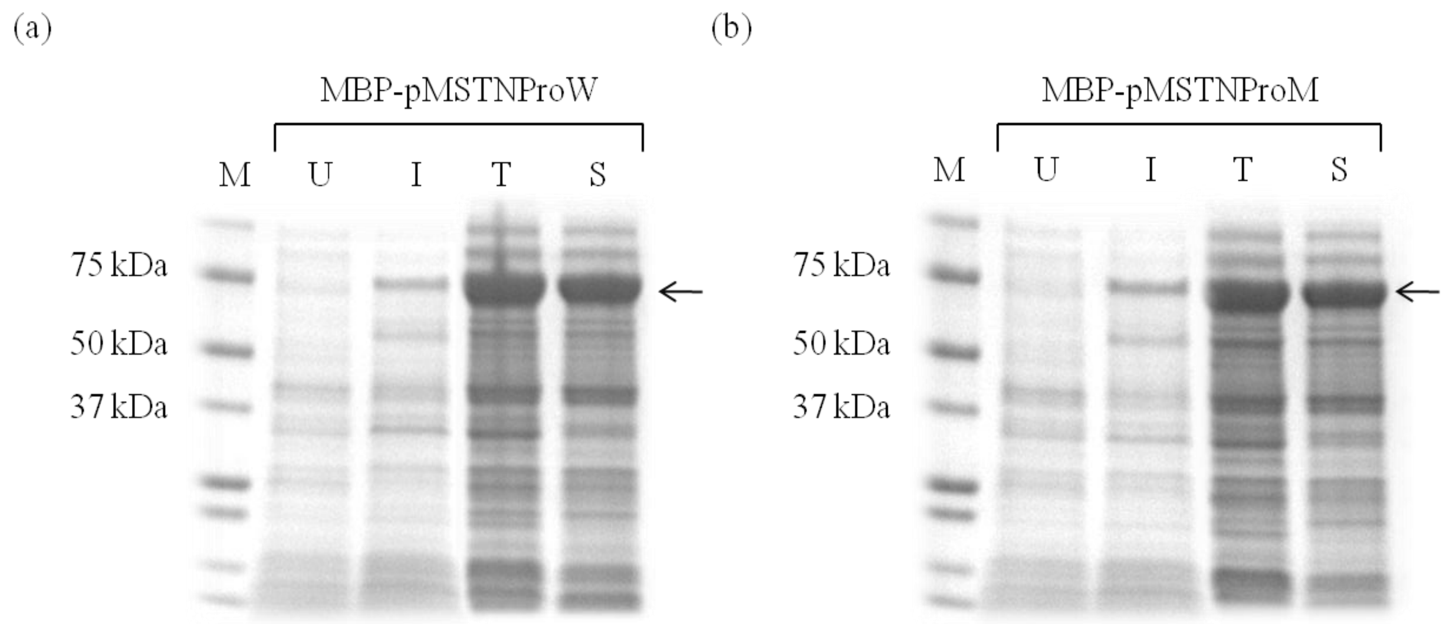

Figure 1. SDS-PAGE analysis of the expression of (a) MBP-pMSTNProW and (b) MBP-pMSTNProM proteins (as indicated by arrows) . Cell culture was sampled before (U) and after induction by IPTG (I). After collected the cell pellet by centrifugation, the total cell lysate (T) was prepared by sonication at 15 second pulses for 20 minutes in cold column buffer. Soluble fraction (S) was obtained by centrifugation of cell lysate. The fractions were then applied to a 12.5% SDS-PAGE gel, and protein expression was examined by Coomassie blue staining.

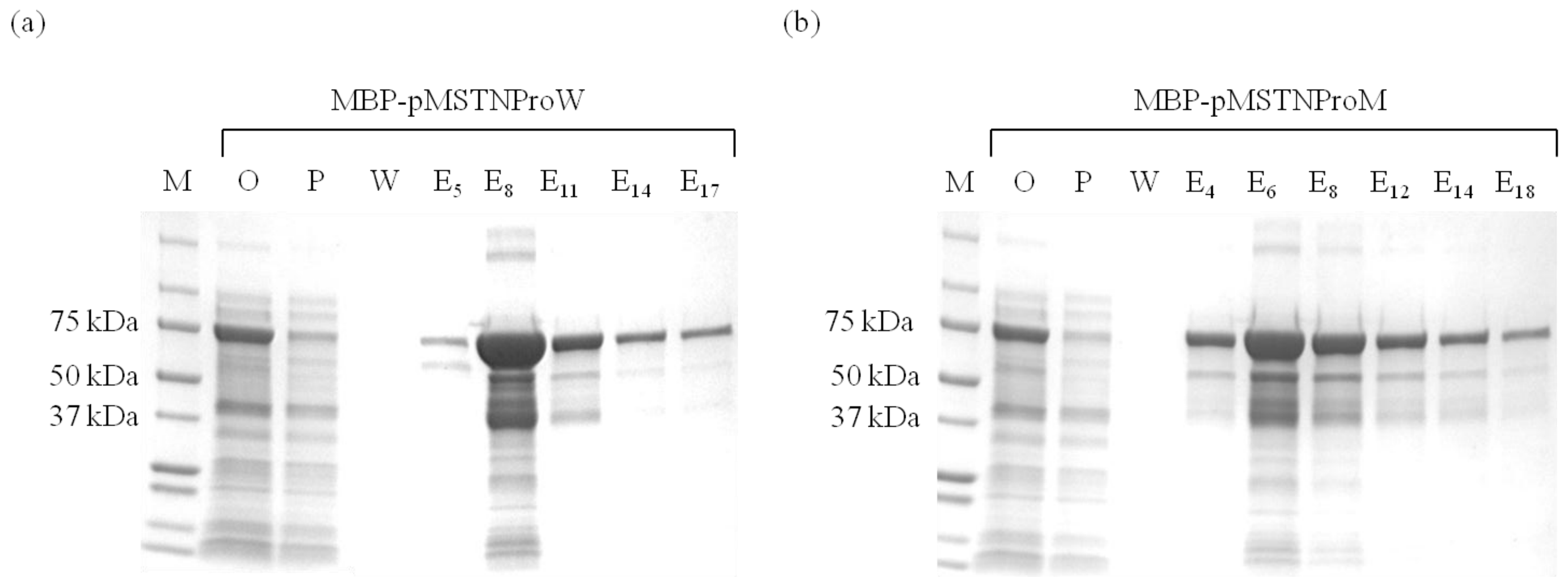

Figure 2. Purification of MBP-pMSTNProW and MBP-pMSTNProM fusion proteins by amylose-resin affinity chromatography. Original 1:6 diluted soluble fraction (O), pass-through (P), washing (W), and elution. (E) fractions were applied to a 12.5% SDS-PAGE gel for electrophoresis followed by Coomassie blue staining. The MBP-MSTNPro fusion proteins (75 kDa) were semi-purified by amylose-resin affinity chromatography. Other protein bands indicated the presence of protein degradation in the elution fractions collected. M, protein standard (Biorad).

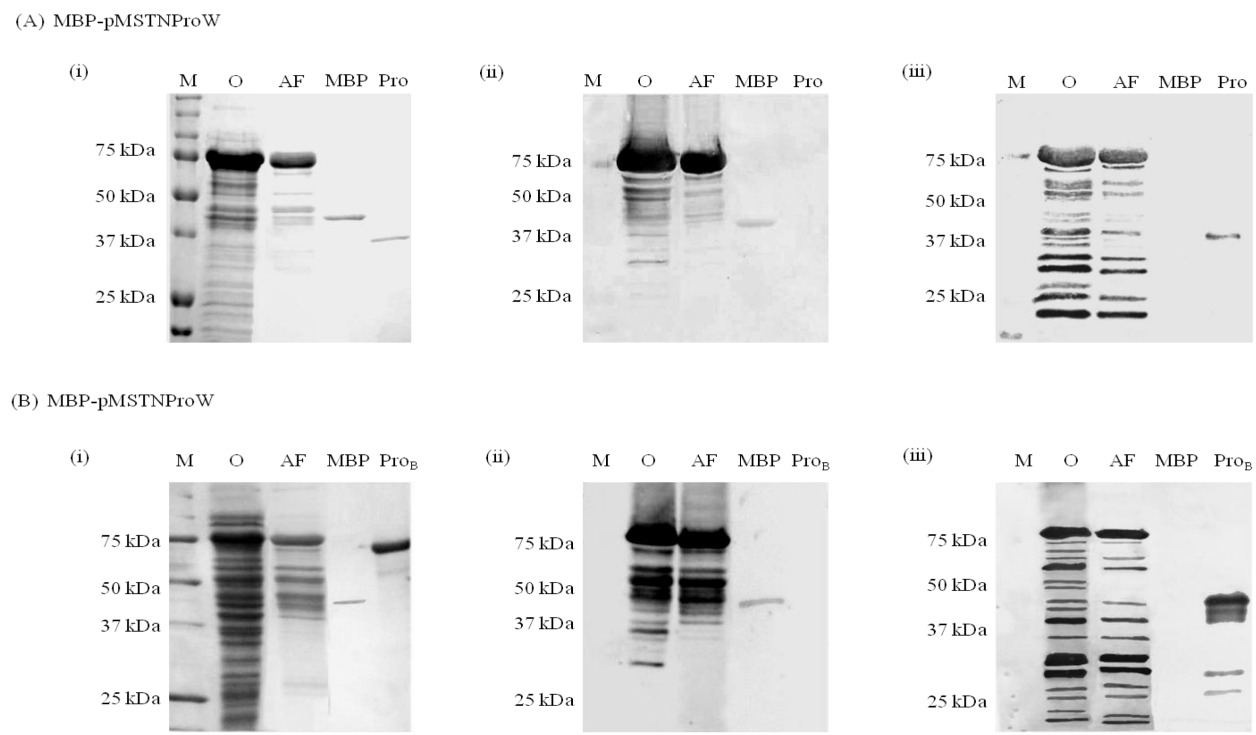

Figure 3. SDS-PAGE and Western blot analysis of the (A) MBP-pMSTNProW and (B) MBP-pMSTNProM soluble fractions before and after purified by amylose resin affinity chromatography. (A-i) & (B-i) SDS-PAGE using 12.5% gels stained by Coomassie blue. (A-ii) & (B-ii) Western blot with anti-MBP primary antibody (1:10,000), followed by anti-mouse IgG antibody conjugated to alkaline phosphatase (1:20,000); (A-iii) & (B-iii) Western blot with anti-MSTNPro primary antibody (1:10,000), followed by anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:20,000). M, Prestain protein standard (Biorad); O, Original 1:6 diluted soluble fraction from cell lysate before purification; AF, combined elution from affinity chromatography; MBP, Maltose binding protein; Pro, commercial mouse MSTN propeptide (R&D Systems, Minneapolis, MN); Pro$_B$, commercial mouse MSTN propeptide with 1% BSA (R&D Systems).

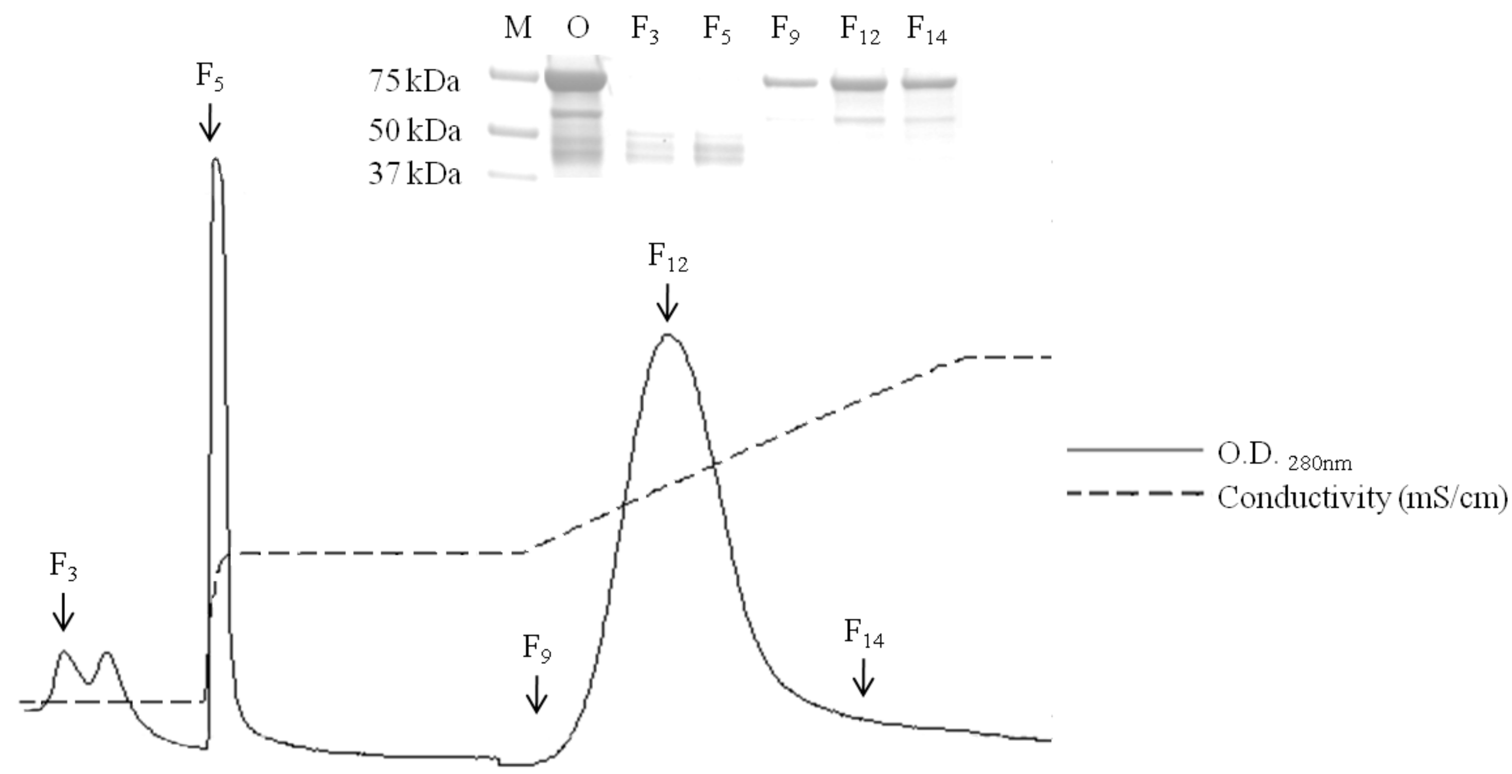

Figure 4. Anion exchange chromatogram of the MBP-pMSTNProW proteins. The affinity purified elution fractions of MBP-pMSTNProW protein were pooled and were further purified by anion exchange chromatography using the FPLC system with Mono Q HR 5/5 column. The eluted MBP-pMSTNProW protein was collected in the fractions 9 to 14, which were analyzed by SDS-PAGE. M, Protein standard (Biorad); O, original affinity purified elution; F, fraction collected from anion exchange chromatography.

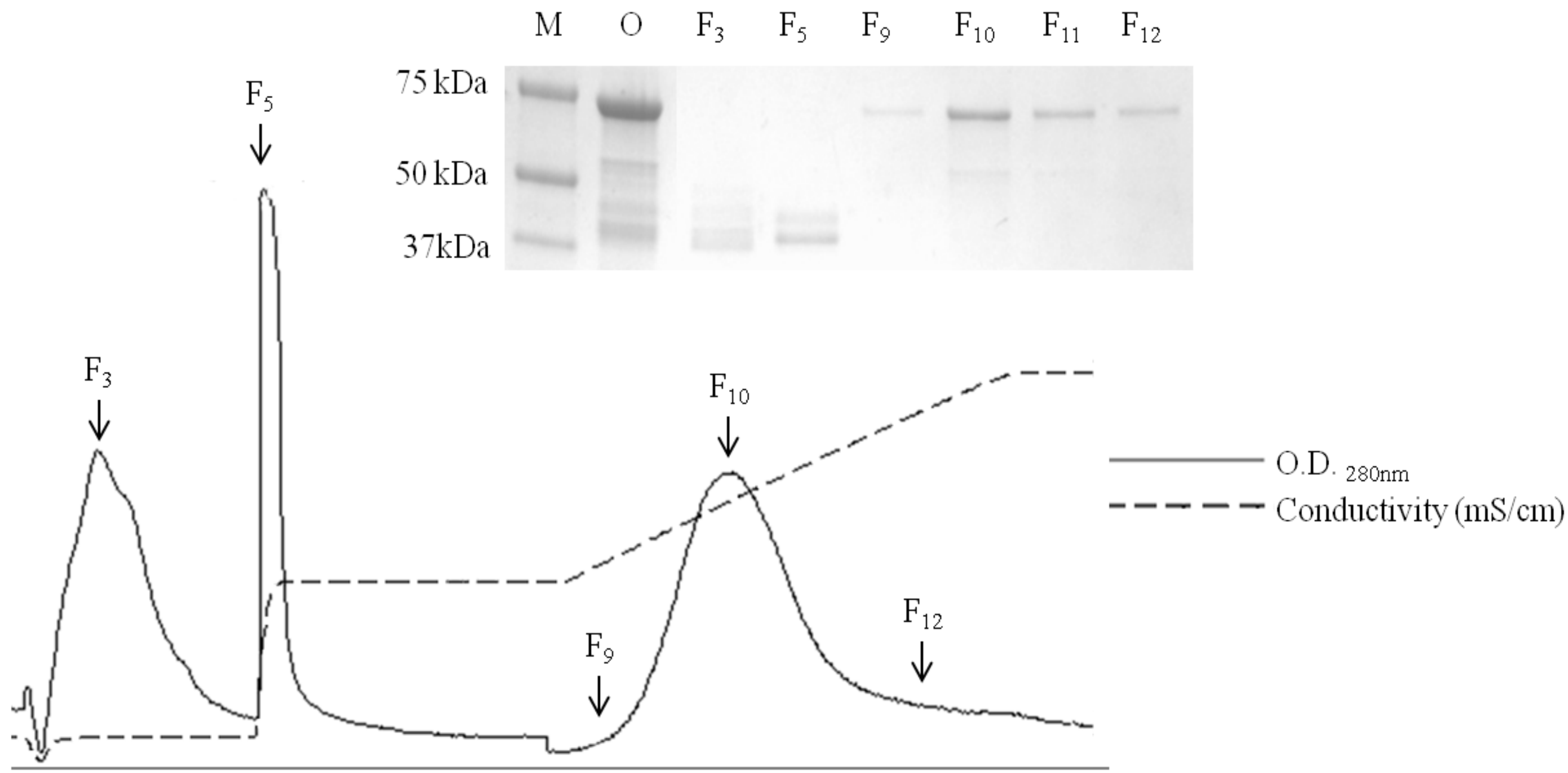

Figure 5. Anion exchange chromatogram of the MBP-pMSTNProM proteins. The affinity purified elution fractions of MBP-pMSTNProM protein were pooled and were further purified by anion exchange chromatography using the FPLC system with Mono Q HR 5/5 column. The eluted MBP-pMSTNProM protein was collected in the fractions 9 to 12, which were analyzed by SDS-PAGE. M, Protein standard (Biorad); O, original affinity purified elution; F, fraction collected from anion exchange chromatography.

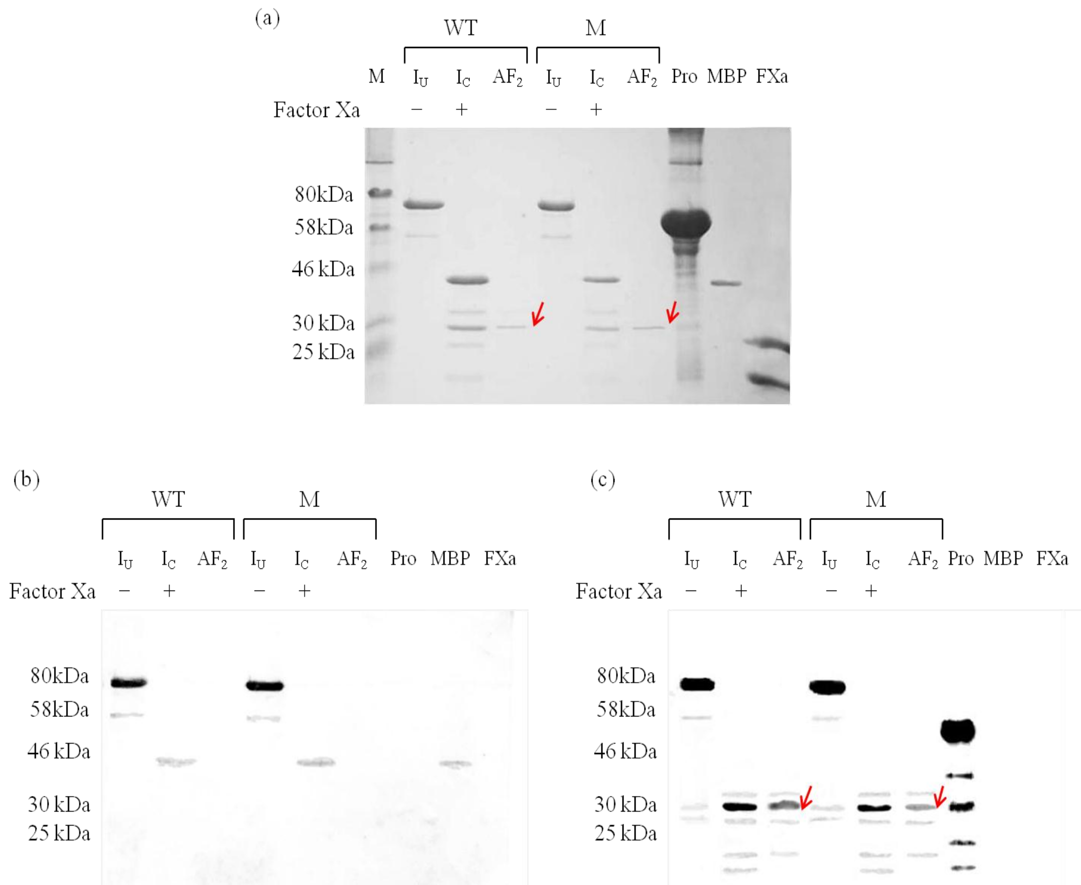

Figure 6. SDS-PAGE and Western blot analysis of the pMSTNPro proteins after Factor Xa cleavage and the 2nd round of amylose resin affinity chromatography. (a) SDS-PAGE of the proteins using a 12.5% gel stained by Coomassie blue. (b) Western blot with anti-MBP primary antibody (1:10,000), followed by anti-mouse IgG antibody conjugated to alkaline phosphatase (1:20,000); (c) Western blot with anti-MSTNPro primary antibody (1:10,000), followed by anti-rabbit IgG antibody conjugated to alkaline phosphatase (1:20,000). The purified pMSTNPro proteins were indicated by the arrows. M, Prestained Protein Marker, Broad Range (7-175 kDa) (NEB); I_U, combined elutions from anion exchange chromatography; I_C, combined elutions from anion exchange chromatography after Factor Xa cleavage; AF2, purified pMSTNPro proteins after the 2nd round of amylose resin affinity chromatography; Pro, commercial mouse MSTN propeptide with 1% BSA (R&D Systems, Minneapolis, MN); MBP, maltose binding protein; FXa, Factor Xa.

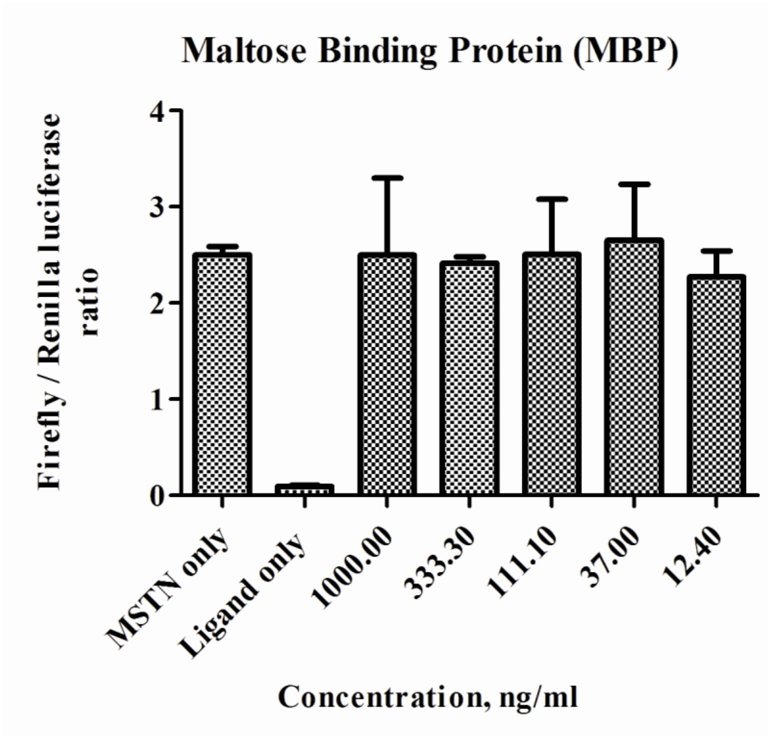

Figure 7. Bioactivity test result of the maltose binding protein (MBP) purified by amylose-resin affinity chromatography and anion exchange chromatography. Error bars represent the standard error of the mean.

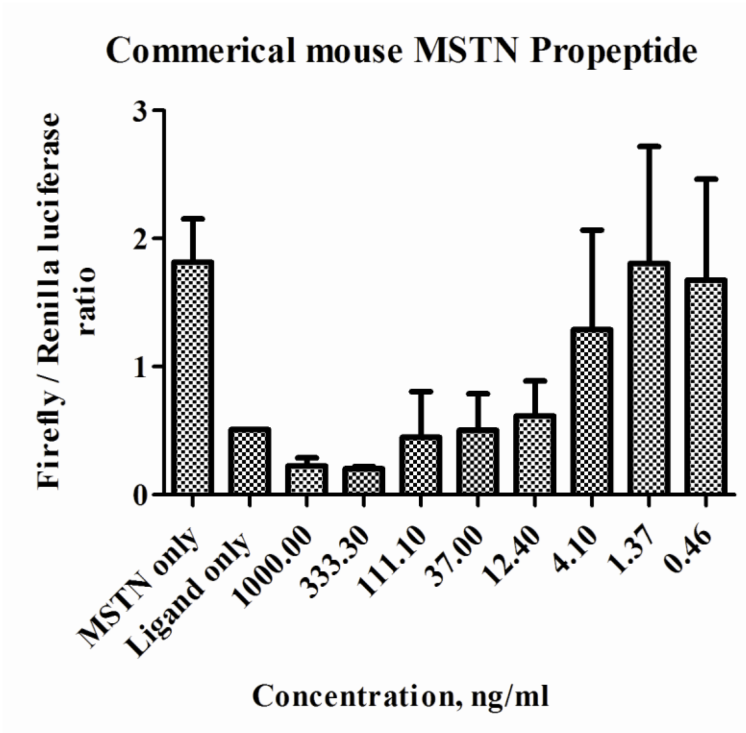

Figure 8. Bioactivity test result of the commerically avaiilable mouse MSTN propeptide (R&D Systems, Minneapolis, MN). Error bars represent the standard error of the mean.

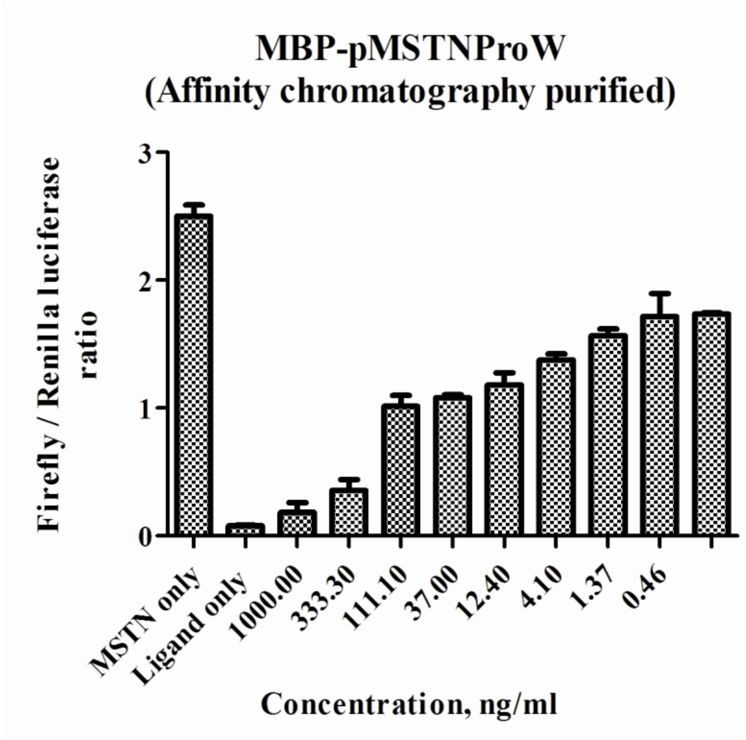

Figure 9. Bioactivity test result of the MBP-pMSTNProW protein purified by amylose-resin affinity chromatography. Error bars represent the standard error of the mean.

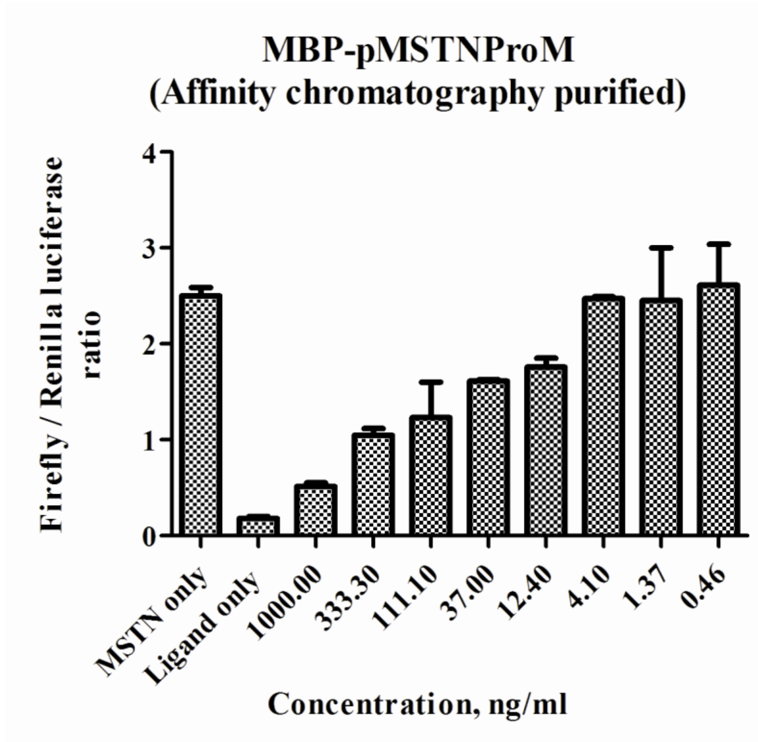

Figure 10. Bioactivity test result of the MBP-pMSTNProM protein purified by amylose-resin affinity chromatography. Error bars represent the standard error of the mean.

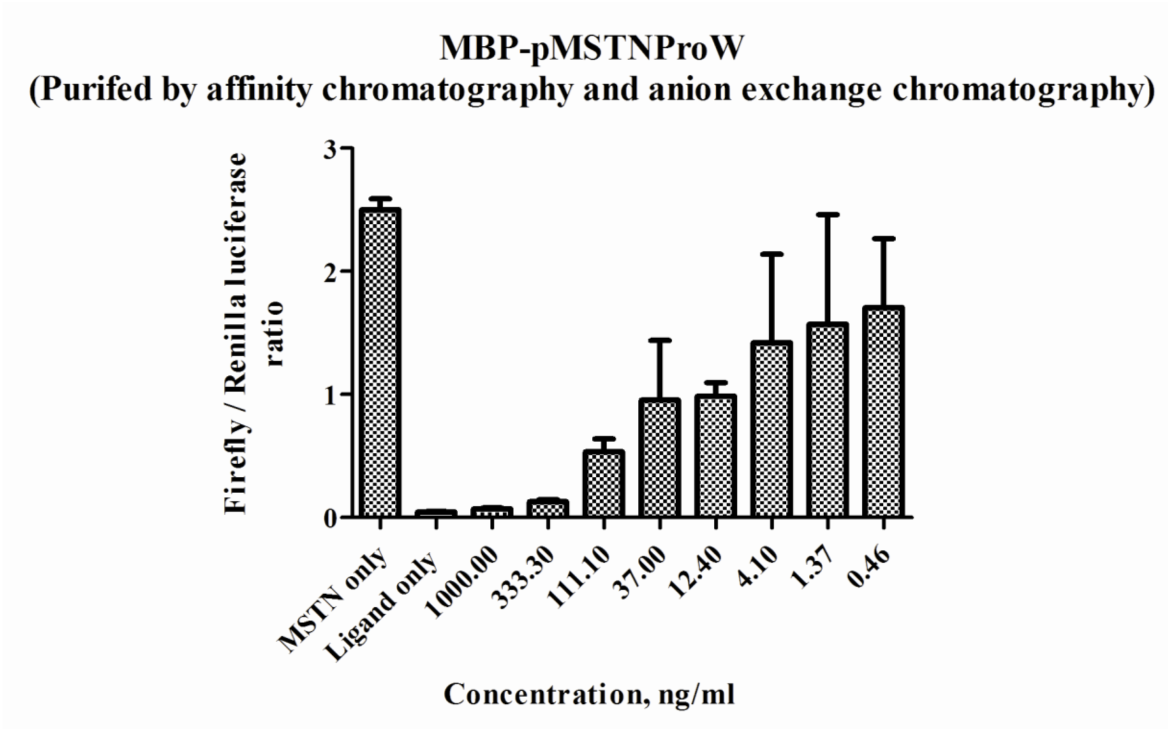

Figure 11. Bioactivity test result of the MBP-pMSTNProW protein purified by amylose-resin affinity chromatography followed by anion exchange chromatography. Error bars represent the standard error of the mean.

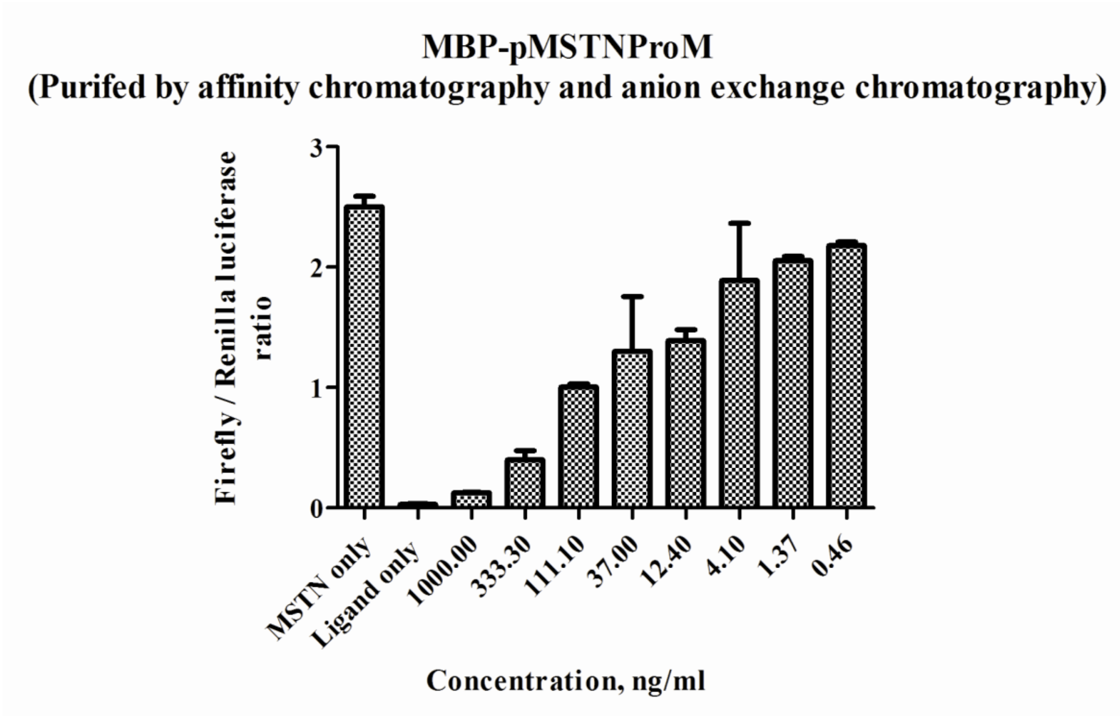

Figure 12. Bioactivity test result of the MBP-pMSTNProM protein purified by amylose-resin affinity chromatography followed by anion exchange chromatography. Error bars represent the standard error of the mean.

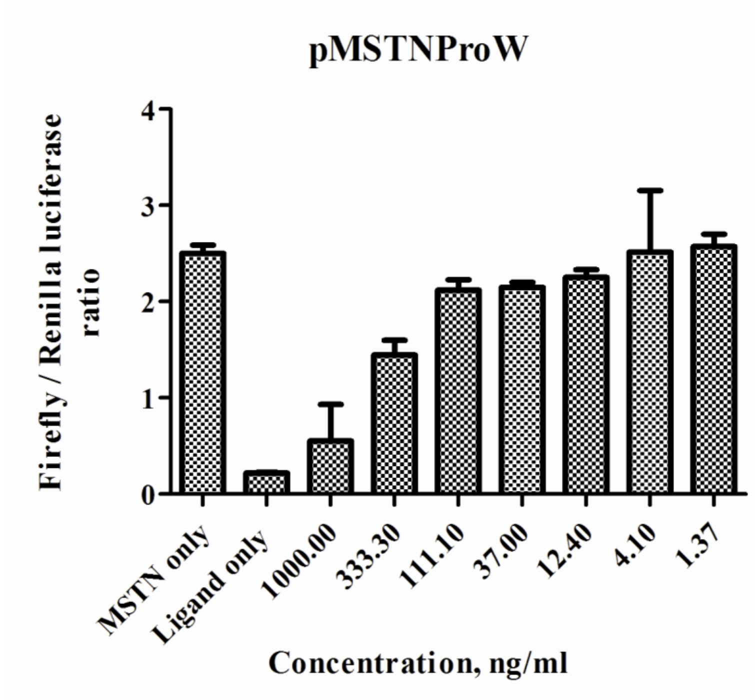

Figure 13. Bioactivity test result of the pMSTNProW protein. The MBP-pMSTNProW protein was first purified by amylose-resin affinity chromatography and anion exchange chromatography. The MBP was then removed from the pMSTNPro proteins by Factor Xa digestion followed by the 2nd round of amylose-resin affinity chromatography. Error bars represent the standard error of the mean.

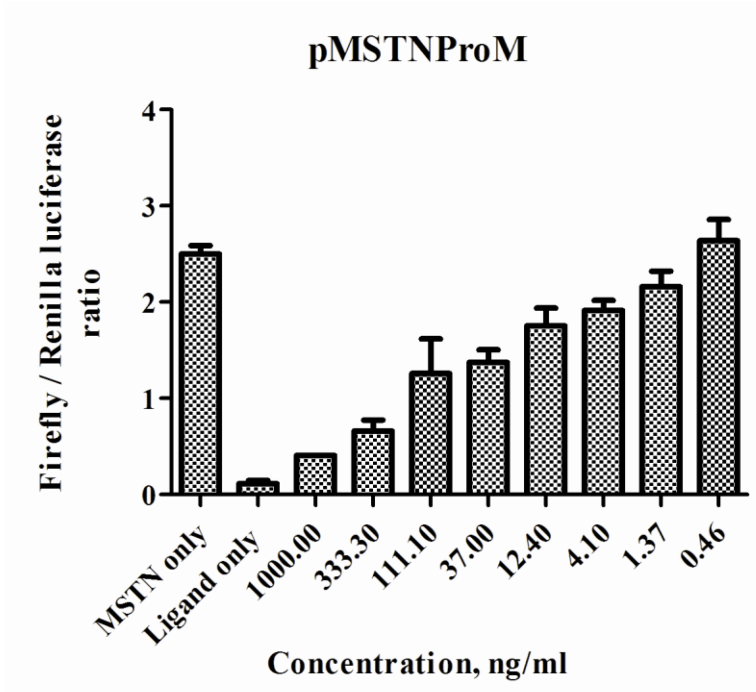

Figure 14. Bioactivity test result of the MBP-pMSTNProM protein purified by amylose-resin affinity chromatography and anion exchange chromatography. The MBP was removed from the pMSTNPro proteins by Factor Xa digestion followed by the 2nd round of amylose-resin affinity chromatography. Error bars represent the standard error of the mean.