

High-Yield Production of Bioactive Flatfish (*Paralichthys olivaceus*) Myostatin-1 Prodomain in *Escherichia coli*

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ABSTRACT

Myostatin Propeptide (MstnPro), the N-terminal part of Mstn precursor molecule, binds to Mstn and suppress its activity. Mstn is a potent negative regulator of skeletal muscle growth in many animal species, thus MstnPro has a great potential to be used as an agent to enhance skeletal muscle growth in animal species. Bioactive fish Myostatin-1 Propeptide (Mstn1Pro) proteins were recently expressed in soluble forms in *E. coli* using Maltose Binding Protein (MBP) as a fusion partner. However, the yield of purified protein was too low to realize the benefit of large production capacity of the *E. coli* expression system. The objective of this study was to examine whether the yield of bioactive, recombinant flatfish (*Paralichthys olivaceus*) Mstn1Pro (poMstn1Pro) production can be improved through a proper selection of *E. coli* strain and other expression parameters. The poMstn1Pro gene construct, previously cloned into pMAL-c2 vector downstream of the Maltose-Binding Protein (MBP) gene, were transformed and expressed in *E. coli* K12BT1 strain. The MBP-poMstn1Pro protein was purified by the amylose-resin affinity chromatography. About 51 mg of soluble MBP-poMstn1Pro were purified by affinity chromatography from a liter culture. Pull-down assay and bioactivity test demonstrated the binding of MBP-poMstn1Pro to Mstn and its Mstn-suppressing capacity, indicating that the N-terminal fusion of MBP in the flatfish Mstn1Pro does not affect either the binding of flatfish Mstn1Pro to Mstn or its Mstn-suppressing activity. Results of this study demonstrate that a large production of MBP-poMstn1Pro is possible using an *E. coli* system to investigate the potential of MBP-poMstn1Pro to enhance muscle growth in aquaculture species or the binding characteristics of Mstn1Pro with its partners.

Keywords: *Paralichthys Olivaceus*, Prodomain, GDF-8, Myostatin, Maltose-Binding Protein (MBP)

1. INTRODUCTION

Myostatin (Mstn), a member of the Transforming Growth Factor- β (TGF- β) superfamily, is a potent negative regulator of skeletal muscle growth in mammalian species (Lee, 2004). Recent studies have also indicated that muscle growth in fish is negatively

regulated by fish Mstn (Acosta *et al.*, 2005; Amali *et al.*, 2004; Lee *et al.*, 2009; 2010; 2011; Medeiros *et al.*, 2009; Xu *et al.*, 2003). Unlike mammalian species, at least two types of Mstn (1 and 2) are expressed in fish (Ostbye *et al.*, 2007; Rodgers and Garikipati, 2008) because of the dramatic role of Mstn in regulating muscle mass, there has been much interest in developing

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Mstn-based strategies to improve skeletal muscle growth in agriculturally important animal species, including fish (Rodgers and Garikipati, 2008; Tsuchida, 2008). Mstn Propeptide (MstnPro), the N-terminal part of Mstn precursor molecule, has been shown to be a potent suppressor of Mstn activity in mammalian species (Hu *et al.*, 2010; Lee and McPherron 1999; Li *et al.*, 2010; Matsakas *et al.*, 2009; Yang *et al.*, 2001). Fish Mstn peptide also appears to suppress Mstn activity. Mstn-1 Propeptide (Mstn1Pro) of the marine fish Sparus aurata suppressed Mstn activity in vitro (Rebhan and Funkenstein, 2008). An increase in fiber number was observed in transgenic zebrafish over expressing Mstn1Pro (Xu *et al.*, 2003). An improved growth of rainbow trout was observed by treatment with flatfish (*Paralichthys olivaceus*) Mstn1Pro expressed an *E. coli* system (Lee *et al.*, 2010), indicating the potential of fish Mstn1Pro as an agent to improve the growth of commercially important aquaculture species. It is, therefore, desirable to develop a method for economic production of large quantity of fish Mstn1Pro.

If soluble expression of a recombinant protein is induced, *E. coli* remains a popular choice for recombinant protein production due to its relative low cost and high yields (Chou, 2007). An earlier study reported an insoluble expression of seabream Mstn1Pro in an *E. coli* system, thus a series of time-consuming refolding procedures were performed to obtain bioactive soluble Mstn1Pro (Funkenstein and Rebhan, 2007). Recently, however, bioactive fish Mstn1Pro proteins were expressed in soluble forms in an *E. coli* system using Maltose Binding Protein (MBP) as a fusion partner (Lee *et al.*, 2010; 2011), suggesting that MBP may serve as an useful fusion partner to induce soluble expression of MstnPro proteins of various species. However, the yield of purified protein was too low (about 700 $\mu\text{g L}^{-1}$ culture) to realize the benefit of large production capacity of the *E. coli* expression system. The *E. coli* strain used in the above studies was Rosetta-gami 2(DE3) pLysS (Novagen, WI, USA), a strain that contains chromosomal copy of the T7RNA polymerase gene under the lacUV5 control. Since the pMAL expression vectors (New England BioLabs, MA and USA) use tac promoter and carry the lacI^q gene for induction with IPTG, any competent *E. coli* strain would be suitable to express recombinant proteins using the pMAL expression vectors. The present work was designed to examine whether the yield of bioactive, recombinant flatfish (*Paralichthys olivaceus*) Mstn1Pro (poMstn1Pro) production can be improved through a proper selection of *E. coli* strain and other expression parameters. Results of the current study show that up to 51 mg of bioactive,

affinity-purified MBP-poMstn1Pro protein can be produced per liter culture in an *E. coli* system.

2. MATERIALS AND METHODS

2.1. Cloning of Expression Vector

To express flatfish (*Paralichthys olivaceus*) Mstn1Pro (poMstn1Pro), we used an expression construct of poMstn1Pro (pMALc2x-poMstn1Pro) prepared previously (Lee *et al.*, 2010). K12TB1 strain (New England BioLabs) of *E. coli* was transformed with the expression construct. The newly transformed *E. coli* were then plated on a Luria Broth (LB) agar plate containing ampicillin (100 $\mu\text{g mL}^{-1}$). The following day, a single colony from the transformation plate was inoculated in 5 mL of LB broth (100 $\mu\text{g mL}^{-1}$ ampicillin). After an overnight growth (16 h, 37°C), plasmids were extracted to confirm correct insertion of the expression plasmid by DNA sequence analysis.

2.2. Cytoplasmic Protein Expression from *E. coli* Transformed with pMALc2x-poMstn1Pro Expression System

A selected colony harboring pMALc2x-poMstn1Pro was inoculated in 5 mL Luria-Bertani (LB) (1.2% tryptone, 0.6% yeast extract and 0.8% NaCl) medium containing 100 $\mu\text{g mL}^{-1}$ ampicillin and grown overnight with vigorous shaking. The seed culture was then transferred into 50 mL fresh LB medium in 250 mL flask. When the culture reached to an optical density of 0.3-0.4 a (600 nm) at 37°C, protein expression was induced for different period by adding Isopropyl β -D-1-Thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM under vigorous shaking. At the completion of induction, cells were harvested by centrifugation at 4,000 g, then resuspended in affinity column buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.5) and disrupted by sonication. The soluble and insoluble fractions were prepared by centrifugation at 10,000 g for 30 min at 4°C. For each sample, the supernatants (soluble fraction) were collected and the same volume of column buffer was used to resuspend the pellets (insoluble fraction). SDS-PAGE analysis of the cell lysates, soluble and insoluble fractions was performed to examine the extent of soluble expressions of the MBP-poMstn1Pro protein.

2.3. Affinity-Purification of Soluble MBP-poMstn1Pro Protein

After induction, cell pellets were harvested by centrifugation at 4,000 g for 20 min at 4°C. Each gram (wet weight) of the cell pellets was resuspended in 5 mL

of column buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.5) containing 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, cells were lysed by sonication in short pulses of 15 sec for 15 min in ice-water bath. Cell lysates were then centrifuged at 20,000 g for 20 min at 4°C and the supernatants (soluble fraction) were collected. The soluble fractions were subjected to amylose resin (New England BioLabs) affinity chromatography following the procedure described previously (Lee *et al.*, 2010). Fractions were analyzed by SDS-PAGE and Western blot to examine the purified proteins.

2.4. Protein Assay

Protein concentration was determined by the Modified Lowry method (Pierce, IL, USA) using BSA as a standard.

2.5. SDS-PAGE Analysis

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.

2.6. Western Blot Analysis

Proteins on SDS-PAGE gels were electrophoretically transferred to PVDF membranes. After blocking with TBST (100 mM Tris-HCl, pH 7.5, 0.9 NaCl and 0.1% Tween 20) with 3% BSA, the membranes were incubated with either monoclonal anti-MBP antibody (New England BioLabs) or monoclonal anti-Mstn antibody (Kim *et al.*, 2006) at 1:10,000 dilution for 1 h at room temperature. After washing with TBST, the membrane was incubated with a goat anti-mouse IgG antibody conjugated to alkaline phosphatase for 1 h at room temperature, followed by color development with the BCIP/NBT substrate (Sigma, St. Louis, MO, USA).

2.7. Examination of the Binding of MBP-poMstn1Pro to Mstn by a Pull-Down Assay

A pull-down assay was performed to analyze the ability of MBP-poMstn1Pro to bind to Mstn. 200 ng of Mstn (R and D Systems, MN, USA) was added to either 1 μ g of affinity purified MBP-poMstn1Pro or 1 μ g of affinity-purified MBP and mixed separately for 2 h at room temperature with 10 μ L 4,000 g for 1 min.

After removing the pass-through, the resin was washed with 1 mL of column buffer three times. The resin was then mixed with 20 μ L of reducing SDS-PAGE loading buffer. After heating the sample at 100°C for 5 min, the loading buffer was carefully removed from the resin. The eluted protein was then analyzed by SDS-PAGE and Western blot.

2.8. Bioactivity Test Using a Luciferase Reporter Assay

A204 rhabdomyosarcoma cells (ATCC, HTB-82) were seeded in a 96 well plate at 20,000 cells 100^{-1} μ L well in DMEM media with 10% fetal bovine serum and penicillin-streptomycin plus fungizone for 24 h at 37°C with 5% CO₂. Cells were then transfected with 0.1 μ g of pGL3-(CAGA)₁₂-luciferase plasmid carrying a firefly luciferase gene under the control of a Smads-binding promoter (CAGA)₁₂ box using FuGENE6 Transfection Reagent (Roche, Mannheim, Germany), as well as 0.05 μ g of pRL-TK plasmid (Promega, Madison, WI, USA) 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 h, medium was replaced with serum-free medium and incubated for 9 h. In each well, 9 ng mL⁻¹ of mouse Mstn (R and D Systems, Minneapolis, MN) and various concentrations of MBP-poMstn1Pro were added and incubated for 6 h. 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) following the manufacturer's suggested procedures. For each protein subjected to bioactivity test, the firefly/renilla luciferase ratio was calculated.

3. RESULTS AND DISCUSSION

3.1. Expression and purification of MBP-poMstn1Pro

The effect of post-induction time at 37°C on soluble expression of MBP-poMstn1Pro was examined in a fixed condition of inducer (IPTG, 0.4 mM) and cell mass concentration (0.3-0.4 at OD₆₀₀). SDS-PAGE analysis showed that increasing the induction time generally increased the proportion of insoluble expression of MBP-poMstn1Pro (**Fig. 1**). The soluble fraction was not visibly increased beyond 3 h after induction, thus 3 h post-induction was used for subsequent expression experiments.

MBP binds to amylose, thus amylose resin affinity chromatography was used to purify MBP-poMstn1Pro

from the soluble fraction of cell extract. The result of SDS-PAGE analysis of the affinity purified MBP-poMstn1Pro is shown in **Fig. 2**. Compared to the crude extract (lane 1), eluted fractions contained predominantly MBP-poMstn1Pro (lane 3-6) with minor amounts of proteins at various sizes. About 51 mg of affinity-purified MBP-poMstn1Pro were harvested per liter of *E. coli* culture (**Table 1**). This amount is more than 150 fold increase as compared to the amount from our previous study in which about 320 μg of affinity purified MBP-poMstn1Pro was obtained per liter of culture (Lee *et al.*, 2010).

The host strain used in this study was K12TB1, while Rosetta-gami 2(DE3) pLysS strain was used as a host strain in the previous study (Lee *et al.*, 2010) because this strain showed the most soluble expression of MBP-poMstn1Pro among five tested strains. At the same time, in the previous study, the expression was induced at 12.5°C for 24 h because that 12.5°C induction resulted in the most soluble expression of the recombinant protein while 37 and 25°C inductions resulted in a large proportion of insoluble expression in the strain. Unlike the Rosetta-gami 2(DE3) pLysS strain, the K12TB1 strain was able to express the MBP-poMstn1Pro in soluble forms at 37°C in the current study, indicating that host strain is a factor affecting the yield of soluble expression of MBP-poMstn1Pro.

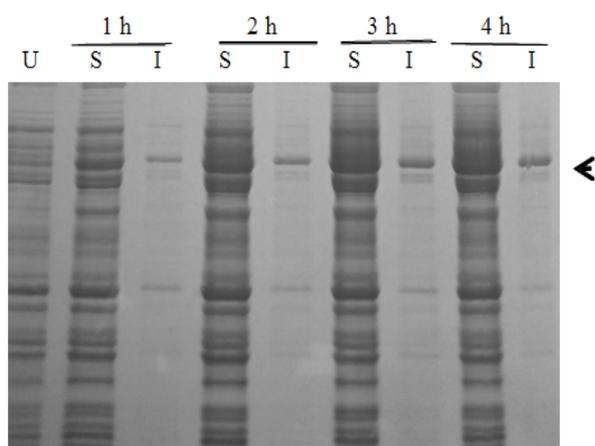


Fig. 1. Effect of post-induction time (1, 2, 3 and 4 h) on soluble expression of MBP-poMstn1Pro. Arrow head indicates the MBP-poMstn1Pro protein. U, uninduced; S, soluble fraction (supernatant); I, insoluble fraction (pellet)

Induction temperature is known to be an important factor affecting soluble expression of recombinant protein in *E. Coli* and lowering induction temperature usually helps soluble expression by providing more time for proper folding due to delayed translational process (Blank *et al.*, 2006; Larsen *et al.*, 2008; Liu *et al.*, 2006). Given that the total protein of cell pellet was only 21.2 mg L⁻¹ culture in the 12.5°C induction condition as compared to 281 mg L⁻¹ culture in the current study, it appears that lower induction temperature can be a limiting factor in achieving a high level of expression of recombinant proteins in *E. coli* even though lower induction temperature support the soluble expression of recombinant proteins.

3.2. Binding of MBP-Pomstn1pro to Mstn and Suppression of Its Activity

MstnPro inhibit Mstn activity by complex formation with the active form of Mstn, thus we performed a pull-down assay with Mstn to determine the binding of MBP-poMstn1Pro to Mstn.

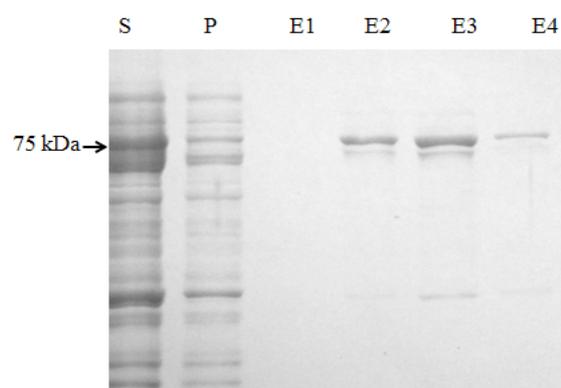


Fig. 2. SDS-PAGE analysis of affinity purification of MBP-poMstn1Pro. The soluble crude extract (S) obtained from 250 mL culture was applied to 10 mL of amylose affinity matrix. After washing, proteins immobilized to the amylose column were then eluted with an elution buffer containing maltose (E1, E2, E3 and E4). P, pass-through

Table 1. Yields of poMstn1Pro protein recovered from each purification step

Steps	$\mu\text{g mL culture}$	Yield (%)
Total protein	281±8.2	100.0
Soluble protein	230±4.6	81.9
Affinity chromatography	51±1.2	18.1

Protein concentration was measured by modified Lowry method using BSA as a standard. The protein recovery was calculated as the mean±SEM from the purifications of a triplicate of 250 mL culture

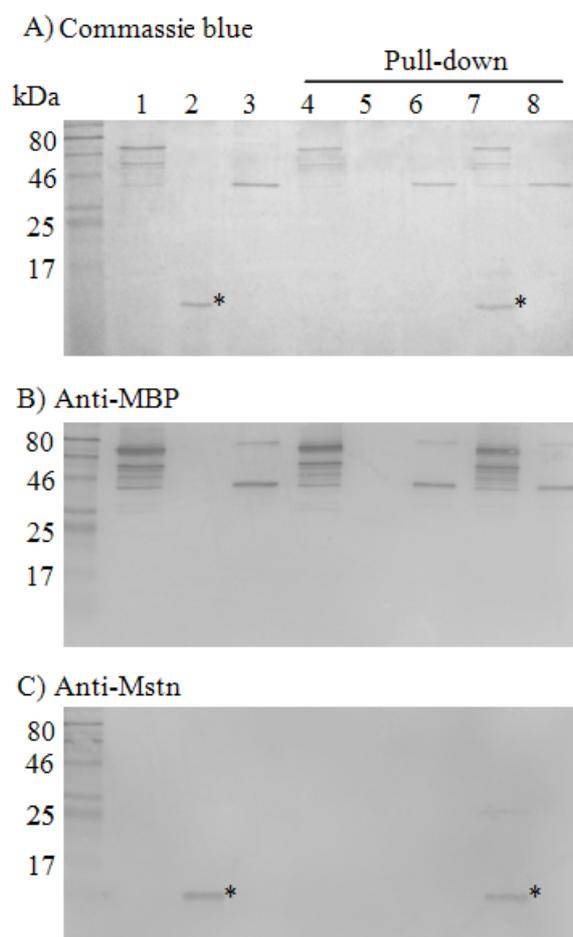


Fig. 3. SDS-PAGE (A) and Western-blot analysis (B, C) of MBP-poMstn1Pro binding to Mstn in a pull-down assay. Amylose resin was mixed with either 1 μ g of MBP-poMstn1Pro (lane 4), 200 ng of Mstn (lane 5), 1 μ g MBP (lane 6), 1 μ g of MBP-poMstn1Pro plus 200 ng of Mstn (lane 7) or 1 μ g of MBP plus 200 ng of Mstn (lane 8). The column was then washed with column buffer three times. Proteins bound to the amylose resin were then eluted with reducing SDS-PAGE loading buffer and subjected to SDS-PAGE analysis. Western-blot analysis was performed with either anti-MBP (B) or anti-Mstn (C) antibodies. Lane 1, MBP-poMstn1Pro (72 kDa); lane 2, Mstn (12.5 kDa); lane 3, MBP (42 kDa). Asterisks (*) indicate Mstn monomer

Mstn was mixed with either MBP-poMstn1Pro or MBP and incubated with amylose resin. After washing, the eluted fractions from the amylose resin were analyzed by SDS-PAGE and Western-blot analysis.

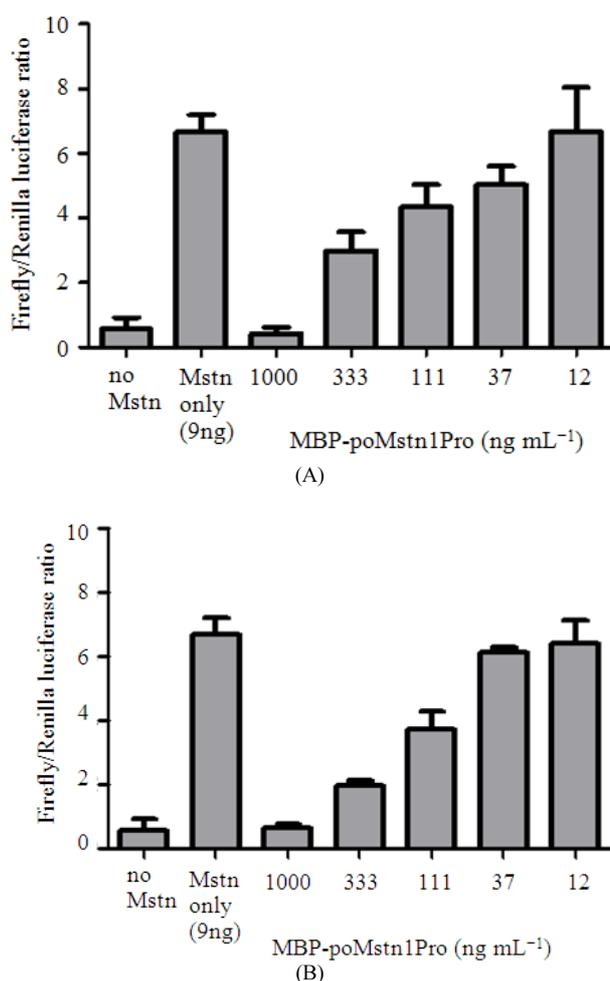


Fig. 4. Inhibition of Mstn activity by (A) MBP-poMstn1Pro produced in K12TB1, (B) MBP-poMstn1Pro produced in Rosetta-gami2 (DE3) pLysS

Figure 3 shows the results of SDS-PAGE and Western-blot analysis of the pull down assay. Mstn by itself were not able to bind to the amylose resin (Lane 5). However, Mstn was in the eluted fraction of amylose resin when the mixture of Mstn and MBP-poMstn1Pro was loaded into the resin (lane 7), indicating the binding of MBP-poMstn1Pro to Mstn. When the mixture of Mstn and MBP was loaded into the amylose resin, Mstn was not in the eluted fraction while MBP was in the eluted fraction, demonstrating that MBP has no binding affinity to Mstn. The above results, thus, together indicate that N-terminal fusion of MBP to poMstn1Pro does not affect the binding of poMstn1Pro to Mstn.

MstnPro is proteolytically cleaved off from Mstn precursor proteins during post-translational processing and binds to active Mstn to suppress Mstn activity by preventing its binding to its receptor (Hill *et al.*, 2002; Thies *et al.*, 2001; Zimmers *et al.*, 2002). Since the binding of MBP-poMstn1Pro to Mstn was demonstrated in a pull-down assay, we examine the ability of affinity-purified MBP-poMstn1Pro to inhibit the biological activity of Mstn using the pGL3-(CAGA)₁₂-luciferase reporter assay system in which various concentrations of MBP-poMstn1Pro plus 9 ng mL⁻¹ Mstn were added to A204 cells transiently transfected with the pGL3-(CAGA)₁₂ plasmid. Twelve CAGA sequences, a transcriptional regulatory element responsive to TGF- β family proteins including MSTN, are inserted into the pGL3-(CAGA)₁₂ luciferase reporter plasmid construct in the upstream of the promoter region of firefly luciferase. Thus, A204 cells transiently transfected with the pGL3-(CAGA)₁₂ luciferase reporter plasmid are responsive to Mstn signaling, exhibiting a quantitative luciferase activity proportional to the amount of Mstn being added to the culture (Thies *et al.*, 2001). Current assay result showed that the MBP-poMstn1Pro produced in this study suppressed Mstn activity (**Fig. 4A**). The MBP-poMstn1Pro produced in the Rosetta-gami 2(DE3) pLysS strain also demonstrated its Mstn-inhibiting activity when examined in the same experiment (**Fig. 4B**). The concentration for half inhibition of Mstn was at around 111 ng mL⁻¹ for both proteins, indicating that the folding of MBP-poMstn1Pro was not affected by the two different induction conditions with the two *E. coli* strains.

4. CONCLUSION

Significant improvement has been made in the yield (51 mg L⁻¹ *E. coli* culture) of bioactive, affinity-purified flatfish Mstn1Pro fused to MBP, illustrating that a large-scale production of bioactive fish Mstn1Pro is feasible in *E. coli*. The N-terminal fusion of MBP in the flatfish Mstn1Pro did not affect either the binding of flatfish Mstn1Pro to Mstn or its Mstn-suppressing activity, indicating that fish Mstn1Pro containing MBP fusion partner can be used directly without removing the MBP fusion tags in suppressing Mstn activity. The ability to produce large quantity of bioactive fish Mstn1Pro in a cost-effective way will be useful in future studies examining the potentials of Mstn1Pro as an agent to enhance muscle growth in aquaculture species or in studies investigating its binding characteristics of Mstn1Pro with its partners.

5. ACKNOWLEDGEMENT

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