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
Mstn-based strategies to improve skeletal muscle growth in agriculturally important animal species, including fish (Rodgers and Garikipati, 2008; Tsuvida, 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 reported in transgenic zebrafish over expressing MstnPro proteins of various species. However, the yield of purified protein was too low (about 700 µg 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 lacI⁰ 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 µg mL⁻¹). The following day, a single colony from the transformation plate was inoculated in 5 mL of LB broth (100 µg 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 µg 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 vigourous 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-Cl, 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 µg of affinity-purified MBP-poMstn1Pro or 1 µg of affinity-purified MBP and mixed separately for 2 h at room temperature with 10 µL 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 µL 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 µl 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 µg 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 µg 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.

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. 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)](image)

![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](image)

![Table 1. Yields of poMstn1Pro protein recovered from each purification step](image)
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.
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-$\beta$ 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.

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