PRODUCTION AND BIOACTIVITY POTENTIAL OF THREE RECOMBINANT GROWTH HORMONES OF FARMED FISH

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ABSTRACT

This study was aimed to produce recombinant growth hormone (rGH) from giant grouper (*Epinephelus lanceolatus*), giant gouramy (*Osphronemus gouramy*) and common carp (*Cyprinus carpio*) and compare their bioactivity potential by means of inducing the growth hormone of juvenile Nile tilapia (*Oreochromis niloticus*) as the model. DNA fragment encoding mature GH protein of giant grouper (*El*-mGH), giant gouramy (*Og*-mGH) and common carp (*Cc*-mGH) was amplified by PCR method. The purified PCR products were ligated to pCold-1 to generate pCold/*El*-mGH, pCold/*Og*-mGH, and pCold/*Cc*-mGH protein expression vector, respectively. Each of the expression vectors was transformed into the *Escherichia coli* BL21. *E. coli* BL21 was cultured using 2xYT medium and protein production was induced by cold shock at $15\pm1^{\circ}$C for overnight. The inclusion bodies of *E. coli* transformants containing protein expression vector were isolated by sonication method, and rGH production was analyzed by SDS-PAGE. Juvenile of Nile tilapia of average body weight of 12.41 ± 3.28 g was intramuscularly injected once a week for 4 weeks with 1 µg inclusion body containing rGH per gram fish body weight. The result showed that rGH in molecular weight of about 25 kDa was obtained. Fish injected with rGH of *El*-mGH, *Cc*-mGH and *Og*-mGH grew 20.94%, 18.09%, and 16.99% faster, respectively, compared with the control. This result indicated that the three rGH produced in *E. coli* possessed biological activity when tested on Nile tilapia and further research is needed to find its effect on the growth of other aquaculture fish species.

KEYWORDS: bioactivity, recombinant protein, growth hormone, Nile tilapia

INTRODUCTION

Various genetic manipulation techniques have been developed to improve the growth of farmed fish such as breeding selection, protein recombination, and transgenesis. The process of selective breeding requires a relatively long time to achieve significant results because the genetic gain per generation is relatively low, as reported by Bolivar & Newkirk (2002) on selective breeding of tilapia where 12 generations were required to double the growth rate. Over-expression of growth hormone (GH) gene using transgenesis technology can drastically improved fish growth (Nam

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et al., 2001; Kobayashi *et al*., 2007). Transgenesis needs shorter time to achieve a significant result (three generations) compared to selection method (more than three generations). However, transgenesis can only be performed on fish which their eggs can artificially be fertilized, or one cell stage embryo can be collected before gene construct is transferred.

Numerous studies have demonstrated that injection of native or recombinant GH (rGH) to different fish species resulted in growth enhancement (Funkenstein, 2006), indicating that increased levels of GH have the potential to improve growth of fish. GH can also be administered to fish by oral (Moriyama *et al*., 1993; Tsai *et al*., 1997, Jeh *et al*., 1998) and immersion method (Moriyama & Kawauchi, 1990). Enhancement of growth on several fish species after rGH protein administration had been reported such as rainbow trout (*Onchorhynchus mykiss*) using salmon rGH (Moriyama *et al*., 1993), Japanese flounder (*Paralichtys olivaceus*) using flounder rGH (Jeh *et al*., 1998) and common carp using giant catfish (*Pangasianodon gigas*) rGH (Promdonkoy *et al*., 2004).

In this study, we designed three protein expression vectors producing rGH by *Escherichia coli* and then analyzed their bioactivity by injecting them in juvenile of Nile tilapia as the model. This study is a first step to find an alternative way in order to increase the growth rate of farmed fish in Indonesia to support the Ministry of Marine Affairs and Fisheries aquaculture production target on 2014.

MATERIALS AND METHODS

Fish Maintanance

Juveniles of Nile tilapia (average body weight of 12.41±3.28 g) were obtained from Freshwater Aquaculture Research Center, Bogor. They were maintained in indoor aquaria (90 L) under natural photoperiod and fed with a commercial diet to satiation three times daily. Water exchange of fish rearing media was 50% of the total volume conducted every day before feeding was performed.

Construction of Protein Expression Vector

DNA fragments coding for signal peptide and mature GH protein (mGH) was predicted by using SignalP 3.0 software (*http://cbs.dtu.dk/ services/signalP*). mGH DNA fragment was amplified by PCR with a template of plasmid containing the full-length of GH cDNA from giant gouramy (Nugroho *et al*., 2008), giant grouper (Mulyadi *et al*., 2008) and common carp (Alimuddin, *unpublished*) using a set of primer for each species. Primer sets for giant grouper mGH (*El*-mGH) DNA fragment amplification were *El*-mGH-F (5'-ctcgag cag cca atc aca gac ggc cag-3') and *El*-mGH-R (5'-aagctt cta cag ggt aca gtt ggc ctc agg-3') containing *Xho* I and *Hind* III recognition sites (underlined) respectively. Primer sets for giant grouper mGH (*Og*-mGH) were *Og*-mGH-F (5'- ggatcc cag cca atc aca gac agc cag-3') and *Og*-mGH-R (5'-gaattc cta cag agt gca gtt agc ttc tgg-3'), containing *Bam* HI and *Eco* RI recognition sites (underlined) respectively. Further, primers for amplification of common carp mGH (*Cc*-mGH) DNA fragment were *Cc*-mGH-F (5'-ggatcc tca gac aac cag cgg ctc ttc-3') and *Cc*-mGH-R (5'-gtcgac cta cag ggt gca gtt gga atc cag-3') containing *Bam* HI and *Sal* I recognition sites (underlined), respectively. The PCR products were purified, cloned into the pGEM-T Easy vector (Promega), and used for DNA sequencing. DNA sequencing was carried out using ABI PRISM® 3100-Avant Genetic Analyzer following the manufacturer's protocols.

Plasmid pGEM-T Easy containing the mGH DNA fragment was digested with two restriction enzymes as described in the cloning of each fish mGH above. The mGH fragment was ligated into the corresponding sites of the expression vector pCold-1 (4.4 kb; Takara) controlled by *cspA* promoter. The plasmid construct was designated as pCold/*El*-mGH, pCold/ *Cc*-mGH and pCold/*Og*-mGH for giant grouper, common carp and giant gouramy mGHs, respectively.

Production and Bioactivity Analysis of rGH Protein

E. coli BL21 (DE3) harboring pCold/*El*-mGH, pCold/*Cc*-mGH, or pCold/*Og*-mGH was incubated at 15ºC for 24 hours in a 2xYT medium (Alimuddin *et al*., 2008) containing 100 μg of ampicillin per mL and 1 mM IPTG (isopropyl-b-D-thiogalactopyranoside) for induction of protein synthesis. The bacterial cells were harvested by centrifugation for 5 min at 13,000 rpm in a refrigerated centrifuge, resuspended in phosphate buffer saline (PBS), washed twice by the same solution, sonicated twice for 30 s on ice, centrifuged again and the resultant pellet of

inclusion bodies was resuspended in PBS containing SDS and β-mercaptoethanol. Sample were heated at 100°C for 5 min, chilled on ice and then centrifuged briefly. An aliquot of 10 µL was analyzed by SDS-PAGE with a concentration of 10% polyacrylamide gel, and protein was visualized using Coomassie Blue dye.

A total of 100 Nile tilapia juveniles were randomly divided into five groups with 20 juveniles per group. The juvenile in each group were stocked in the two separate aquaria. Three fish groups were intramuscularly injected once a week for 4 weeks with 1 µg inclusion body containing rGH per gram fish body weight. The inclusion body was dissolved in phosphate buffer saline (PBS). The last two groups were as controls, i.e. first fish groups was injected with inclusion body from bacteria harboring pCold 1-empty without insertion of mGH DNA fragment, and the second was injected with an equal volume of PBS. The juvenile were fed with commercial diet three times daily at satiation. Feeding and water management were the same for all treatments. Individual body weight was measured every week for 2 months. Data obtained were descriptively analyzed.

RESULTS AND DISCUSSION

Construction of Protein Expression Vector

PCR amplification products of mGH DNA fragment for giant grouper (*Og*-mGH), common carp (*Cc*-mGH) and giant grouper (*El*-mGH) were approximately 576 bp, 579 bp, and 576 bp in length (Figure 1 left), respectively. As predicted and shown in Figure 1 (right), the size of pGEM-T Easy containing mGH DNA

fragment (resulting plasmid referred to as pT-mGH) was bigger than that of pGEM-T Easy (3 kb; T). This indicated that ligation and transformation processes were successful.

In order to isolate mGH DNA fragment from pT-mGH, the plasmid was digested by 2 restriction enzymes as described above. As shown in Figure 2 (left, lane 1), the restriction produced 2 DNA bands, i.e. pGEM-T Easy (3.0 kb) and mGH DNA fragments (~0.6 kb). The purified mGH DNA fragment containing 2 restricted sites at the 5' and 3' end, respectively, could then be used to construct protein expression vector. Further, pCold-1 expression vector was also digested by the same restriction enzymes used in the isolation of mGH DNA fragment from pT-mGH, to prepare a ligation site of mGH DNA fragment into pCold-1. The size of digested pCold-1 (Figure 2, center and lane 3) seemed bigger than the undigested pCold-1 (Figure 2, center and lane 2), though their size was similar. Visually, different in DNA size was caused by difference of DNA mobility in the electrophoresis gel between the linear digested pCold-1 and the supercoiled undigested pCold-1. A linear DNA moves slower than the supercoiled, but it faster than an open circular (Li *et al.*, 1991).

Further, the mGH DNA fragment was ligated with the linear pCold-1 and then transformed into *E. coli* DH5α. DNA plasmid of ligation product from three bacterial clones for each mGH fish was then sequenced to identify the bacterial clones which carry the plasmid containing mGH DNA sequence similar with the references reported earlier. From the sequencing results (data not shown), one clone bacteria for pCold/*Og*-mGH, two clones for pCold/*El*-mGH and pCold/*Cc*-mGH contained mGH DNA
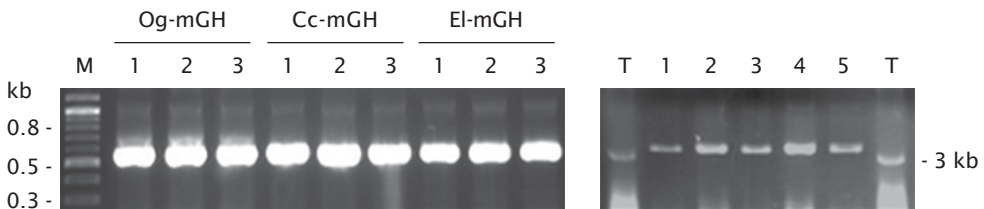

Figure 1.   Left: PCR product of DNA fragment coding for mature GH (mGH) from giant gouramy (*Og*-mGH), common carp (*Cc*-mGH) and giant grouper (*El*-mGH), and right: cracking the bacterium *E. coli* DH5α containing ligation product of pGEM-T Easy (T) and mGH DNA fragment. Lanes 1 through 3 in figure-left and 1 through 5 in figure-right were the number of sample for each fish GH and bacterial colony, respectively. M was 2-log ladder DNA marker (BioLabs Inc., New England)
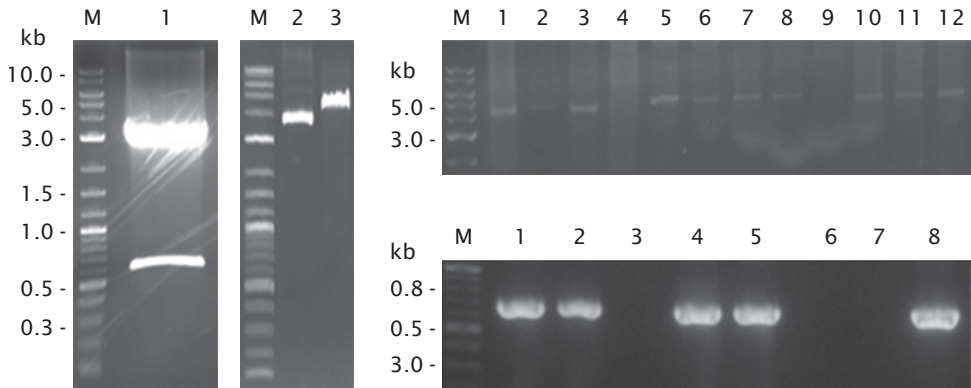
Figure 2.   Left: pT-mGH restriction resulted 3.0 kb pGEM-T Easy (pT) and ~0.6 kb mGH DNA fragments. Center: supercoiled pCold-1 plasmid (lane 2) and linear digested pCold-1 (lane 3). Right-top: cracking product of *E. coli* BL21 carrying pCold-mGH plasmid; lanes 1 through 12 were the number bacterial colonies. Right-bottom: PCR products in analysis of insert orientation in pCold-1 vector; lanes 1 through 8 were the number bacterial colonies. M was 2-log ladder DNA marker (BioLabs Inc., New England)

sequence as in the database. Plasmid from those *E. coli* DH5α clones was transformed into *E. coli* BL21 for recombinant protein expression. As shown in Figure 2 (right-top), almost the transformed *E. coli* BL21 carried the plasmid. The pCold-mGH plasmid was then isolated from the bacteria to verify orientation of DNA ligation using PCR method with pCold forward and mGH-R primers. The size of PCR amplification product was about 0.65 kb consisting of 0.6 kb mGH DNA and 0.05 kb of pCold-1 fragments. Figure 2 (right-below) showed 5 of 8 bacteria clones carrying the pCold-mGH with proper insert orientation. Totally, it was obtained 10 bacterial colonies for pCold/*Og*-mGH, 10 for *pCold/El*-mGH and 6 for *pCold/Cc*-mGH.

Production of Recombinant Protein rGH

The rGH protein for giant gouramy (*Og*-mGH), common carp (*Cc*-mGH) and giant grouper (*El*-mGH) in *E. coli* BL21 using pCold-1 expression vector successfully produced (Figure 3, showed by arrow head). The predicted size of rGH was about 22 kDa, smaller than the size of rGH showed in Figure 3 (25 kDa). The additional size of 3 kDa was from His-tag, Factor Xa site and multi cloning sites located at the upstream of GH sequence in the vector. Further, a 200 mL of liquid culture medium could produce 0.93 grams of bacterial cells containing rGH. Using the software Totallab TL 120,
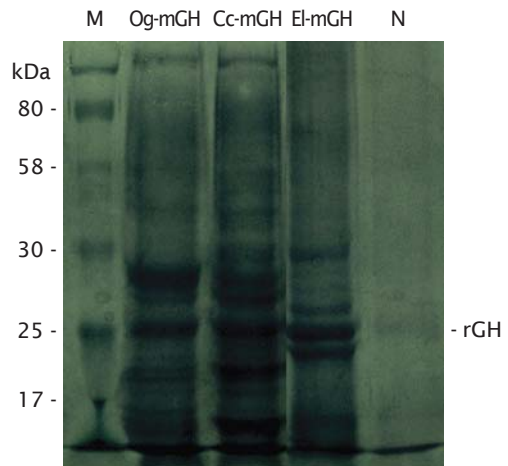

Figure 3.   SDS-PAGE of recombinant protein growth hormone (rGH) for giant gouramy (*Og*-mGH), common carp (*Cc*-mGH) and giant grouper (*El*-mGH). rGH protein was showed by the arrow head. M was prestained protein markers (Biolabs) and N was proteins from *E. coli* BL21 carrying pCold-1 empty (no mGH DNA insertion)

rGH production levels of the three fish species were relatively similar and ranged from 8.00% to 12.04% of the total protein produced
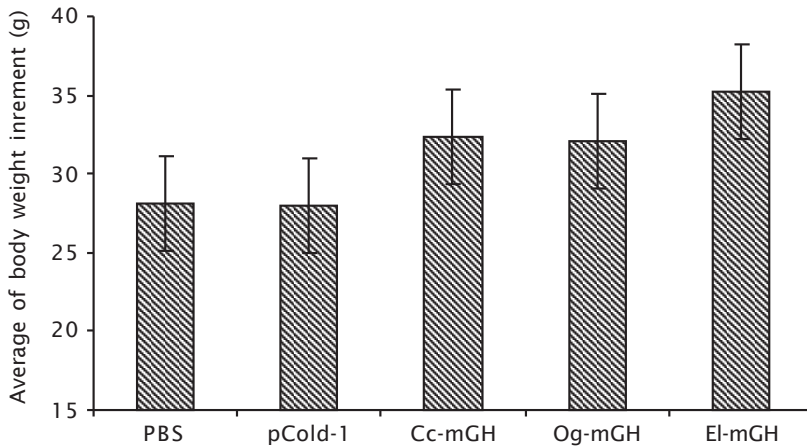
Figure 4. The increment of Nile tilapia body weight (g) after injected with the inclusion bodies containing rGH from giant gouramy (*Og*-mGH), common carp (*Cc*-mGH) and giant grouper (*El*-mGH). Inclusion bodies of 1 μg per gram Nile tilapia body weight was injected once a week for four weeks, and fish growth was measured every week for two months. PBS: fish injected with phosphate buffer saline, and pCold-1: fish injected with inclusion bodies from bacteria containing pCold-1 empty without insersion of mGH DNA fragment

by *E. coli* BL21, though other proteins varied. The rGH production levels obtained in this study were relatively similar with the earlier studies for tuna GH (Sato *et al.*, 1988), tilapia GH (Rentier-Dulue *et al.*, 1989) and yellowfin progy GH (Tsai *et al.*, 1995), i.e. 5%-10% of total protein. In addition, *E. coli* BL21 carrying pCold-1 empty (without mGH DNA insertion) produced no rGH and other insoluble proteins as in *E. coli* BL21 carrying pCold-mGH. However, variation in protein production levels in *E. coli* BL21 carrying pCold-mGH and no other insoluble proteins produced in *E. coli* BL21 carrying pCold-1 empty remained to be elucidated.

Bioactivity of rGH Protein in Nile tilapia

The growths of Nile tilapia injected with rGH, protein from bacteria carrying pCold-1 empty, and PBS as control were compared to determine the bioactivity of rGH protein. As shown in Figure 4, the average increment of fish body weight injected with rGH was sligtly higher compared with the controls (PBS and protein of pCold-1 empty injected fish). This indicated that rGH protein possessed bioactivity in terms of increasing the growth of Nile tilapia as a model. The increment of fish body weight after injected with rGH of *El*-mGH

(20.94%) was slightly higher than the two other rGHs (18.09% for the Cc-MGH and 16.99% for the *Og*-mGH). Thus, the bioactivity of *El*-mGH in Nile tilapia was higher compared to *Gg*-mGH and *Cc*-mGH, while *Gg*-mGH and *Cc*-mGH were relatively similar.

Discussion

Application of recombinant technology for fish GH as a strategy to improve the growth has been reported by various researchers (Tsai *et al.* 1997; Promdonkoy *et al.* 2004). In this study, as a first step towards growth enhancement of farmed fish species, rGH of three farmed fish had been produced in *E. coli*. Expression vector used was pCold-1, while earlier studies were generally used pET as the expression vector (Sekine *et al.*, 1985; Sato *et al.*, 1988; Tsai *et al.*, 1995). Both expression vectors were induced with IPTG to produce recombinant protein, but pCold-1 could also be induced using low temperature incubation (15ºC). Production levels of rGH using pCold-1, 8.0%-12.0% were comparable to that of studies using other vectors, such as pET; 5.0%-15.0% (Sekine *et al.*, 1985; Sato *et al.*, 1988; Tsai *et al.*, 1995). Nucleotide sequences of the three fish mGH used in this study were different; the homology between giant gouramy *versus*

giant grouper was 84.93%, giant gouramy *versus* common carp was 60.73%, and giant grouper *versus* common carp was 61.61%). Thus, variation in rGH production may be caused by *E. coli* preference to codon sequence in translation process (Seetharam *et al.*, 1988), bacterial culture system, and construction of vector expression used. In addition, variation in fish model used in rGH bioactivity analysis may be also taken into account.

The dose of inclusion bodies containing rGH injected in the Nile tilapia was the same among the treatments. Growth of rGH-injected Nile tilapia was higher compared to that of non-injected rGH control. Thus, rGH produced in this study was biologically functioned as a growth stimulator. Growth enhancement of *El*-mGH-injected Nile tilapia was slightly higher compared to that of *Gg*-mGH and *Cc*-mGH, while the last two was similar. As shown in Figure 3, number of protein derived from *El*-mGH recombinant bacteria was less than that of *Gg*-mGH and *Cc*-mGH. Proportion of rGH in *El*-mGH contained inclusion bodies was higher than that of *Gg*-mGH and *Cc*-mGH. Thus, the differences on the growth stimulation of rGH may be resulted from the variation on the actual doses of rGH injected. Injection using a purified rGH needs to be performed to obtain a real bioactivity of the three rGHs.

The delivery method of rGH applied in this study was injection. This technique might be impractical for farmers. By technical considerations and the efficiency of rGH application in fish farming, further research needs to be conducted to determine a proper delivery method. Mass delivery method of rGH, such as through supplementation in commercial diet (Moriyama *et al.*, 1993; Tsai *et al.*, 1997; Jeh *et al.*, 1998) and immersion (Moriyama & Kawauchi, 1990; Moriyama *et al.*, 2008) have been developed. Supplementation of rGH in the diet of black snapper juvenile improved the growth up to 60% (Tsai *et al.*, 1997). Immersion of fish juvenile in the medium containing rGH enhances the growth, such as 43% in coho salmon and about 60% in chum salmon (Moriyama & Kawauchi, 1990). In addition, immersion of Nile tilapia larvae in the water containing rGH-produced *Pichia pastoris* can drastically enhanced the growth; 171% (Acosta *et al.*, 2007). In our ongoing study, immersion of giant gouramy larvae in the water containing giant gouramy rGH (*Og*-mGH) improved the body weight; almost 2-fold higher compared to the control. Thus, immersion method may be as an effective and suitable way to deliver rGH to improve farmed fish growth for fish farmers.

CONCLUSIONS

1. Recombinant growth hormones (rGH) of giant gouramy, common carp and giant grouper have been produced in *E. coli.*
2. The three rGHs possessed bioactivity in terms of accelerating the growth in Nile tilapia.

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