Pakistan J. Zool., vol. 46(4), pp. 975-981, 2014.

Pichia pastoris Expressing Recombinant *Misgurnus anguillicaudatus* Growth Hormone Promotes the Growth of *M. anguillicaudatus* Larvae

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Abstract.- Growth hormones (GH) can promote fish growth, however, it is extremely low in fish body and difficultly to purify. The study attempted to address this problem by introducing gene engineering technology. The HIS4 mutant yeast *Pichia pastoris* GS115 strain was transformed with a constructed vector pPIC9K containing *misgurnus anguillicaudatus* Growth Hormone (maGH) cDNA under the control of alcohol oxidase (aox1) gene promoter. PCR analysis was employed in verifying the stable integration of introduced DNA. SDS-PAGE analysis indicated that recombinant *M. anguillicaudatus* GH (rmaGH) had been expressed and exported into the culture medium after methanol induction. The production peaked at 72 h after induction, and the yield was as high as 300 mg/L in shaking-flask fermentation medium, accounting for 53% of the total secreted proteins. By feeding experiment to larvae, rmaGH showed the significant growth-promoting effects in body weight and length on *M. anguillicaudatus* compared with the control group ($P<0.01$) at 6 weeks. These results were valuable to develop a new fish growth promoting agent.

Key Words: *Misgurnus anguillicaudatus*, growth hormone, *Pichia pastoris*, secretion expression, growth-promoting effect, recombinant protein.

INTRODUCTION

The growth hormones (GH), prolactin, placental lactogen, and somatolactin, belong to the family of pituitary hormones, which share several similarities in structure, function, and gene organization (Acosta *et al*., 2008). GH are single-chain polypeptide hormones of about 22 kDa, secreted by the anterior pituitary gland, which regulate diverse and essential physiological processes. They are involved in the regulation of somatic growth, development (Xu *et al*., 2013), and reproduction through modulation of steroidogenesis, gametogenesis, ovulation, and gonadal differentiation (Hull and Harvey, 2001), and also govern gonadotropin secretion and responsiveness (Munakata *et al*., 2007), osmoregulation (Almeida *et a*., 2013), appetite (Johnsson and Bjornsson, 1994), behavior (Ojima and Iwata, 2009), and immunologic function (Shved *et al*., 2011) across vertebrates.

Previously, a number of cDNAs of fish GH have been cloned and expressed in *Escherichia coli* (Sekine *et al*., 1985; Bai *et al*., 1999; Wang *et al*., 2001). The recombinant GH expressed in *E. coli* largely contribute to several physiological processes in fish, and have been widely used in aquaculture (Poen and Pornbanlualap, 2013). However, as *E. coli* is a prokaryote and its intrinsic characteristics differ from those of eukaryotes, the protein product may be typically obtained as insoluble, misfolded inclusion bodies, requiring subsequent solubilization and re-folding steps. Therefore, *E. coli* is generally not suitable for the expression of proteins that contain a high level of disulfide connectivity or proteins that require post-translational modifications such as glycosylation, proline cis/trans isomerization, disulfide isomerization, lipidation, sulfation, or phosphorylation (Daly and Hearn, 2005; Macauley-Patrick *et al*., 2005; Zhu *et al*., 2014). These limitations have prompted biotechnologists to seek new production systems. With the development of eukaryotic expression systems, several fish GH have been expressed in the yeast *Pichia pastoris* (Acosta *et al*., 2007; Li *et al*., 2003). *P. pastoris* is an efficient host for heterologous gene expression using the promoter from the methanol-induced

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0030-9923/2014/0004-0975 $ 8.00/0

alcohol oxidase 1 gene. Owing to its extensive application in the commercial production of various foreign proteins (Daly and Hearn, 2005; Acosta *et al*., 2007; Peng *et al*., 2013), in the present study, *P. pastoris* was selected as the expression system, and the *Misgurnus anguillicaudatus* GH (maGH) was cloned and expressed in it.

The objective of this study was to establish a system with high-level expression of maGH in *P. pastoris* and evaluate the effect of the recombinant *P. pastoris* on the growth of *M. anguillicaudatus* fry that were immersed into water containing this recombinant maGH. The results showed that the recombinant *P. pastoris* had significant growth-promoting effects on *M. anguillicaudatus.* The present study provides important information relevant to the use of recombinant GH for the enhancement of the growth rate of fish in aquaculture.

MATERIALS AND METHODS

Preparation of total RNA and RT-PCR

M. anguillicaudatu pituitary was dissected and immediately homogenized in the medium provided by the RNA isolation kit (TaKaRa, Japan), The cDNA synthesis was carried out with the PrimeScript^{TM} RT Reagent Kit (TaKaRa, Japan), A forward primer
(FP: 5'GCTGGAATTCTCAGAGAACCAAAGGCTCTT3')
and a reverse primer
(RP: 5'CACTGCGGCCGCTTACTACAGGGTGCAGTTG3')
were designed from *M. anguillicaudatu* cDNA growth hormone sequence (GenBank Accession No.DQ350433) for the amplification of the mature GH. The forward and reverse primers contain the restriction sites for *EcoRI* and *NotI* restriction endonuclease (underlined), respectively. The reaction mixture of PCR was first kept at 94°C for 5 min, then 35 cycles of PCR (94°C for 40 s, 50°C for 40 s, 72°C for 1 min) were done, and the sample finally kept at 72°C for 10 min. PCR products were separated in 1% agarose gels.

Construction of the expression vector pPIC9K-maGH

The PCR product of approximately 570 bp was digested by *BamHI* and *NotI* and subcloned into the *P. pastoris* shuttle expression vector pPIC9K, which was previously digested with the same enzymes to generate the expression plasmid. The vector contains the alcohol oxidase (aox1) promoter from *P. Pastoris* and the histidine4 gene (HIS4) as selection marker. As the resultant plasmid, pPIC9K-maGH was sequenced using α-factor pPIC9K Primer. Insertion of the PCR product was verified by restriction enzyme digestion, electrophoresis; PCR and sequencing (Shanghai Sangon Biotech, China).

Transformation of Pichia pastoris *and screening for Mut^{+} phenotype*

The expression plasmid was linearized with *Sal I* and transformed into *P. pastoris* strain GS115 by lithium chloride method. The transformation mixture was plated on selective medium MD [1.34 % (w/v) yeast nitrogen base, 4×10^{-5} biotin, 2% (w/v) agar, 2% (w/v) glucose]. Colonies were visible after 2-3 days at 30°. The target gene in the recombinants was detected by a genomic PCR assays using the
5'AOX1 (5'-GACTGGTTCCAATTGACAAGC-3') and
3'-AOX1 (5'-GCAAATGGCATTCTGACATCC-3')
primers (Shanghai Sangon Biotech, China) to screen for methanol utilization plus (Mut^{+}) and methanol utilization slow (Mut^{S}) phenotypes. The GS115 clone recombinant with pPIC9K plasmid was used as negative control (GS115/pPIC9K).

Methanol-induced maGH expression in Pichia pastoris *and SDS–PAGE analysis*

Fermentations of selected clones were done in 250 ml shake-flasks. Recombinant colonies (GS115/pPIC9K-maGH) with methanol utilization plus (Mut^{+}) phenotype were inoculated into 10 ml of yeast peptone glucose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone and 1% (v/v) glucose as carbon source] for 24 h under the condition of 30□ in a shaking incubator (250 rpm). Then, clones of Mut^{+} yeast were inoculated in liquid BMGY medium (1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% yeast nitrogen base; 4×10^{-5} biotin; 1% glycerol) and grown at 30°C in a shaking incubator for 30 h until the culture reached an absorbance at 600 nm (A600) of 10 units. Cells were harvested by centrifugation

and gently resuspended in 25 ml of Buffered Methanol-complex Medium (the ingredient was same as BMGY but containing 0.5% methanol instead of 1% glycerol as carbon source) to induce expression of recombinant maGH through the AOX1 promoter. Absolute methanol was added continuously to maintain a final concentration of 0.5% (V/V) for 120 h at 30°. The proteins of the culture supernatant were precipitated by trichloroacetic acid (TCA) and resuspended in reducing sample loading buffer. They were analyzed by Coomassie stained 15% SDS-polyacrylamide gel with 5% stacking gel and the protein lane scanned by gel image analysis system BandScan to calculate the percentage of rmaGH.

Biological activity assays

M. anguillicaudatus larvae at 2 days post hatching were acclimatized in tanks (500 l) with fresh water for one week prior to the experiment. Five experimental groups (n = 200 for each group) were immersed into the water which containing the following ingredient: (1) culture supernatant of transformed *P. pastoris* containing the rmaGH at a dose of 0.1 mg/l (rmaGH culture supernatant groups), (2) cell lysates of transformed *P. Pastori* expressing rmaGH at a dose of 0.1 mg/l (rmaGH cultures groups), (3) cell lysates of non-transformed *P. pastoris* (yeast culture groups), (4) culture supernatant of non-transformed *P. pastoris* expressing total protein at a dose of 0.1 mg/l (yeast culture supernatant groups), (5) non-treated group (the control groups). The treatment was done for 90 min without water recirculation, it was repeated three times in a week for 6 weeks. Sampling of 30 fishes per group was taken at two weeks, four weeks and six weeks from the beginning of the experiment. Growth promoting effect was evaluated by measuring of body weight and fork length increase. Data were expressed as mean ± SD and analyzed by a Student's *t*-test.

RESULTS

Molecular cloning of maGH gene and construction of expression plasmid pPIC9K/maGH

The PCR products amplified with FP1 and RP2 were approximately 590 bp. The amplicon was recovered by double digestion with *EcoRI* and *NotI*. Then, it was ligated into plasmid pPIC9K, giving rise to the expression plasmid pPIC9K-maGH. The construct was used to transform the *E. coli DH5α.* The plasmid extracted from Ampicillin-resistant transformants was digested with *EcoRI* and *NotI*, and a 570 bp fragment was obtained as expected, indicted that the insertion direction was correct. In addition, sequencing showed that the open reading frame of maGH cDNA was completely in frame with that of the α-factor signal peptide.

Transformation of P. pastoris *and* Mut^+ *phenotype selection*

Transformation with the *Sal I*-linearized version of pPIC9-maGH favored its insertion into the yeast genome by homologous recombination, positive transformants (His^+ Muts) were selected on MD plates. Transformant *P. pastoris* were analyzed by PCR with the primers of aox1 gene. The PCR products of transformants have two expected amplification bands typical of Mut^+ clones. One of 1062 bp pertaining to the growth hormone expression "cassette" flanked by aox1 sequences, and the other of 2105 bp corresponding to the native aox1 gene of the yeast genome (clone N2-N4 in Fig.1). The clone N5 (Mut^s) only 1062 bp appeared meaning absence of aox1 endogenous gene. Mut^+ clones were chosen for the subsequent expression study.

Selection of high expression strain and production of rmaGH

Expression cassette-positive clones of each type were fermented in YPD medium and then induced with methanol to a final concentration of 0.5% for a total induction time of 120 h. After induction with methanol, all clones expressed an extracellular protein of approximately 24 kDa with the same size as the standard maGH, which were visualized by Coomassie brilliant blue stained SDS-PAGE. The protein band was absent in the culture supernatant of non-transformed GS115 host cells (Fig.2). There was one clone expressing the highest levels about 300 mg/L recombinant maGH in shake-flask fermentation medium, which accounted for 53% of all the protein in the culture supernatant. Therefore, it was designated as GS115/(pPI9K-

rmaGH) and was selected for further analysis. The production increased along with the increase of duration time after methanol induction, then, the peak of the production was obtained at 72 h (Fig.2).

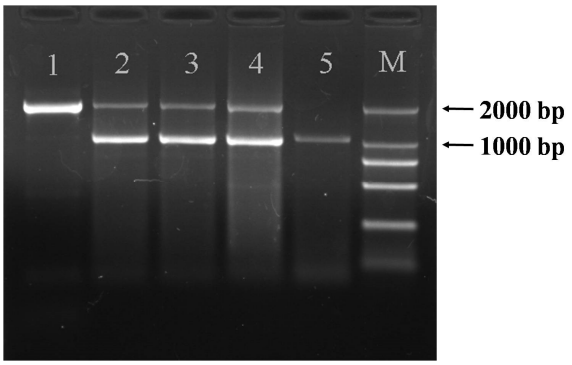

Fig. 1. PCR detection of yeast genome DNA for positive transformants. Transformant *P. pastoris* were analyzed by PCR with the primers of alcohol oxidase 1 (aox1) gene. Lane 1: GS115，amplification bands of PCR have 2105 bp corresponding to the native aox1 gene of the yeast genome; 2-4 Lane: N2, N3 and N4 clones are Mut^+ GS115/(pPI9K-rmaGH) strains, Mut^+ phenotypes have two bands, one of 1062 bp pertaining to the growth hormone gene flanked by aox1 sequences, and the other of 2105 bp corresponding to the native aox1 gene of the yeast genome; Lane 5: N5 clone is Mut^s phenotypes, which only have 1062 bp band; M: DNA Marker DL-2000.

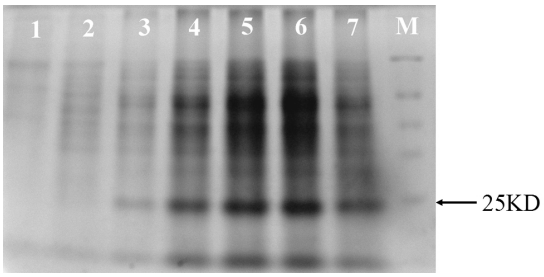

Fig 2. SDS-PAGE analysis of recombinant rMaGH expressed after methanol induction. Lane 1: Culture supernatants of GS115 strain resolved by SDS-PAGE. Lane 2: GS115/pPIC9K strain as negative control. Lane3-7: Culture supernatants of GS115/ (pPI9K-rMaGH) strains, rMaGH expressed at 24-, 48-, 72-, 96-, 120-h post-induction, respectively. M: Standard Protein Molecular Weight Markers. The arrow refer to Protein Marker.

Assessing biological activity of rmaGH

The wet body weight of larvae were recorded at two weeks, four weeks and six weeks from the beginning of the experiment. Statistically significant differences in the growth rate were found in larvae treated with culture supernatant of recombinant *P. pastoris*, compared with the control group ($P < 0.01$) and with the other groups ($P < 0.05$) at 4 weeks. At six weeks from the beginning of the experiment, larvae were treated with recombinant *P. pastoris* culture supernatant had a weight increase of 2.79, 1.99, 1.88 and 2.09-fold higher than the control group ($P<0.01$), the rmaGH culture groups ($P<0.01$), the yeast culture supernatant groups ($P<0.01$), and the yeast culture groups ($P<0.01$), respectively (Fig. 3A). In addition, fork length of the rmaGH culture supernatant groups had a great increase of 2.1-fold higher than the control group after 6 weeks (Fig.3B).

DISCUSSION

Although many recombinant GH had been produced by using *E. coli* (Sekine *et al*., 1985; Bai *et al*., 1999; Wang *et al*., 2001), the product expressed in the inclusion bodies requires additional modification, which weakens its bioactivity. Accordingly, the present study attempted to address this problem by introducing a new host to express recombinant maGH (rmaGH). An easy expression system, which was capable of producing biologically active rmaGH in large quantity, was developed and the rmaGH bioactivities were analyzed by vitro experiments.

The GH cDNA of *M. anguillicaudatus* was cloned, and the open reading frame of the maGH encoded a precursor of 210 aa, including a 22 aa signal peptide and a 188 aa mature protein. To achieve secretion of a particular target protein, the preferred approach is to select appropriate secretion signal. This selection can be based on the protein's own native secretion signal, such as the *S. cerevisiae* alpha-mating factor (α-MF) pre-pro leader sequence, the acid phosphatase signal sequence, or the invertase signal sequence (Macauley and Patrick, 2005; Li *et al*., 2001). The most commonly used signal sequence in *P. pastoris* secretion system is

the *S. cerevisiae* α-MF (Daly *et al*., 2005; Acosta *et al.*, 2007; Peng *et al*., 2013). Similar to *S. cerevisiae*, linear DNA can generate stable transformants of *P. pastoris* via homologous recombination between the transforming DNA and regions of homology within the genome. Therefore, in the present study, the native maGH leading signal was removed and the *S. cerevisiae* α-MF secretion signal was used for the secretion of the recombinant hormone. PCR and sequence analyses indicated that the maGH genes were transformed into *P. pastoris* GS115, and SDS-PAGE analysis showed the presence of rmaGH in the culture supernatant, with a molecular weight of around 24 kDa, similar to that of the standard maGH, suggesting that the α-MF signal peptide was recognized and processed appropriately. Gel scanning of the protein band revealed that the secreted rmaGH accounted for 53% of all the protein in the culture supernatant, which could minimize time-consuming and laborious downstream purification process.

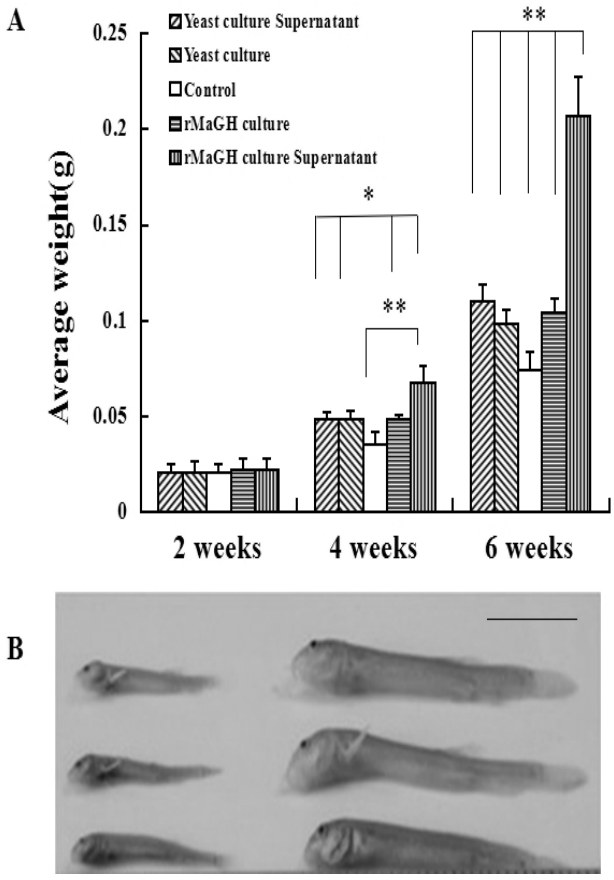

Fig. 3. Effects of recombinant maGH on growth in body weight and fork length of the *M. Anguillicaudatus* larvae. The treatments were conducted three times in a week for 90 min each. The graphic represents the average body weight ± SE. (A) Significant differences in the growth rate were found in larvae of the rmaGH culture supernatant groups compared with the control groups ($P < 0.01$) and with the other groups ($P < 0.05$) at 4 weeks. There are significant differences between larvae of the rmaGH culture supernatant groups and the other groups ($P < 0.01$) at 6 weeks. Data were analyzed by a Student's *t*-test, * $P < 0.05$, ** $P < 0.01$. (B) Phenotype of the larvae in rmaGH culture supernatant groups and the control groups at 6weeks.Three *M. anguillicaudatus* larvae located along the left of the picture belong to the control group and the right belong to the rmaGH culture supernatant group. Scale bar = 10 mm.

Numerous studies have been conducted to develop high-copy recombinants to obtain large quantities of products. As the number of integrated copies of the expression cassette could affect the amount of protein expressed, in the present study, the expression level of rmaGH was different. The results indicated that multicopy recombinant plasmid containing the maGH cDNA was integrated into the genome of *P. pastoris*, and that a large quantity of biologically active rmaGH (300 mg/l rmaGH) was secreted into the culture supernatant, which was higher than that of carp and tilapia GH obtained in *P. pastoris* (Wang *et al*., 2003; Acosta *et al*., 2008). Furthermore, the expression of rmaGH in *P. pastoris* was noted to be time-dependent and the optimal time period was 72 h after induction with methanol, similar to the expression of the recombinant carp GH in *P. pastoris*, which also peaked at 72 h of induction (Li *et al*., 2003). The above-mentioned results were obtained in shake-flask culture in the laboratory, and as *P. pastoris* is well suited for fermentation (Peng *et al*., 2013), a tenfold higher recombinant GH production could be achieved through high-density fermentation.

Similar to the synthesis of rmaGH in *P. pastoris* and its secretion into the extracellular environment, the production of other teleost GH has also been reported in the literature (Wang *et al*., 2003; Li *et al*., 2003). Li observed the growth-promoting effect of recombinant carp GH, which

was secreted into the culture supernatant of recombinant *P. pastoris* (Li *et al*., 2003), while Acosta reported that the recombinant tilapia GH expressed in *P. pastoris* cells could significantly increase the body weight of tilapia (Acosta *et al*., 2007). These findings indicate that irrespective of whether the recombinant GH are expressed in the cells or secreted into the culture medium, their effect may be the same. The present study was conducted to better understand the role of *P. pastoris* rmaGH in fish, and is the first to compare the growth-promoting effects of rmaGH obtained from recombinant *P. pastoris* culture supernatant, recombinant *P. pastoris* cells, normal *P. pastoris* culture supernatant, and normal *P. pastoris* cells on *M. anguillicaudatus* fry (the control group was treated with water alone). The results showed that the weight and body length of *M. anguillicaudatus* fry treated with rmaGH obtained from recombinant *P. pastoris* culture supernatant were 2.79- and 2.1-fold higher than those of the control group ($P < 0.01$) after 6 weeks, respectively. Furthermore, the culture supernatant containing rmaGH had a stronger effect on the growth of the larvae than the rmaGH obtained from *P. pastoris* cells, indicating that extracellular rmaGH could be assimilated more easily by the larvae, when compared with rmaGH obtained from *P. pastoris* cells.

Recent studies had focused on the growth-promoting effects of GH on genetically modified fish and found that GH-treated transgenic fish exhibited accelerated growth rates, when compared with non-transgenic fish (Cao *et al*., 2014; Gopal *et al*., 2014; Duan *et al*., 2013). However, numerous problems were noted to be associated with transgenic fish, such as high predation mortality (Duan *et al*., 2013), impairment of the immune system (Batista *et al*., 2014), delayed gonadal development (Cao *et al*., 2014), smaller and lighter bone development (Zhu *et al*., 2013), etc. Research on this topic conducted in Europe has suggested that the potential environmental effects of transgenic fish that escape into the wild are ambiguous. (Zhang *et al*., 2014). With improved living standard, increasing numbers of people now prefer animal food products with high quality than quantity. In many countries, people avoid transgenic foods because of the controversy associated with the effects of such foods on human health (Frewer *et al*., 2013). Accordingly, the method of immersion of larvae into recombinant *P. pastoris* culture supernatant appears to be a more efficient and safe approach for GH administration. Nevertheless, further detailed studies are required to determine the optimal conditions for rmaGH production through large-scale fermentation, the optimal larval period for treatment with rmaGH, and the optimal dose of rmaGH.

ACKNOWLEDEGMENTS

This work is supported by grants from the National Natural Science Foundation of China (no. 31200923 and U1204329), Tianjin Key Laboratory of Animal and Plant Resistance Open Fund (no. 01046651012), the Henan Scientific and Technological Research Projects (no. 142300410164), the Natural Science Project of the Education Department of Henan Province (no. 14A180031 and 2010B360002).

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(Received 27 January 2014, revised 12 May 2014)