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Recombinant production of biologically active giant grouper (*Epinephelus lanceolatus*) growth hormone from inclusion bodies of *Escherichia coli* by fed-batch culture

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ABSTRACT

Growth hormone (GH) performs important roles in regulating somatic growth, reproduction, osmoregulation, metabolism and immunity in teleosts, and thus, it has attracted substantial attention in the field of aquaculture application. Herein, giant grouper GH (ggGH) cDNA was cloned into the pET28a vector and expressed in Shuffle® T7 Competent *Escherichia coli*. Recombinant N-terminal 6× His-tagged ggGH was produced mainly in insoluble inclusion bodies; the recombinant ggGH content reached 20% of total protein. For large-scale ggGH production, high-cell density *E. coli* culture was achieved via fed-batch culture with pH-stat. After 30 h of cultivation, a cell concentration of 41.1 g/l dry cell weight with over 95% plasmid stability was reached. Maximal ggGH production (4.0 g/l; 22% total protein) was achieved via mid-log phase induction. Various centrifugal forces, buffer pHs and urea concentrations were optimized for isolation and solubilization of ggGH from inclusion bodies. Hydrophobic interactions and ionic interactions were the major forces in ggGH inclusion body formation. Complete ggGH inclusion body solubilization was obtained in PBS buffer at pH 12 containing 3 M urea. Through a simple purification process including Ni-NTA affinity chromatography and refolding, 5.7 mg of ggGH was obtained from 10 ml of fed-batch culture (45% recovery). The sequence and secondary structure of the purified ggGH were confirmed by LC–MS/MS mass spectrometry and circular dichroism analysis. The cell proliferation-promoting activity was confirmed in HepG2, ZFL and GF-1 cells with the WST-1 colorimetric bioassay.

Introduction

Growth hormone (GH)[1] is a single-chain polypeptide hormone secreted by somatotrophs in the anterior portion of the pituitary gland and performs an important role in regulating somatic growth and vertebrate development. The hormonal action of GH is directly initiated via binding to GH receptors (GHRs) located in the liver or other tissues to induce hepatic IGF-I secretion (endocrine mode) or local IGF-1 production (autocrine and/or paracrine mode) [1]. In the past two decades, GH has been investigated extensively in a variety of mammalian species, such as human, rat, porcine, bovine and ovine [2,3]. Moreover, cDNA cloning has allowed our structural knowledge of GH in vertebrates to gradually improve. Sequence analyses have suggested that despite amino acid differences among species, GHs from all species share a similar protein structure composed of approximately 50–60% α-helices and four cysteines for the formation of two intramolecular disulfide bonds [4]. Reports have shown that the α-helix bundle motif is associated with the oligomerization of its receptor upon binding, thereby activating cytosolic components for signal delivery [5]. The importance of GH as a potential growth-promoting agent has been recognized for applications in the fields of medical therapy and animal husbandry. For instance, human GH has been used for therapeutic treatments in the regulation of normal growth in GH-deficient children [6], and pigs treated with porcine GH exhibit a marked increase in feed utilization with increased weight gain [7]. The growth-promoting performance of

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[1] *Abbreviations used:* GH, growth hormone; GHRs, growth hormone receptors; Trx, thioredoxin; GST, glutathione S-transferase; IUCN, International Union for Conservation of Nature; ggGH, giant grouper growth hormone; DCW, dry cell weight; ZFL, zebrafish liver; BSA, bovine serum albumin; TCA, trichloroacetic acid.

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mammalian GH facilitates the investigation of GH introduced into aquaculture.

Among teleosts, the GH gene has been cloned from diverse fish species and heterologously expressed in recombinant systems [8–13]. Several reports have shown that recombinant teleost GH can enhance the growth rate of a number of fish species via immersion, oral or injection administration [10,14–17]. Recently, genetic engineering studies have made efforts to produce fast-growing transgenic fish species, such as Atlantic salmon, common carp, coho salmon, mud loach and tilapia, by introducing the GH gene into their chromosomes [18–22]. Subsequently, these GH transgenes induce dramatically enhanced growth and reveal the potential of GH application in aquaculture. In addition to promoting growth and development, teleost GHs have also been shown to be involved in diverse physiological functions, such as reproduction, osmoregulatory adaptation, metabolism improvement and the immune response [23,24]. For example, GH participates in reproductive functions in Japanese eel by regulating early spermatogenesis [25,26]. In addition, GH acts in synergy with cortisol to improve seawater tolerance in salmonids by upregulating the gill cortisol receptors [27]. Furthermore, GH transgenesis in coho salmon enhances metabolic reactions by emphasizing carbohydrate degradation for energy production and lipid synthesis and by increasing the utilization of lipids and proteins in synthetic roles to accelerate growth [22]. Finally, the administration of recombinant truncated tilapia GH can enhance growth and innate immunity in tilapia [28]. Given these biological functions, teleost GH has attracted attention in the field of aquaculture for development as a growth-promoting agent or immune stimulator. With this goal, GHs from diverse fish species have been produced in various recombinant systems [15,16,29–31]. Bacterial expression systems are commonly used due to their advantages, including easy manipulation, inexpensive culture, high-level expression and quick generation of a recombinant protein. Reports have shown that the recombinant production of diverse fish GHs in an *E. coli* prokaryotic system usually results in expression in inclusion bodies due to their complex structures [12,32–35]. Although a few studies have attempted to express GH in a soluble form by tagging with a highly soluble protein such as thioredoxin (Trx), glutathione S-transferase (GST) or maltose-binding protein, the biological activity of GH is limited as a fusion protein, and the cleavage step to separate the fusion protein is usually expensive [36,37]. Furthermore, the production of recombinant proteins from inclusion bodies has several advantages, such as resistance to proteolytic degradation and simple primary recovery from the total protein. Thus, most studies attempting to produce biologically active fish GH are efficiently performed with inclusion bodies via solubilization and refolding processes prior to purification by chromatography [17,32,34,35].

Giant grouper (*Epinephelus lanceolatus*) is the largest bony fish found in coral reefs throughout the Indo-Pacific region. It is an economically important marine fish species that can weigh up more than to 400 kg and grow more than 2.7 m in length, and it has been listed by the International Union for Conservation of Nature (IUCN) as a vulnerable species. Giant grouper is considered a promising species for aquaculture because its price tends to be higher than other fishes due to high demand; however, the long culture time (approximately 3–4 years) for the commercialization of giant grouper restricts the supply. The administration of recombinant GH has been considered by the aquaculture industry as a solution to enhance the growth rate of this fish. Recently, giant grouper (*E. lanceolatus*) GH (ggGH) cDNA encoding 204 amino acids, including a putative signal peptide with 17 amino acids and a mature GH polypeptide with 187 amino acids, was isolated from the pituitary [38]. The purpose of this study is to investigate the high-level production of giant grouper GH in a fed-batch culture of *E. coli*. In addition, a purification process was also developed to obtain biologically active ggGH. We expected that the results will be useful for the application of recombinant ggGH to growth enhancement in the aquaculture industry.

Materials and methods

Plasmid construction, bacterial strain and culture media

Total RNA was isolated from giant grouper (*Epinephelus lanceolatus*) brain tissue using Trizol reagent according to the manufacturer's instructions, and first-strand cDNA was synthesized in a 20-μl RT reaction from 2.5 μg of total RNA using SuperScriptIII (Invitrogen-Life Technologies). The mature sequence of giant grouper growth hormone (ggGH) cDNA (GenBank accession, EU280321) was amplified using the forward primer 5′-ACCATGGGCCATCATCATCATCATCAT c agccaatcacagacggccagcgt ctgttc (underline indicates *Nco*I site) and the reverse primer 5′-ACTCGAGCTACAGGGTACAGTTGGCCTCAGGAG (underline indicates *Xho*I site). PCR reactions were performed in an Applied Biosystems 9700 Thermal Cycler using a temperature cycle profile of 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 45 s, with a final extension of 72 °C for 7 min. The final holding temperature was 4 °C. The PCR products were digested with *Eco*RI and *Xho*I and then cloned into the same enzyme sites in the pET28a vector to create the pET-ggGH plasmid for expression. *E. coli* DH5α was used as the host strain for maintenance of the plasmid. The pET-ggGH plasmid was transformed into Shuffle T7 Expression Competent *E. coli* (NEB C3029H) for expression.

Seed cultivation and flask cultivation

E. coli Shuffle T7 cells containing pET-ggGH were stored in 20% glycerol at −20 °C. Five hundred microliters of frozen glycerol stock was inoculated into a 250-ml flask containing 50 ml Luria–Bertani (LB) medium supplemented with 50 μg/ml kanamycin in a shaking incubator at 37 °C at 175 rpm for 16 h cultivation as the seed culture. Shake flask experiments were performed in modified SSP medium [39] (15 g/l peptone, 5 g/l yeast extract, 5 g/l glucose, 8 g/l K_2HPO_4, 2 g/l KH_2PO_4; pH 7.5). One milliliter of seed culture was inoculated into a 500-ml flask containing 100 ml SSP medium and incubated at 37 °C at 175 rpm. Following 3.5 h cultivation (OD_{600} 1), ggGH expression was induced using 0.1 mM IPTG. After 6 h cultivation, the cells were harvested for estimation of expression levels by 15% SDS–PAGE.

Fermentation by fed-batch cultivation

For large-scale production of recombinant ggGH, a fed-batch culture of *E. coli* cells was performed at 37 °C in a 5-l bioreactor (Winpact FS-02, Taiwan) equipped with a built-in digital controller for pH, temperature, agitation, dissolved oxygen (DO) and peristaltic pumps for adding acid, base, antifoam and nutrients. The set point for pH and DO concentration was controlled by on-line monitoring using a pH sensor (Mettler-Toledo InPro3030/325, Urdorf, Switzerland) and a DO sensor (Mettler-Toledo InPro6800/12/320, Urdorf, Switzerland), respectively. The initial work volume was 2 L of modified R medium. The medium contained 6.75 g/l KH_2PO_4, 3 g/l $Na_2HPO_4{\cdot}12H_2O$, 5 g/l $(NH_4)_2SO_4$, 1.5 g/l $MgCl_2$, 0.1 g/l NH_4Cl, 3 g/l citric acid, 20 g/l glucose, 20 g/l yeast extract, 30 g/l peptone and 10 ml of a trace metal solution. The composition of the trace metal solution (per ml of 5 M HCl) was 10 mg $FeSO_4{\cdot}7H_2O$, 2.25 mg $ZnSO_4{\cdot}7H_2O$, 1.35 mg $CaC_{l2}{\cdot}2H_2O$, 0.5 mg $MnSO_4{\cdot}5H_2O$, 1 mg $CuSO_4{\cdot}5H_2O$, 0.3 mg $AlCl{\cdot}6H_2O$, 0.1 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.2 mg H_3BO_3, and 2 mg thiamine-HCl. The entire