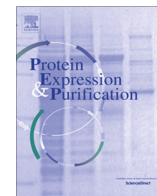




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Recombinant production of biologically active giant grouper (*Epinephelus lanceolatus*) growth hormone from inclusion bodies of *Escherichia coli* by fed-batch culture

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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.

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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.

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- μ l RT reaction from 2.5 μ g 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'-ACCATGGGCCATCATCATCATCATC agccaatcacagacggccagctctgttc (underline indicates *Nco*I site) and the reverse primer 5'-ACTC-GAGTACAGGGTACAGTTGGCTCAGGAG (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* DH5 α 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 μ g/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 K₂HPO₄, 2 g/l KH₂PO₄; 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 (OD₆₀₀ 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 KH₂PO₄, 3 g/l Na₂HPO₄·12H₂O, 5 g/l (NH₄)₂SO₄, 1.5 g/l MgCl₂, 0.1 g/l NH₄Cl, 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 FeSO₄·7H₂O, 2.25 mg ZnSO₄·7H₂O, 1.35 mg CaCl₂·2H₂O, 0.5 mg MnSO₄·5H₂O, 1 mg CuSO₄·5H₂O, 0.3 mg AlCl₃·6H₂O, 0.1 mg (NH₄)₆Mo₇O₂₄·4H₂O, 0.2 mg H₃BO₃, and 2 mg thiamine-HCl. The entire

content of the seed culture (5% v/v) was inoculated. The agitation speed and flow rate of aeration were set at 900 rpm and 3 l/min. The pH was maintained at 7.0 by adding 28% (v/v) ammonium hydroxide. The DO concentration was maintained above 20% air saturation. A nutrient feeding solution was added using the pH-stat feeding strategy [39]. The nutrient solution contained 750 g/l glucose, 50 g/l yeast extract, and 75 g/l peptone. When the pH rose above the set point (pH 7.0) by 0.1 U due to the depletion of glucose, nutrient feeding solution was automatically added to increase the glucose concentration in the culture broth. When fed-batch feeding was activated by the initial glucose consumption, the cultivation temperature was shifted from 37 °C to 30 °C to avoid acetate accumulation resulting from the high growth rate. Expression of the ggGH gene was induced by adding IPTG to a final concentration of 0.1 mM. Foam was suppressed as necessary by the addition of sterilized antifoam (Sigma A-5758). Culture samples were withdrawn periodically for quantitative analysis during the cultivation. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600nm}) on a spectrophotometer (Thermo, G10S UV-Vis, USA). Dry cell weight (DCW), estimated plasmid stability, and glucose and acetate concentrations were determined as reported by Hu et al. [39].

Purification of ggGH from the inclusion body

Recombinant *E. coli* cells were harvested from 10 ml of culture (around 2 g of wet cell weight) of a fed-batch culture induced at mid-log stage by centrifugation at 4000×g for 15 min at 4 °C and washed twice in 50 mL PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·12H₂O, 1.8 mM KH₂PO₄; pH 7.3), then centrifuged again. The washed cell pellet was resuspended thoroughly in 25 ml PBS buffer and lysed by sonication using a sonicator (Chrom Tech. UP-800, Taiwan) at 20% of power output. To determine the optimal conditions for the isolation of inclusion bodies, 1 ml of lysed mixture samples were put in 1.5 ml microcentrifuge tube and centrifuged at different centrifugal forces for 15 min at room temperature. The ggGH content in the supernatant and pellet was evaluated by 15% SDS-PAGE. To determine the optimal conditions for the solubilization of inclusion bodies, the inclusion bodies from 1 ml of lysed mixture samples were isolated by centrifugation at the 10,000×g for 15 min at room temperature, and washed twice with 2 mL PBS buffer to remove cell debris, then centrifuged again. The inclusion bodies were treated with 1 ml PBS buffers containing different pH values or urea concentrations for 2 h at room temperature, and then were centrifuged at 10,000×g for 15 min. The ggGH content in the supernatant and pellet was evaluated by 15% SDS-PAGE. The inclusion bodies isolated from 10 ml of fed-batch broth were completely soluble in 50 ml of denaturing buffer (PBS buffer containing 3 M urea and 0.1 mM dithiothreitol, pH 12) at room temperature for 2 h, and the solubilized solution was clarified by centrifugation at 17,500×g for 30 min at 4 °C. The supernatant fraction was collected and then loaded on a 5 mL Ni-NTA agarose column (Qiagen, Valencia, CA, USA). The column was washed with 3 column volumes of wash buffer (PBS buffer, pH 12, containing 30 mM imidazole) and eluted with 3 column volume of elution buffer (PBS buffer, pH 12, containing 250 mM imidazole). Fractions containing purified ggGH were pooled together (~15 ml, protein concentration is around 0.4 mg/ml) and diluted by addition of an equal volume of PBS buffer (pH 7.3) containing 2 mM reduced glutathione and 0.2 mM oxidized glutathione, and then dialyzing against 500 ml of pH 7.3 PBS buffer containing 1 M urea at 4 °C for 4 h using a Slide-A-Lyzer Dialysis Cassette G2 (Thermo) dialysis membrane. The dialysis buffer was subsequently replaced with 500 ml of pH 7.3 PBS buffer containing 0.5 M urea and 50 mM glucose for 4 h, and finally, the purified ggGH protein was dialyzed against 500 ml of pH 7.3 PBS buffer

containing 50 mM glucose at 4 °C overnight to gradually remove urea and detergent from protein. Around 30 ml of refolded protein was centrifuged at 17,500×g for 30 min at 4 °C to remove aggregated protein. The supernatant was concentrated by centrifugation using a Microsep Advance Centrifugal Device (Life Science) and stored at 4 °C prepared for bioactivity assays.

Bioactivity assays with recombinant giant grouper GH

The biological activity of recombinant ggGH was evaluated by determining its growth-promoting activity on human liver hepatocellular carcinoma (HepG2) cells, grouper fin (GF-1) cells, and zebrafish liver (ZFL) cells. The sources of the cell lines, culture medium and culture conditions in this assay are shown in Table 1. The cells were seeded onto a 96-well plate at a density of 1 × 10⁴/well and cultured in growth medium with optimal individual culture conditions. After 24 h of cultivation, the growth medium was discarded, and the cells were arrested in the G₀/G₁ phase by replacing the medium with 100 μl growth medium without serum. For the activity assay, the medium without serum was replaced with 100 μl of growth medium with 0.1% FBS containing various concentrations of purified ggGH, 10 ng/ml of commercial human GH (Sigma H5916, USA) (positive control) and 10 ng/ml bovine serum albumin (BSA) (negative control). The bioactivity assay was repeated in three independent experiments, and each sample was included in triplicate. Cell growth based on the enzymatic cleavage of the tetrazolium salt WST-1 (Roche 11644807001, IN, USA) to a water-soluble formazan dye was quantitated by detecting absorbance at 440 nm after 48 h of incubation. Multiple-group comparisons were examined using one-way analysis of variance (ANOVA), and Tukey's test was used to evaluate significant differences between groups. The differences were defined as significant at *p* < 0.05.

Protein analysis and yield calculation

The expression levels of recombinant ggGH were analyzed on a 15% SDS-PAGE gel stained with Coomassie brilliant blue R-250 (Bio-Rad). The ggGH was quantified using a densitometer (BioSpectrum 500 Imaging System, UVP, CA, USA) to scan the ggGH band in the gel. The protein concentration was determined by the Bradford protein assay method using BSA as the standard [40]. The purity of purified ggGH was analyzed by reverse-phase HPLC equipped with a betabasic-C18 column (4.6 mm × 150 mm, 5 μm, Keystone Scientific Inc., Bellefonte, PA, USA). Proteins were separated using a linear gradient from 30% in buffer A (0.1% TFA in H₂O) to 100% in buffer B (0.1% TFA in acetonitrile) at a flow rate of 0.2 ml/min, and detection was achieved by monitoring the UV absorbance at 220 nm.

Table 1

Cell lines, culture medium and culture conditions used in the giant grouper GH bioactivity assay.

Cell line	Species and type	Culture medium	Culture condition	Reference and source
HepG2	Human hepatocellular carcinoma	DMEM + 10% FBS	37 °C, 5% CO ₂	[50]
GF-1	Grouper fin cell	L15 + 10% FBS	28 °C	[51]
ZFL	Zebrafish liver cell	50% L-15 + 35% DMEM + 15% F-12 + 5% FBS ^a	28 °C	[52]

^a Culture medium supplemented with 0.15 g/l sodium bicarbonate and 15 mM HEPES, 0.01 mg/ml insulin and 50 ng/ml EGF (unfiltered).

306 Spectroscopic analysis

307 A circular dichroism (CD) spectrum was determined at 25 °C in
 308 the wavelength range of 190–250 nm using a JASCO spectropolarimeter. Purified ggGH was resuspended in deionized water to
 309 a final concentration of 1 mg/ml. CD spectra were recorded with the following parameters: 10 mm cell length, 0.1 nm data pitch,
 310 standard sensitivity, 1 s D.I.T., 1 nm bandwidth, 50 nm/min scanning speed. The sample was scanned 5 times for data accumula-
 311 tion, and the average spectrum was plotted.

315 LC-MS/MS analysis

316 Sixty microliters of deionized water containing 5 µg of purified
 317 ggGH was treated with 10 µl of denaturation solution containing
 318 0.7 mg SDS and 70 mM DTT at 95 °C for 5 min and then incubated
 319 on ice to yield reduced purified ggGH. After 10 min of DTT treat-
 320 ment on ice, 10 µl of 0.5 M iodoacetamide (IAA) was added to
 321 the sample for alkylation, and the sample was then precipitated
 322 with 10% trichloroacetic acid (TCA) on ice for 15 min. The pre-
 323 cipitated pellet was collected by centrifugation at 13,000 rpm at
 324 4 °C for 30 min, washed three times with ddH₂O, and then treated
 325 with 100 µl of buffer (pH 8.5) containing 50 mM NH₄HCO₃, 2% N-
 326 octyl-glucoside and 0.1 µg trypsin at 4 °C for 16–18 h. After over-
 327 night incubation, 10 µl of 2% formic acid was added to the solution
 328 mixture and analyzed by LC-MS/MS. Mass spectrometry data were
 329 analyzed using Data Explorer software (Applied Biosystems, South
 330 San Francisco, CA, USA). The obtained peptide mass results were
 331 then searched against the NCBI database using the Mascot peptide
 332 mass fingerprinting search program (Matrix Science, Boston, MA).

333 Results

334 Expression and characterization of the recombinant ggGH fusion
335 protein

336 The expression plasmid pET-ggGH encoding the mature ggGH
 337 was derived from pET28a and used to transform *E. coli* Shuffle T7
 338 for expression. The N-terminal of ggGH was tagged with six histi-
 339 dines for purification by Ni-NTA affinity chromatography (Fig. 1). To
 340 produce the recombinant ggGH protein, cells were grown in mod-
 341 ified SSP medium and induced with 0.1 mM IPTG at an OD₆₀₀ of 1.
 342 As shown in Fig. 2, the *E. coli* culture produced a protein that was
 343 absent under non-induced conditions (lanes 1 and 2). This protein
 344 had a molecular weight slightly lower than 26 kDa according to the
 345 molecular mass standard in SDS-PAGE. The expected molecular
 346 mass of recombinant ggGH was ~22 kDa, which corresponds to
 347 the fusion of the 6×-His tag (~0.9 kDa) and the ggGH
 348 (~20.6 kDa). The recombinant ggGH expressed in *E. coli* after IPTG
 349 induction was further characterized by western blotting using a
 350 6×-His epitope tag antibody. A single signal match with the mole-
 351 cular mass of ggGH and its absence in the uninduced sample clarify
 352 the identity of the recombinant ggGH (Fig. 2B). The proportion of
 353 recombinant ggGH among total protein content was further ana-
 354 lyzed, and we determined that more than 91% of the recombinant
 355 ggGH was expressed as inclusion bodies, even when the culture
 356 temperature was reduced to 27 °C or 16 °C (Fig. 2A). Recombinant
 357 ggGH content with induction at 37 °C was approximately 20% of
 358 total protein.

359 Fermentation of *E. coli* Shuffle T7/pET-ggGH by fed-batch cultivation

360 To mass produce the ggGH protein, fed-batch cultivation using
 361 pH-stat was performed to achieve high-level production of recom-
 362 binant *E. coli*. When the initial glucose concentration of 20 g/l in

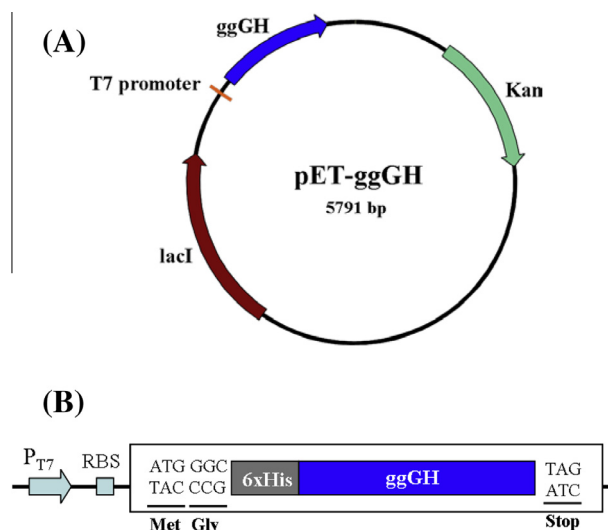


Fig. 1. Construction of the expression plasmid pET-ggGH. (A) The PCR fragment that encodes mature ggGH with a 6×-His tag was digested with *Nco*I and *Xho*I and then cloned into pET28a at the same restriction sites. Kan, kanamycin resistance; lacI, lac repressor. (B) Diagram illustrating the main features of pET-ggGH. Expression of the ggGH gene is controlled by the T7 promoter (P_{T7}). RBS, ribosome binding site.

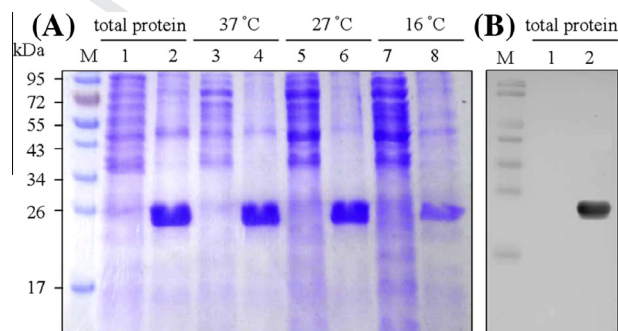


Fig. 2. Recombinant expression of ggGH in *E. coli* Shuffle T7 by flask culture. (A) SDS-PAGE analysis of proteins from the *E. coli* Shuffle T7 host. (B) The recombinant ggGH fusion protein was identified by western blotting using a 6×-His epitope tag antibody. Lane: M, molecular mass standard; 1, total protein without IPTG induction at 37 °C; 2, total protein with IPTG induction at 37 °C; 3, 5 and 7 are soluble proteins with IPTG induction at 37 °C, 27 °C and 16 °C, respectively; 4, 6 and 8 are insoluble proteins with IPTG induction at 37 °C, 27 °C and 16 °C, respectively.

the bioreactor was exhausted after 7.5 h cultivation time, the addi-
 tion of feeding solution was activated due to the pH rising above
 the set value of 7.1. Concurrently, the cultivation temperature
 was reduced to 30 °C to reduce the growth rate and thus prevent
 byproduct acetate accumulation. Feeding with 28% ammonium
 hydroxide (v/v) was initiated to maintain a neutral pH, and a nitro-
 gen source was supplied when the pH was below 6.9. With glucose
 as the limiting parameter during the batch phase, the specific
 growth rate and the yield coefficient for glucose were 0.52/h and
 0.415 g/g, respectively. The glucose concentration was maintained
 below 1.6 g/l during fed-batch cultivation to avoid growth inhibi-
 tion due to the accumulation of acetic acid. Thus, the acetate con-
 centration was maintained below 1.2 g/l during the cultivation
 process. In the fed-batch phase, the specific growth rate declined
 to 0.018/h due to glucose limitation (<1.6 g/l), and the yield coeffi-
 cient for glucose was maintained at approximately 0.38 g/l. Thus, a
 biomass of 41.1 g/l DCW was achieved after 30 h of cultivation, and
 more than 90% of the recombinant *E. coli* cells containing the pET-
 ggGH expression plasmid were maintained (Fig. 3A). To evaluate

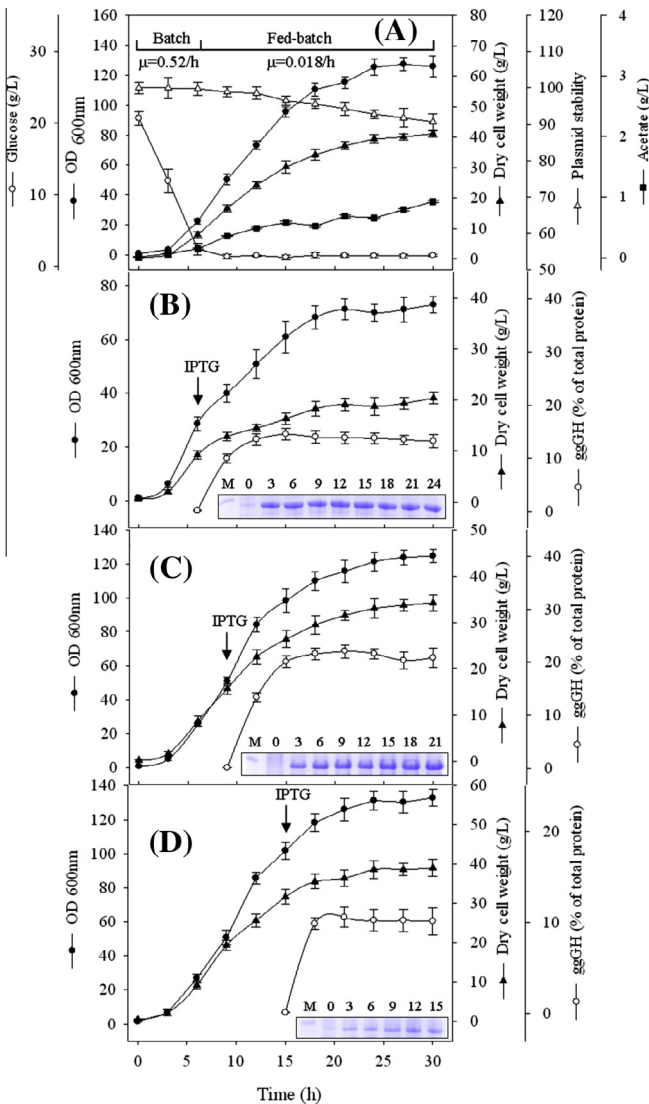


Fig. 3. (A) Fed-batch cultivation of recombinant *E. coli* Shuffle T7/pET-ggGH with pH-stat without IPTG induction. Cell density OD₆₀₀ (●), dry cell weight (▲), glucose (○), plasmid stability (△), and acetic acid (■). Fed-batch cultivation with IPTG induction at (B) early log phase (6 h), (C) mid-log phase (9 h), and (D) late log phase (15 h). Cell density OD₆₀₀ (●), dry cell weight (▲), and ggGH content (○). The arrow indicates the time of IPTG induction. SDS-PAGE for ggGH content analysis is shown in the bottom right corner. Lane M indicates the molecular mass standard; the numbers indicate the hours post-induction.

the expression efficiency of recombinant ggGH at the time of induction, cells were induced with 0.1 mM IPTG at the early log stage (6 h), mid-log stage (9 h) and late log stage (15 h). When cells were induced at the early log phase, the fraction of recombinant ggGH compared to the total protein content increased for 3 h and then slightly decreased. The DCW and maximal ggGH content were 20.4 g/l and 15% of total protein, respectively (Fig. 3B). When cells were induced at mid-log phase, the fraction of recombinant ggGH increased for 6 h after induction and then maintained a constant expression level until the end of cultivation. The DCW and maximal ggGH content were 34.2 g/l and 22% of total protein, respectively (Fig. 3C). When cells were induced at the late log phase of the growth curve, the ggGH content increased rapidly for 3 h and then maintained the expression levels with little variation. The DCW and maximal ggGH content were 38.7 g/l and 11% of total protein, respectively (Fig. 3D). Although the DCW induced in late log phase was higher than that induced in mid-log phase, the ggGH

content induced in mid-log phase was higher compared to early log phase and late log phase. Thus, the maximal amount of ggGH was produced when cells were induced in the mid-log phase, and the concentration of ggGH reached as high as 4 g/l.

Purification and characterization of recombinant ggGH

Purification of recombinant ggGH was performed from cells induced in the mid-log phase of fed-batch cultivation via isolation, solubilization, Ni-NTA affinity chromatography and refolding. The optimal conditions for centrifugal force were determined for the isolation of inclusion bodies from the cell-disrupted mixture. The results indicated that as centrifugal force increased, the amount of recombinant ggGH distributed in the pellet increased gradually. Most recombinant ggGH content could be recovered with centrifugal forces greater than 4000×g for 15 min, and the greatest efficiency for isolating ggGH was a centrifugal force of 10,000×g for 15 min (Fig. 4A); thus, 10,000×g was used to isolate inclusion bodies during the purification process. The optimal pH and urea concentration conditions to denature recombinant ggGH from inclusion bodies were evaluated. The results indicated that solubilization efficiency did not obviously change at different pH values until the PBS buffer had a high alkaline pH of 12 (Fig. 4B). The amount of recombinant ggGH distributed in the supernatant increased gradually accompanying with the increasing of urea concentration in pH 7 PBS buffer. Most recombinant ggGH content could be soluble in pH 7.0 PBS buffer containing 5 M urea (Fig. 4C). Subsequently, PBS buffer at pH 12 containing different concentrations of urea to enhance the solubility of ggGH from the inclusion bodies was examined. The results indicated that the most recombinant ggGH from the inclusion bodies were converted into the soluble form in the presence of urea concentrations greater than 3 M urea (Fig. 4D). Thus, PBS buffer at pH 12 containing 3 M urea was used to solubilize recombinant ggGH during the purification process. Recombinant ggGH was purified from 10 ml of a fed-batch culture induced at the mid-log phase using Ni-NTA affinity chromatography (Fig. 5A). The recovery yields from each purification step are summarized in Table 2. The final amount of ggGH and recovery yield were 5.7 mg and 45%, respectively. The purity of the purified ggGH as determined by HPLC was 92% (Fig. 5B). CD analysis was performed to determine the structural integrity of the purified ggGH. The results revealed that the CD spectrum of purified ggGH comprised three major peaks at 193, 208 and 223 nm, which are features of proteins with a high α-helix content and are also exhibited by other members of the GH family of proteins (Fig. 5C). Finally, LC-MS/MS analysis was performed to further confirm the identity of purified ggGH. The results showed that identity of tryptic peptide derived from purified ggGH corresponded to the trypsin-digested fragment of ggGH (Fig. 6). This result demonstrated the identity of purified ggGH and indicated that secondary structures of purified ggGH had been successfully refolded.

Bioactivity of purified recombinant ggGH

To determine whether the purified ggGH was biologically active, its growth-promoting action in the HepG2, ZFL, and GF-1 cell lines was examined using a WTS-1 assay. Cells in starvation media were treated with various concentrations of purified ggGH, commercial human GH and BSA, and its growth-stimulating activity was evaluated. The results showed that purified ggGH significantly stimulated the proliferation of HepG2, ZFL, and GF-1 cells compared to the control group, and this proliferation occurred in a dose-dependent manner for concentrations ranging from 1 ng/ml to 10 ng/ml. Moreover, for mammalian HepG2 and teleost ZFL and GF-1 cells, purified ggGH demonstrated a significantly higher

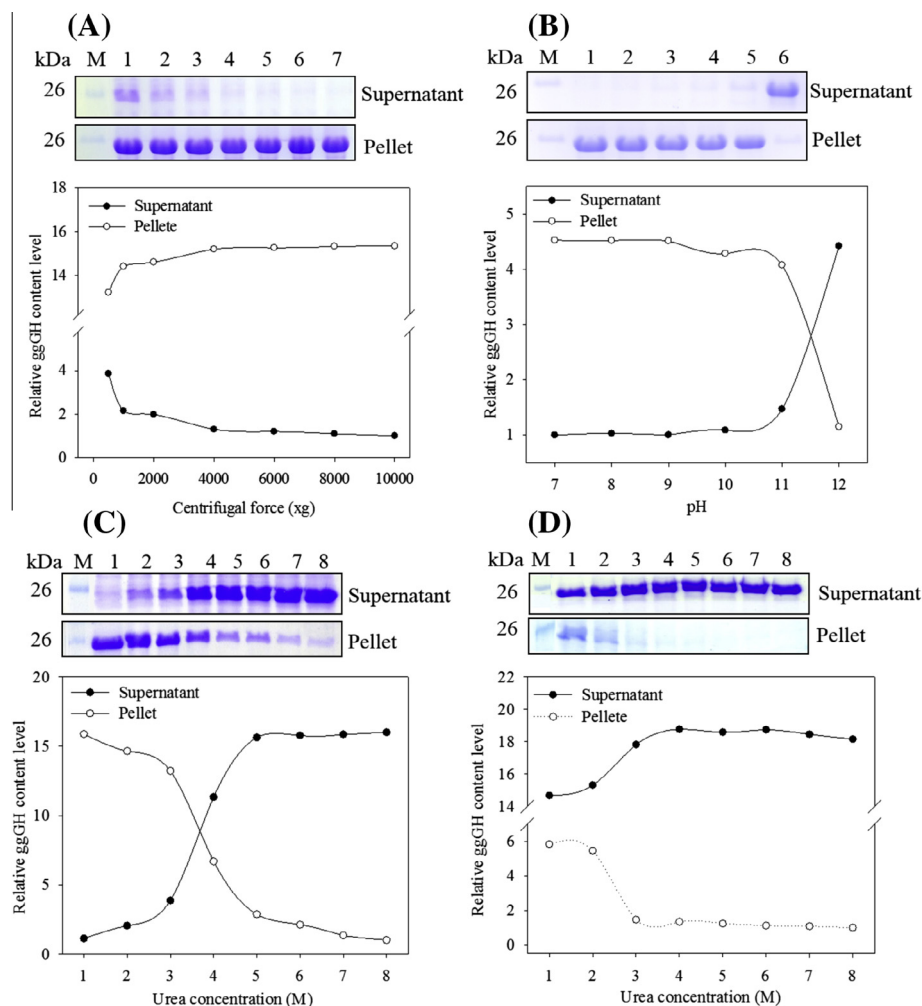


Fig. 4. (A) Effect of centrifugal force on the isolation of ggGH inclusion bodies. A fixed amount of lysed cell mixture was centrifuged at different centrifugal forces for 15 min. Lane M: molecular mass standard; 1: 500×g; 2: 1000×g; 3: 2000×g; 4: 4000×g; 5: 6000×g; 6: 8000×g; 7: 10,000×g. (B) Effect of pH on the solubility of ggGH inclusion bodies. A fixed amount of ggGH inclusion bodies was solubilized in PBS buffer with different pH values for 2 h. Lane M: molecular mass standard; 1: pH 7; 2: pH 8; 3: pH 9; 4: pH 10; 5: pH 11; 6: pH 12. Effect of urea on the solubility of ggGH inclusion bodies. A fixed amount of ggGH inclusion bodies was solubilized in (C) pH 7.0 and (D) pH 12 PBS buffer with different urea concentrations for 2 h, respectively. Lane M: molecular mass standard; (1) 1 M urea; (2) 2 M urea; (3) 3 M urea; (4) 4 M urea; (5) 5 M urea; (6) 6 M urea; (7) 7 M urea; (8) 8 M urea. The ggGH content in the supernatant and pellet was quantified using a densitometer to scan the ggGH band in the gel. The ggGH contents of pellets centrifuged at 10,000×g for 15 min, solubilized in pH 7.0 PBS buffer for 2 h, and solubilized in pH 7.0 buffer or pH 12 PBS buffer containing 8 M urea for 2 h were set as 1.

461 growth-stimulating efficiency than commercial human GH at a
462 concentration of 10 ng/ml, and the highest absorbance at 440 nm
463 was demonstrated for GF-1 cells. In contrast, no growth-stimulating
464 function was observed with BSA (negative control) compared
465 to the control group (Fig. 7). These results indicated that the
466 recombinant ggGH protein purified under the established process
467 retained its biological activity.

468 **Discussion**

469 The production of recombinant growth hormone (GH) in an
470 *E. coli* expression system has been widely performed in the
471 biotechnology and pharmaceutical industries. In general, high-
472 level expression of GH often resulted in inclusion bodies due to
473 the complex disulfide structure of GH. In this study, a pET-ggGH
474 plasmid was transformed into *E. coli* Shuffle T7 for the production
475 of recombinant ggGH. Although lower temperatures to slow down
476 protein synthesis and folding have been described as an efficient
477 approach to increase the soluble proportion of 6-his-tagged human
478 growth hormone (hGH) [37,41], this strategy was unable to

479 improve the solubility of ggGH in this study, even when the
480 temperature was lowered to 16 °C. There was a similar case for
481 striped catfish growth hormone (scGH) tagged with 6×-His, which
482 was expressed as an inclusion body in *E. coli* even when the culti-
483 vation temperature had been reduced. The differences in the
484 expression results between hGH and teleost GH may be caused
485 by the diversity of its component amino acids and structural char-
486 acteristics [8]. To mass-produce bioactive ggGH protein from
487 recombinant *E. coli*, an alternative to shaker cultivation is required.
488 Fed-batch culture of *E. coli* with a pH-stat strategy is an efficient
489 approach to improve biomass concentration and product yield
490 [39]. The feeding of nutrients is activated based on the increase
491 in pH when glucose is depleted; thus, a lower glucose concentra-
492 tion (below 1.6 g/l) was maintained to prevent the accumulation
493 of acetic acid. It is well known that acetate byproducts are overpro-
494 duced in *E. coli* under oxygen-limiting conditions and suppress cell
495 growth and recombinant protein expression. Several studies have
496 attempted to prevent acetate accumulation by supplying pure oxy-
497 gen, and they achieved high-density culture of recombinant *E. coli*
498 (>150 DCW/l) [39,42]. However, the high production costs and

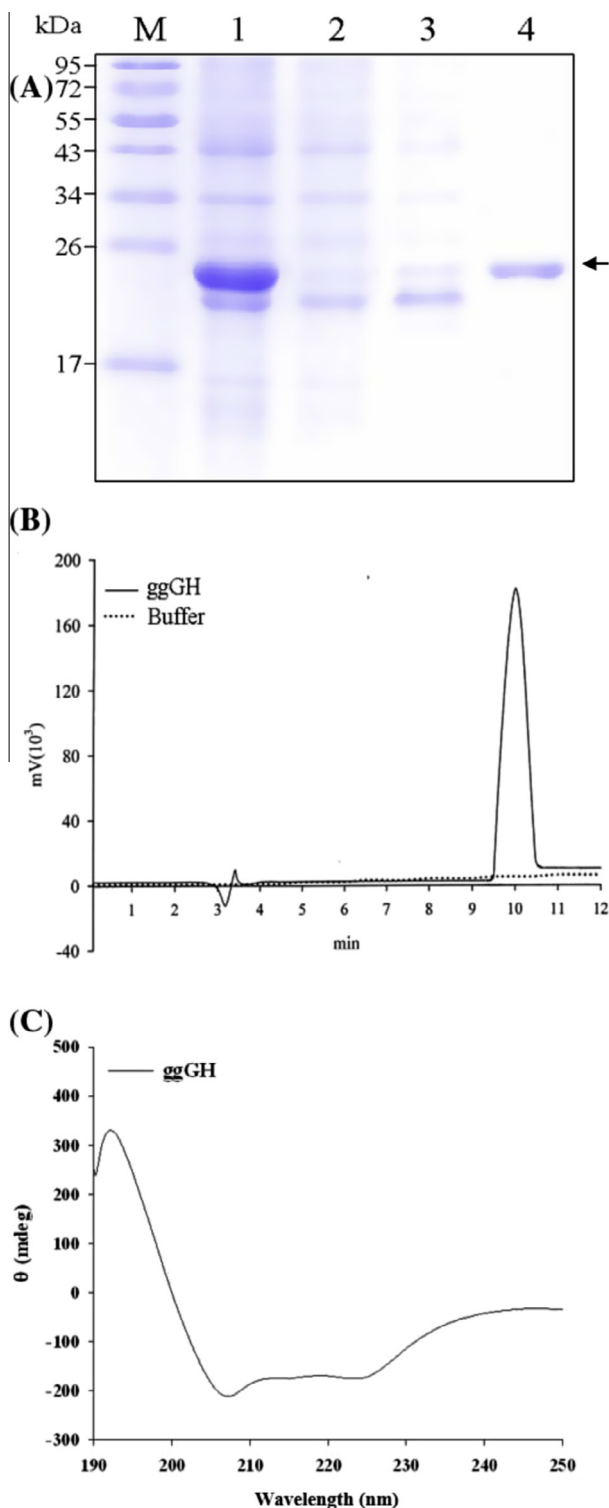


Fig. 5. (A) Purification of recombinant ggGH by Ni-NTA affinity chromatography. Lane M, molecular mass standard; lane 1, supernatant after 3 M urea and pH 12 denaturation and centrifugation; lane 2, flow through after binding nickel; lane 3, flow through after washing with PBS buffer containing 30 mM imidazole; lane 4, sample after Ni-NTA affinity chromatography and refolding. (B) Reverse phase HPLC analysis of purified ggGH. (C) CD spectrum of purified ggGH showing a typical profile of an α -helix rich protein.

saturation was maintained by decreasing the cultivation temperature during fed-batch cultivation, and the acetic acid concentration could be maintained effectively below 1 g/l. A biomass density of 41.1 g/l DCW was achieved after 30 h of cultivation by maintaining these optimal growth conditions. Although certain teleost GH genes have been cloned and expressed in *E. coli*, to the best of our knowledge, this study is the first report of production of teleost GH using fed-batch fermentation and represents the highest biomass achieved to date by fed-batch culture without pure oxygen supplementation. The efficiency of ggGH expression was estimated by IPTG induction at different cell concentrations. Due to the metabolic burden resulting from the expression of ggGH, cell mass was decreased compared to the absence of induction, regardless of when the induction was performed; thus, cell growth was strictly restricted by metabolic stress, and the lowest biomass values were obtained when induction was performed at an early stage of the log phase. Although higher biomass was obtained when induction was performed in the late log phase of the growth curve, cells at high density may undergo a stress response due to a lack of nutrients, the accumulation of metabolic waste and elevated osmotic pressure, which are all unfavorable for the production of recombinant proteins [42]. Therefore, the lowest ggGH content was obtained when induction was performed at higher cell concentrations. For these reasons, when cells were induced at mid-log phase, the maximum amount of ggGH produced was 4 g/l (22% of total protein). This value is the highest concentration of recombinant GH reported to date using an *E. coli* expression system.

Solubilization and refolding are required processes to obtain bioactive ggGH protein because ggGH is expressed mainly as an inclusion body in *E. coli*. Isolation of ggGH from inclusion bodies by centrifugation is the first step to purify the ggGH protein. High centrifugal force (10,000 \times g) was used to increase the recovery yield in this study; however, higher centrifugal force increases costs and is difficult to scale up in industry. Indeed, most ggGH can be isolated from inclusion bodies under a moderate centrifugal force (4000 \times g), and thus the isolation of ggGH in this manner is easy to practice in industry. Reports have showed that improved solubilization of recombinant GH inclusion bodies can be performed in high alkaline pH [43]. In this study, a high alkaline pH of 12 was responsible for the higher solubilization of insoluble ggGH. The result is similar to solubilization of insoluble caprine and human GH in *E. coli* and suggested that pH plays a crucial role in destabilizing the aggregation of inclusion bodies [44,45]. Changing the charge distribution within the protein molecule by changing the pH generally leads to the unfolding of the native protein and prevents multiple monomer proteins from forming aggregates, which may increase the solubility of ggGH from inclusion bodies occurring at high alkaline pH 12. Urea generally possesses the ability to diminish hydrophobic interactions between water and protein molecules. The increased solubility of ggGH in the alkaline pH buffer containing 3 M urea indicated the existence of both ionic and hydrophobic interactions in the inclusion bodies. A previous report indicated that striped catfish GH could be efficiently purified by affinity Ni-NTA chromatography and refolded into a bioactive form at alkaline pH [8]. In this study, 5.7 mg of biologically active ggGH that was more than 92% pure was obtained from a 10-ml fed-batch culture after Ni-NTA chromatography and refolding (recovery yield, 45%). In the case of fed-batch cultivation with induction at the mid-log stage, a total of 1.4 g of biologically active ggGH could be obtained from 2.5 l of final culture broth after purification. Inclusion bodies fused to an appropriate partner have been suggested to retain native-like secondary structures, with the potential to render *E. coli* hosts more efficient as microbial cell factories for protein production [46]. Furthermore, secondary structure predictions for striped catfish GH and other catfish

499 safety risks of pure oxygen make scale-up of this production
500 procedure in industry difficult in practice. Here, we offer an effi-
501 cient fermentation process for the mass production of recombinant
502 *E. coli* without the use of pure oxygen. Twenty percent air

Table 2
Protein recovery in a representative experiment: extraction from inclusion bodies.^a

Purification step	Volume (ml)	Concentration of protein (mg/ml)	Amount of total protein (mg)	Amount of ggGH (mg)	ggGH recovery (%)	Purity (%)
Solubilized of inclusion body	20	1	20	12.8	100	64 ^b
Ni-NTA affinity	15	0.4	6	5.9	46	91 ^c
Dialysis and refolding	18	0.3	6	5.7	45	92 ^c

^a A 10 ml fed-batch culture was induced at mid-log phase that corresponded to 2 g of wet cell weight.

^b Determined by densitometric scanning of SDS–PAGE gel.

^c Determined by HPLC.

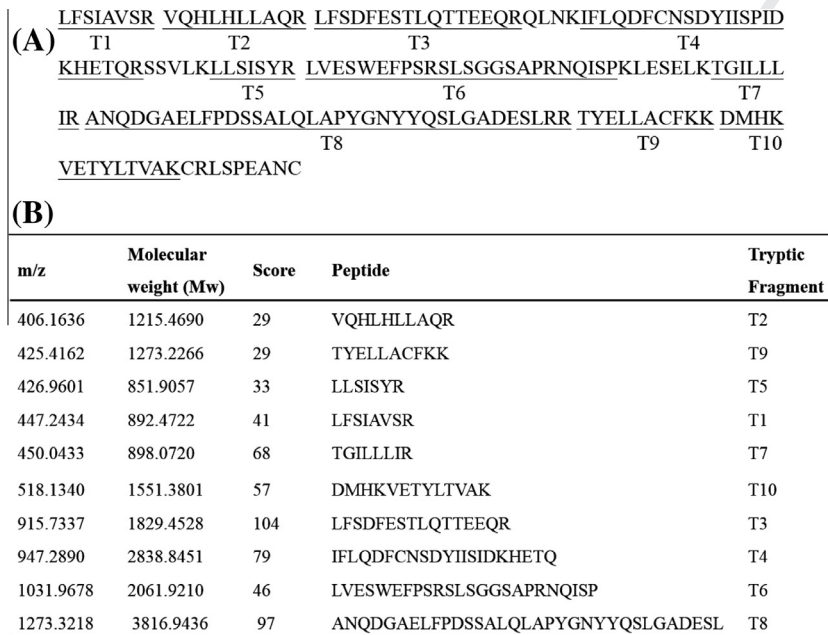


Fig. 6. LC–MS/MS analysis of purified ggGH from *E. coli*. (A) Tryptic peptide map of ggGH. (B) Identify tryptic peptide derived from purified ggGH. Peptide with ion scores more that identity threshold (score > 45) were regarded as identified peptide.

polypeptides suggested that fish GH forms four stable α -helices and may contain two helix binding sites similar to the ones in human GH [8], and the binding site for the two helices is associated with the binding affinity of the GH receptor for the transmission of cell signaling [5]. Recombinant hGH can activate signaling transduction in fish cell lines or mammalian GH-expressed transgenic fish, revealing that a high growth rate is also possible [47,48]. LC–MS/MS analysis revealed that the produced ggGH is identical to putative ggGH, while peptide mapping confirmed the identity of purified ggGH. This data confirmed the sequential validity of purified ggGH. The CD spectrum curve of purified ggGH consists of three major bands that are characteristic of α -helix rich proteins, suggesting the correct secondary structures of purified ggGH were refolded. However, correct secondary structures cannot imply purified ggGH completely has correct disulfide bonds and biological activity. In the present study the growth-promoting activity of purified ggGH proteins were demonstrated in HepG2, ZFL and GF-1 cells, indicating partial purified ggGH proteins are refolded as native confirmation.

GH-mediated gene regulation begins with signal transduction pathways activated downstream of the GH receptor, which is ubiquitously expressed in diverse tissues of vertebrates [49]. Recombinant ggGH showed significant growth-promoting efficacy in HepG2, ZFL and GF-1 cells, indicating that the process for purifying biologically active ggGH and retaining the binding affinity of ggGH

for three different GH receptors is effective. A previous report showed that recombinant human GH is biologically active in mammalian cells at concentrations of 1–50 ng/ml [36]. In our study, the growth-promoting activity of produced ggGH functioned in this range of concentrations and had higher growth stimulation efficiency compared to commercial hGH in the presence of a concentration of 10 ng/ml in mammalian hepG2 cells or teleost ZFL and GF-1 cells. Presumably, the purified ggGH has higher binding affinities for the three different GH receptors than commercial hGH. Most studies examining the growth-stimulating activity of recombinant fish GH were performed with *in vivo* intraperitoneal injection or oral administration in fish [8,32]; however, *in vitro* growth-stimulating function still has not been demonstrated in fish cell lines. In this study, we provide the first description of recombinant ggGH with biological activity to stimulate teleost cell proliferation. Given the above results, we suggest that it should be possible to develop recombinant ggGH as a growth-promoting agent in livestock or the aquaculture industry. In conclusion, we have described an efficient production system to obtain large quantities of recombinant giant grouper GH by fed-batch cultivation and a simple process for the purification and refolding of insoluble ggGH into a biologically active form. This study is the first to report an approach for high-level production of teleost GH. The strategies described may be useful for the efficient production of other teleost GH proteins expressed as inclusion bodies in *E. coli*.

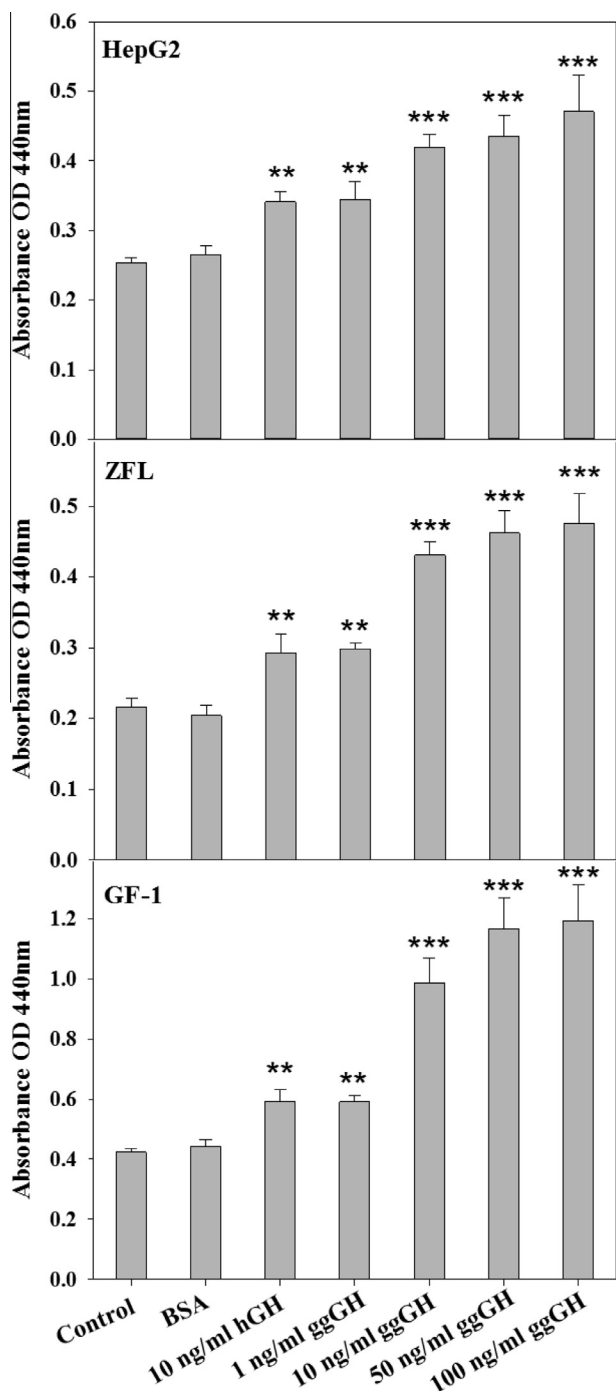


Fig. 7. Cell proliferation assay with the purified ggGH in HepG2, ZFL and GF-1 cell lines. The bioactivity of ggGH was measured in a WST-1 assay as described in "Materials and methods". The absorbance at 440 nm was measured after 48 h of cultivation in medium supplemented with different concentrations of purified ggGH, 10 ng ml⁻¹ of commercial human GH (positive control), and 10 ng ml⁻¹ of BSA (negative control). All data are presented as the mean ± standard deviation (S.D.). The results represent three independent experiments in triplicate and were analyzed by ANOVA followed by Tukey's test with a significance level of $p < 0.05$.

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