Highly Efficient Expression of Fish Growth Hormone by Escherichia coli Cells

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A PCR product encoding the mature segment of fish pregrowth hormone (pre-GH) was inserted into an Escherichia coli expression vector, pET, in which the ori site was replaced by that of pUC19. The yield of recombinant GH (rGH) was as high as 44 to 47% of total protein. This rGH was immunoreactive to GH antibody. After renaturation, rGH was used to inject fish with 0.1 μg of rGH per g once every 2 weeks, and this resulted in increases in weight (65%), percent weight gain (16%), and length (22%) relative to those of an untreated control group at week 16 and onward.

Growth hormone (GH) is one of the polypeptide hormones secreted by somatotrophs in the anterior portions of the pituitary glands of vertebrates. It is involved in the regulation of somatic growth and maintenance of protein, lipid, carbohydrate, and mineral metabolisms (4).

The GH cDNAs of fish have been cloned and sequenced, and their recombinant GHs (rGHs) have also been shown to be potent in accelerating the growth rate of fish (2, 15, 18, 21, 23). These findings make the use of GH in fish farming attractive since GH enhances the appetite, feed efficiency, and growth rate of fish (3). However, the expression efficiency, and growth rate of fish (3). However, the expression level of fish GH cDNA in Escherichia coli cells are not high: 15% of cellular proteins for chum salmon (18), less than 1 (1) or 7.7% (24) for rainbow trout, 5% for eel (12), and 20% for tilapia (10) and tuna (16). Here we describe a plasmid construct in which the GH cDNA of yellowfin porgy (Acanthopagrus latus), which is a major mariculture fish, was expressed at an extremely high level, and the resultant rGH enhanced the growth of fish.

Plasmid construct. The full-length cDNA (915 bp) encoding the pre-GH of yellowfin porgy has been studied previously (23). The cDNA fragment encoding the mature region of pre-GH (mGH cDNA) was amplified by PCR (7). A forward primer, 5′-ATATCATATGGCAGGAGATCATGAGCGCC-3′ (ATAT and an NdeI site preceded the sense strand of pre-GH from +104 to +126), and a reverse primer, 5′-ATATAAGCTTTTACTACAGGGTGCAGTTGGC-3′ (ATAT and a HindIII site preceded the antisense strand of pre-GH from +667 to +650 and one more stop codon [TAA]), were used to amplify 2 ng of template DNA. The 600-bp PCR product was ligated to the Smal site of pUC19. The DNA sequence of this amplified insert in the resultant plasmid, pUCYP, was determined by chain termination sequencing (13) with a synthesized primer, 5′-GTGCATGGGCTG-3′ (corresponding to the antisense strand of pre-GH from +289 to +277). The results show that the sequence is the same as that of the antisense strand of pre-GH cDNA from nucleotides +277 to +104 except that it is preceded by NdeI, ATAGTG, KpnI, SstI, and EcoRI cutting sites. The mGH cDNA fragment recovered from the digestion of pUCYP with NdeI and SstI was ligated into the corresponding sites of the expression vector, pET-3a (4.6 kb; Novagen), which was controlled by the promoters of lacUV5 and bacteriophage T7 φ10 (11, 19). The resultant construct was designated pETYP (Fig. 1).

A 1.4-kb DNA fragment containing the origin of the plasmid replication site, recovered by digesting pUC19 with PvuII and PstII, was ligated into the corresponding sites of the 3.2-kb DNA fragment from pET-3a. The resultant plasmid (pKL), cut with NdeI and BamHI, was then ligated with the 600-bp DNA fragment (consisting of mGH cDNA) that had NdeI and BamHI recovered from pUCYP. This plasmid construct was designated pKLYP (Fig. 1).

Induction and expression of rGH. E. coli BL21(DE3)(pLysS) (Novagen) harboring pETYP or pKLYP was grown in a superbroth medium (9) with 50 μg of ampicillin per ml and 25 μg of chloramphenicol per ml at 37°C until the optical density at 600 nm reached 0.4; a final concentration of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added for induction. After induction, grown cells were harvested, lysed, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5, 8). A protein band of 22 kDa was shown after IPTG induction for 1, 2, 3, and 4 h (Fig. 2, lanes 1 to 4, respectively). The longer the induction time, the higher the intensity of the rGH band. On the basis of scanning the intensities of different bands on gels with a Densito-Pattern Analyzer EPA-3000 (Maruzen Petrochemical Co., Tokyo, Japan), the rGH expression level increased from 10 to 25.3% for cells containing pETYP and from 19.4 to 47.4% for cells containing pKLYP (Fig. 2). However, the efficacy of rGH synthesis decreased from 47.4 to 41, 39, and 30% when cells harboring pKLYP reached A600 of 0.5, 0.6, and 0.7, respectively, after 4 h of induction. The rGH band was absent in the lysates of cells containing either the vector alone (Fig. 2, lane C) or the vector with an mGH cDNA insert before induction (Fig. 2, lane O).

The amount of rGH expressed in cells harboring pETYP and pKLYP ranged from 21 to 25% and from 44 to 47% (n = 5) of total bacterial protein, respectively. The expression level of rGH expressed by pETYP was around twofold higher than that expressed by pETYP. We suggest that higher copy numbers of plasmids are involved in enhancing the expression level. The replication site of pETYP was located on the larger PvuII-PstII fragment. We replaced it with an Rop- pUC19 replicon in pKLYP, resulting in a high copy number of plasmids (17). Thus, the copy number of pKLYP presented in a host cell
might be much higher than that of pETYP, although the exact copy numbers of these two plasmids have not been determined. This is consistent with the results of Sato et al. (16), who reported that using a high-copy-number expression vector resulted in an increased level of bluefin tuna rGH expression.

Compared with the levels of fish rGH expressed in the other microorganisms studied (1, 6, 10, 15, 18, 24), the E. coli harboring pKLYP in this study biosynthesized rGH at the highest expression level.

Western blot (immunoblot) analysis. Protein bands were blotted to a nitrocellulose membrane (20) and detected with polyclonal antiserum raised against natural bonito GH by using alkaline phosphatase-conjugated secondary antibodies with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium as substrates (23). Immunoblot analysis showed that the protein band of 22 kDa reacted specifically.

Amino acid analysis. N-terminal sequencing of the rGH polypeptide was carried out by automated Edmán degradation with a model 477A protein sequencer and an on-line phenylthiohydantoin 120A analyzer (Applied Biosystems, Inc.). The results showed that the 30 amino acid residues of the N terminus are M-Q-P-I-T-D-G-Q-R-L-F-S-I-A-V-S-R-V-Q-H-L-H-L-A-Q-R-L-F-S. They are exactly the same residues that were deduced from codons 18 to 46 of yellowfin porgy pre-GH cDNA (23), except that methionine was introduced by genetic engineering in order to initiate translation. The signal peptide of pre-GH (from codons 1 to 17) was not included.

Isolation of inclusion bodies. The inclusion bodies of E. coli transformants containing pETYP or pKLYP were isolated by treatment with 1.5 M guanidine hydrochloride (2). After the inclusion bodies were resuspended in 8 M urea, the sample buffer (8) was added and analyzed by SDS-PAGE. We found that the major band shown on the gel was a protein located at 22 kDa, which constituted at least 90% of total protein. However, this protein band was absent in the cells containing only the vector.

Denaturation and renaturation of rGH. The inclusion bodies were solubilized and renatured by the method of Sato et al. (14) with some modifications. After induction, cells were collected, washed with TE buffer (10 mM Tris, 1 mM EDTA [pH 8]), and sonicated. The inclusion bodies were collected from precipitates. When the supernatant was analyzed by SDS-PAGE, an extremely small amount of rGH remained in the supernatant (Fig. 3, lane 3). This indicates that most of the biosynthesized rGH aggregates in the inclusion bodies in an insoluble form.

The resultant pellet was resuspended in 1 M sucrose, centrifuged, and washed with 1% Triton X-100 containing 50 mM EDTA (pH 8). The pellet was solubilized with 6 M guanidine hydrochloride and dialyzed against 167 mM ammonium bicarbonate (50 mM; pH 10) containing 2 mM EDTA overnight at 4°C. After centrifugation, the supernatant was continuously dialyzed against 30 mM sodium bicarbonate (pH 8.8) overnight. The resultant supernatant was lyophilized and dissolved in 30 mM sodium bicarbonate at the desired concentration. rGH was analyzed by gel electrophoresis to detect renatur-
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