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Expression of Gilthead Seabream (*Sparus aurata*) Growth Hormone in *Escherichia coli* Using Alginate Lyase Gene Promoter of *Pseudomonas* sp.

Jong-Hee Lee, Sun-Young Choi, Sang-Bong Lee, Cheal-Ho Jin, Sung-Hoi Huh[1] and In-Soo Kong*

Department of Biotechnology and Bioengineering, and
[1]Department of Oceanography, Pukyong National University, Pusan 608-737, Korea

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The promoter region of alginate lyase gene (*aly*) from *Pseudomonas* sp. W7 was used for the high expression of gilthead seabream (*Sparus aurata*) growth hormone (GH) gene in *Esherichia coli*. PCR product encoding the premature segment of the growth hormone was cloned to the downstream of *aly* promoter. GH was overexpressed with 46 amino acid of alginate lyase as fusion protein. GH was immunoreactive and production of GH was repressed with supplementation of 0.4% glucose into culture media.

Key words: gilthead seabream, growth hormone, alginate lyase promoter, overexpression

Introduction

Growth hormone (GH) is one of polypeptide hormones secreted by somatotrophs in anterior portions of pituitary glands of vertebrates and plays an important role in the regulation of somatic growth and maintenance of protein, lipid, carbohydrate and mineral metabolism (Ganong et al., 1983). The cDNA of preGH is consisted of 612 nucleotides and 204 amino acids, which had a predicted molecular weight of 22kDa (Funkenstein et al., 1991). There have been a number of studies demonstrating the growth promoting effect of recombinant growth hormone on fish via several delivery routes. Injection (Agellon et al., 1988; Tsai et al., 1994; Tsai et al., 1995), immersion (Schulte et al., 1989; Moriyama et al., 1990), constant infusion (Down et al., 1989) and oral administration (McLean et al., 1990; Moriyama et al., 1993) of GH showed the increase of body length and body weight of fishes. Among the various routes of growth hormone delivery to fish oral administration appears to be practical method so far (Jeh et al., 1998).

The choice of an expression system for production of recombinant proteins depends on many factors such as growth characteristics, expression levels, orientation of expression and the cost of production including purification yield. The essential component for production of protein is promoter. The most applicable promoters for large-scale protein production used thermal induction or chemical inducers in *E.coli* system. However, not only these chemical inducers are so expensive and toxic (Makrides, 1996), and also conditions for preventing repression is quite cumbersome. So it needs a system for producing of protein with inexpensive and effective way. We already reported the promoter region of alginate lyase gene (Kim et al., 1996; Lee et al., 1998). From previous report, *aly* promoter has strong ability for the expression of alginate lyase in *E.coli* (Kim et al., 1996; Lee et al.,1998). In this study, we have constructed an expression system for fish growth hormone (gilthead seabream) connected with *aly* promoter. The expression system was constructed by assembling the DNA fragments containing the promoter region of alginate lyase gene.

Materials and Methods

Plasmids

pGSBGH which containing the cDNA of gilthead seabream GH was obtained from Dr B. Cavari

*To whom correspondence should be addressed.
Phone : 82-51-620-6185, Fax : 82-51-620-6180
E-mail : iskong@nuri.net

(Funkenstein et al., 1991). pKAL24 (Kim et al., 19 96; Lee et al.,1998) was used for construction of expression system

Construction of expression vector

To obtain the premature region of fish growth hormone, primers were designed on the exact sequence of growth hormone cDNA (Funkenstein et al., 1991). As shown in Fig. 1, The sense primer; 5′ GGCCGTCGACATGGACAGAGTGG TGCTC 3′ (GTCGAC: *Sal*I: primer OI) and antisense primer; 5′GGCCAAGCTTTACTAC AGGGTGCAGTTGGC 3′ (AAGCTT: *Hind*III: primer OII) were used for obtaining GH region from pGSBGH3 (Funkenstein et al., 1991). The ligated fragment of promoter region from pKAL24 and GH region was amplified by PCR with primer OIII (5′TTGAATTCGCCAC TCGCG-ATGAATCG 3′: GAATTC: *Eco*RI, for promoter region) and OII to introduce *Eco*RI recognition site to 5′ end of promoter region. The reactions were performed in a thermal cycler (Perkin-Elmer Cetus) for 25cycles of 30 sec 94℃, 30 sec 55℃, 1 min 72℃ with final extension at 72℃ for 5 min. The resultant PCR product was cloned to pUC19, which was digested *Eco*RI and *Hind*III. Resultant plasmid, pALN69, was transformed to *E.coli* DH5α.

Southern hybridization

The *Eco*RI and *Hind*III digested fragment of pALN69 was transferred to nitrocellulose membrane. The PCR product of pGSBGH3 with primer OI and OII were labeled with digoxigenin-dUTP according to the manufacture's instruction and used as probe.

Identification of recombinant growth hormone

E.coli cells were harvested from 1000 ㎖ of liquid culture and resuspended in 50 ㎖ of 20 mM Tris-HCl buffer, pH 8.0. The cells were disrupted by sonication and the clear lysate was centrifuged at 15,000×g for 20 min. The pellet was resuspended with 50 ㎖ of 20 mM Tris-HCl buffer, pH 8.0 and centrifuged 3 times to remove soluble components. Recovered inclusion body fractions were solubilized in 10 ㎖ of 6M guanidine hydrochloride solution and dialyzed overnight against 20 mM Tris-HCl buffer. The protein sample was subjected to SDS-PAGE, and followed by western blotting using anti gilthead seabream GH antibody which also provided by Dr. B. Cavari After SDS-PAGE, the proteins were transferred to nitrocellulose membrane at 100 V, 2 h at 4℃ and blocked with 0.5% skim milk in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) to prevent non specific binding. The membrane was incubated with primary GH antibody and secondary antibody (alkaline phosphatase conjugated anti-rabbit IgG), respectively. To investigate the effect of glucose on expression of GH gene, glucose was added to LB medium at the concentration of 0.4%.

Results

Overexpression of recombinant growth hormone

The premature region of gilthead seabream growth hormone (preGH) was amplified by PCR method and promoter region, involving 138 nucleotides of alginate lyase gene, was isolated by restriction endonuclease, *Hind*III and *Sal*I. The promoter region of alginate lyase gene was fused to the upstream of growth hormone gene. The replaced sequence was confirmed by the dideoxy chain termination method (Sanger et al., 1977) and southern hybridization of PCR product of pGSBGH3 and *Eco*RI and *Hind*III fragment of pALN69 was corresponded to the predicted size (Fig. 2). From the result of SDS-PAGE, overexpressed protein was detected approximately as 24kDa protein band (Fig. 3). The added 46 amino acids might be contribute to the increment of molecular mass. To verify that the expressed

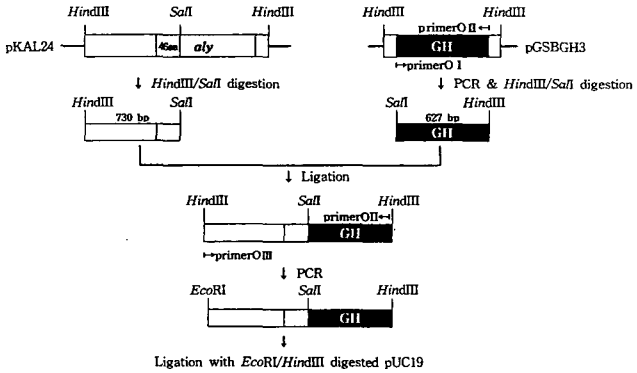

Fig. 1. Construction of gilthead seabream GH expression plasmid, pALN69. The *Hin*dIII-*Sal*I fragment containing *aly* promoter and N-terminal 46 amino acids of alginate lyase from pKAL24 was ligated with PCR product of pGSBGH3. Ligated DNA was amplified with primer OII and primer OIII, and then cloned into a *Eco*RI-*Hind*III site of pUC19, which was named pALN69.

protein was corresponding to fish growth hormone. The western blot analysis was performed. As shown in Fig. 4, a positive band was matched as the protein with the band stained with comassie stained SDS-PAGE gel. However, *E.coli* harboring pUC19 did not show a positive band around the

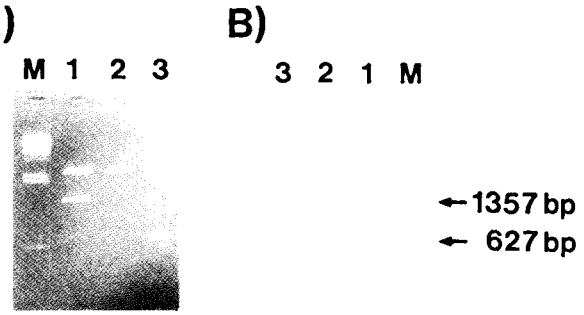

Fig. 2. Southern hybridization of PCR product and pALN69. (A) Agarose gel electrophoresis. Lanes: M, size marker (λ DNA digested with *Hin*dIII); 1, pALN69 digested with *Eco*RI and *Hin*dIII; 2, pUC19 digested with *Hin*dIII; 3, PCR product of pGSBGH3 with primer OI and OII. (B) Southern hybridization. The insert DNA of pGSBGH3 digested with *Eco*RI and *Hin*dIII was used as a probe after labelling with digoxigenin-dUTP by random priming.

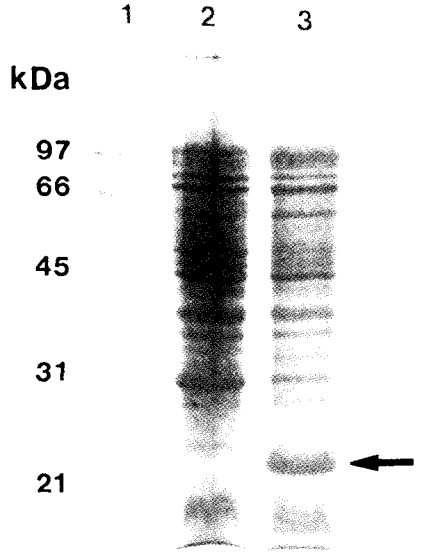

Fig. 3. The expression of gilthead seabream GH in *E.coli*. *E.coli* was cultivated in LB broth with ampicillin at 37℃ for 20 h. Lanes: 1, low molecular marker (BioRad. Co. Ltd. USA); 2, *E.coli* (pUC19); 3, *E. coli* (pALN69).

size of growth hormone. The yield of expressed growth hormone was over 30% and *aly* promoter constitutively expressed this GH in *E.coli*. The overexpressed GH formed inclusion body but well refolded by simple dialysis process. From this simple procedure, the purity of growth hormone reached to more than 90% of total cellular protein (Fig. 4).

Repression of GH production by glucose

We had reported the repression of *aly* promoter by glucose (Kim et al., 1996). The addition of glucose repressed the production of alginate lyase in *E.coli*. In present study, we also found a repression of the fusion protein production by glucose. When 0.4% of glucose was added to medium (LB), we could not identify the expression of growth hormone from SDS-PAGE (Fig. 5).

Discussion

One of the major strengths of recombinant DNA technology is large production of protein from host cell. *Aly* promoter showed the strong ability for overexpressing a foreign protein in *E.coli*. The one important feature of *aly* promoter is the repression for expressing protein by glucose. This might be the clue for understanding and characterization of this promoter. Glucose is a cost effective carbon source for culture of *E.coli* in large-scale production of recombinant protein. Sawers et al. also reported

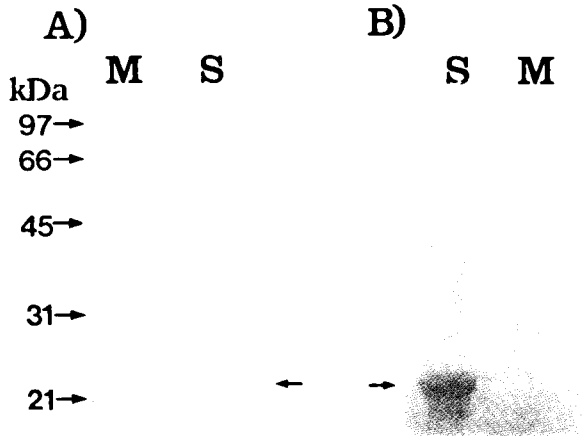

Fig. 4. SDS-PAGE of a partial purified GH and western blot analysis. Panel A and B showed the pattern of SDS-PAGE and western blotting, respectively (Lanes: M, low molecular marker; S, partial purified GH).

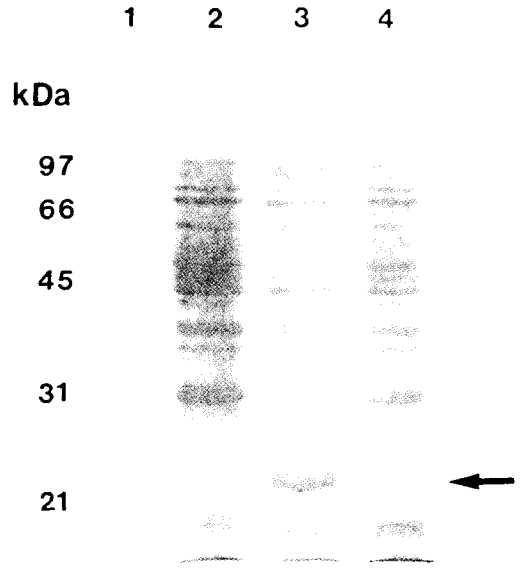

Fig. 5. The repression of *aly* promoter by glucose. Lanes: 1, low molecular marker; 2, *E.coli* (pUC19); 3, *E.coli* (pALN69) cultivated without glucose; 4, *E.coli* (pALN69) cultivated with 0.4% glucose.

that the regulation by glucose as a sole regulator has the advantage in large scale process because it is possible to be readily and cheaply controlled. But this growth hormone was expressed by fusion protein with 46 N-terminal amino acids of alginate lyase in its upstream. When this GH had been attempted to express directly without N-terminal amino acids of alginate lyase, any overexpressed protein was not founded at the size of GH from SDS-PAGE and western blotting (Data not shown). Marston (Marston. 1986) presented the possibility that small part of N-terminus fusion protein may affect the level of transcription, translation or the mRNA stability. We did not confirm that the shorter N-terminal amino acid than used 46 amino acid was possible for expressing the protein under the *aly* promoter. In this paper, we did not determine the biological activity in fish farming. However, data from this research gave us the possibility on the expression of other useful genes with this system

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