Cloning and Characterization of cDNA for Korean Rockfish (*Sebastes schlegeli*) Insulin-like Growth Factor-I

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Abstract To understand the comprehensive mechanisms of biological function for insulin-like growth factor-I (IGF-I) in vertebrates, we have investigated the cDNA sequence of this gene in the korean rockfish (*Sebastes schlegeli*). The mature form of korean rockfish IGF-I was found to be comprised of 67 amino acid residues, showing about a 7 kDa molecular weight. In this study, we used the polymerase chain reaction (PCR) to obtain a korean rockfish IGF-I (KR IGF-I) cDNA fragment, and methods of rapid amplification of cDNA ends (RACE) to obtain a full length of the KR IGF-I sequence.

The KR IGF-I encoded for a predicted amino acid sequence showed identities of 93.6 %, 90.7 %, and 85.4 % in comparison with flounder, chinook salmon, and human IGF-I, respectively. To obtain recombinant biologically active polypeptides, korean rockfish B-C-A-D domains were amplified using the PCR, then the isolated cDNA was expressed in the *E. coli* BL21(DE3). The recombinant KR IGF-I protein biological function was measured by stimulation of [^{3}H] thymidine incorporation, suggesting the cDNA codes for the korean rockfish proIGF-I.

Key words : Insulin-like growth factor-I (IGF-I), korean rockfish (*Sebastes schlegeli*), rapid amplification of cDNA ends (RACE)

Introduction

The insulin-like growth factors (IGF-I and IGF-II) are single chain polypeptides, structurally related to proinsulin, and play a major role in the regulation of normal growth and development. The biological actions of IGFs are mediated by a family of cell surface receptors that induces the type I and type II IGF receptors [4,12]. Furthermore, IGF binding proteins are also important modulators of the biological actions of IGFs [1].

It is generally accepted that IGF-I is produced predominantly in the liver in response to the binding of growth hormone to its specific receptor and mediates the growth-promoting effect of growth hormone [13, 17]. IGF-I contains the NH_2-B-C-A-D-COOH domain; the single peptide and E domain are removed during proteolytic processing to generate the mature form of IGF-I [4,5].

IGF-I cDNA sequences are highly conserved in different vertebrates, including humans [18], chicken [9], rats [19], salmonids [8,10,11], eel [6], and tilapia [2]. In vertebrates, the effects of IGF-I on growth, differentiation, and proliferation are mediated by growth hormone and nutrition conditions [5,7,13]. In spite of the importance of IGF-I for growth and development in vertebrate, there is not a clear understanding of the mechanisms of the IGF-I system in fish since the most diverse group of vertebrates is fish.

Hence, the present study aimed to establish the cDNA sequence of prepro-IGF-I and to investigate the biological role in korean rockfish. As well, we found that the expression of the korean rockfish IGF-I matured peptide by the *Escherichia coli* gene expression system.

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Materials and Methods

Materials

All chemicals were purchased from the Sigma Chemical Company (St. Louis, Mo). Polyacrylamide was purchased from National Diagnostics (Atlanta, USA). Prestained molecular weights standards and [methyl-^{3}H]thymidine (1 μCi /μg) were purchased from Amersham Pharmacia biotech (Little Chalfont, UK). EDTA and Tween 20 were obtained from USB (Little Chalfont, UK). A Mighty Small Ⅱ Apparatus (Hoefer Scientific Instruments, San Francisco, CA) was used for protein electrophoresis. Tissue culture plates were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA).

RT-PCR cloning and sequence analysis

Two degenerate primers were designed from the alignment of IGF-I amino acid sequences from human, salmonide, carp. Liver of korean rockfish (*Sebastes Schlegeli*) was cracked with syringe in TRI reagent (Sigma) and descending size of needles. The RNA sample was suspended in DEPC-treated water after ethanol precipitation, spectrophotometrically quantified at A_{260} and $A_{280\ nm}$, and then analyzed for their integrities on agarose gel (1%) with formaldehyde.

RT-PCR was performed (denaturation at 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 30 s at 52°C, 4 min at 72°C, and finally an elongation step at 72°C for 10 min) with one step RNA PCR kit (TAKARA) and the PCR products were cloned into pT7 Blue T vector (Novagen) for nucleotide sequence determination.

To obtain the full-length sequence of korean rockfish IGF-I (KR IGF-I), rapid amplification of cDNA ends (RACE) methods were performed using gene specific primers (Table 1). 3' and 5' RACE were performed according to the menufacturer's instructions (Clontech). cDNA clones appeared on the LB-ampicillin plate and next the QIAGEN plasmid extraction miniprep kit was used to extract DNA. The entire cDNA, digested with *Pst* I and *Eco R*I restriction enzyme, was subcloned into pT7 Blue T vector and transformed into JM109, and then sequenced by ABI autosequencer. The nucleic acid sequence from the clones was used to search the GenBank database.

Southern blot analysis

The products of RACE were separated by agarose gel electrophoresis, and southern blotting was carried out on amplification products after transfer on nylon membrane (ICN BIOTRANS^{TM}). The blot was prehybridized (1h, 42°C) in a solution of 5X SSC (1X SSC is 0.8% NaCl and 0.4% sodium citrate), 0.02% SDS, 0.1% N-laurysarcosine and 1% blocking reagent. Then, the blot was hybridized (18 h, 42°C) by adding 5 μl of a DIG labeled DNA probe which has been sequenced and identified as part of KR IGF-I. Using PCR and degenerate oligonucleotide primers, which are specific for two highly conserved sequences found in several vertebrates, a resultant cDNA fragment was used as a probe.

The 108 bp DNA probe was synthesized by PCR DIG probe synthesis kit (Roche). The blot was washed at room temperature for 15 min in 2X SSC, 0.1% SDS and for 15 min in 2X SSC, 0.1% SDS at 68°C for 30 min in 0.1X SSC, 0.5% SDS and then at 68°C for 30 min in 0.1X SSC, 0.5% SDS. The hybridized probe was detected with the DIG DNA Detection kit (Roche) and visualized by chemiluminescence reaction.

Expression of recombinant KR IGF-I protein (rKR IGF-I)

rKR IGF-I, from B domain to D domain, was amplified by PCR and two oligonucleotide primers; KR5 primer (sense primer : 5'-GGCCATGGGACCGGAGAC CCTGTGCGGG-3') and KR6 primer (antisense primer : 5'-CCGGATCCAGCTGCCTTGCTAGTCTTGGC-3'). The PCR products were constructed with the histidin (His) gene fusion system (Novagen) of expression vector pET 22b(+) with an N-terminal histidine tag for KR IGF-I. To overexproduce the fusion protein the ex-

Table 1. Primers and used in RACE

Description	Nucleotide sequence
5 ′ RACE primers	5'-GCACGGCGGTCACGCGGCATCGTCGA-3' 5'-CGCGGCATCGTCGACGAATGCTGCTTC-3'
3 ′ RACE primers	5'-AGTAAACCAACAGGTTATGGCCCCAAT-3' 5'-AGAGGCTTTTATTTCAGTAAACCAACAGGTTA-3'

pression vector was transformed into *E. coli* BL21(DE3) cells and selected by ampicillin. Colony hybridization and sequencing were used to identify the correct direction for insertion.

A single colony of BL21(DE3) *E. coli* cells containing a recombinant pET-IGF-I plasmid was incubated in 100 ml of LB broth and incubated at 37°C for 18 h with shaking. The culture was then transferred into 500 ml of LB broth and incubated at 37°C with shaking until the absorbance at 600 nm was 0.6. Then 0.1 mM isopropyl-thio-D-galactoside (Sigma) was added to the culture medium and the culture was incubated at 37°C for 3 h with shaking.

The cells were subsequently pelleted and stored at -80°C. After thawing, the cells were resuspended in 10 ml of binding buffer containing 160 mM Tris-HCl (pH 7.5), 4 M NaCl, and 40 mM imidazole and then lyzed by sonication. The pellet containing insoluble rKR IGF-I was resuspended in 15 ml of the binding buffer supplemented with 6 M guanidine-HCl and incubated on a rotating platform for 24 h at 4°C to denature the protein. rKR IGF-I in the denaturated state was recovered in the supernatant fraction after centrifugation at 10,000g for 30 min. This supernatant was loading on a Ni^{2+} chelation resin column (Novagen) and the elution was carried out with 80 mM Tris-HCl (pH 7.5) containing 2 mM NaCl, 4 M imidazole, and 6 M guanidine-HCl. Proteins were analyzed by 15% SDS-PAGE and stained with Coomassie brilliant blue R-250.

Bioassay

The bioactivity of rKR IGF-I protein was measured with an in vitro assay. [^{3}H]Thymidine incorporation into DNA in a CHES-214 embryonic cell was studied. CHES-214 (3X 10^4 cells/well) were seeded in 24-well plates in DMEM medium supplemented with 10% FBS for 24 h. After 24 h of serum free incubation, the cells were incubated with or without various amounts of IGF-I (0 - 100 nM) for 18 h. The cells were then pulse-labeled with [^{3}H]Thymidine (1 μCi/ml) for 2 h at 25°C. Then 0.5 ml of 3 N NaOH was added, and after 5 min the mixture was transferred to scintillation vials. After addition of 10 ml of aquasol, the solution was counted in a scintillation counter(Phamacia). Every parameter in this experiment was repeted three times.

Results

Isolation and sequence analysis of KR IGF-I cDNA

To identify and isolate the KR IGF-I cDNA, firstly two degenerate primers withen the conserved regions of human, salmonid, and carp IGF-I cDNAs. RT-PCR was performed using the first strand cDNA templates prepared from liver of korean rockfish. Cloning and sequence analysis of the PCR product revealed a 108 bp partial sequence of an unknown IGF-I cDNA (Fig. 1).

To obtain the full-length sequence of KR IGF-I, 5' RACE and 3' RACE were used (Table 1) with two sets of gene-specific primers. The amplification and sequencing of the 3' and 5' RACE revealed a 500 bp sequence of the translated IGF-I mRNA of the korean rockfish liver (Fig. 2). Nucleotide sequence determination of this fragment showed that the full-length sequence described in Fig. 4 is a single mRNA transcript with the same cDNA sequence carrying an ORF of the preproIGF-I sequence.

By southern blotting of the RACE products and hybridization with a KR IGF-I partial DNA probe which had been sequenced before, the RACE products were

5'-	GAC	ACG	CTC	CAA	TTT	GTG	TGC	GGT	GAG	AGA	GGC
	D	**T**	**L**	**Q**	**F**	**V**	**C**	**G**	**E**	**R**	**G**
	TTT	TAT	TTC	AGT	AAA	CCA	GGC	TAT	GGC	CCC	AAC
	F	**Y**	**F**	**S**	**K**	**P**	**G**	**Y**	**G**	**P**	**N**
	GCA	CGG	CGG	TCA	CGT	GGT	ATA	GTC	GAC	GAA	TGC
	A	**R**	**R**	**S**	**R**	**G**	**I**	**V**	**D**	**E**	**C**
	TGT	TTC	CAG	-3'							
	C	**F**	**Q**								

Fig. 1. Nucleotide and deduced amino acid sequence of the partial cDNA obtained by PCR from total RNA of korean rockfish.

verified (Fig. 3).

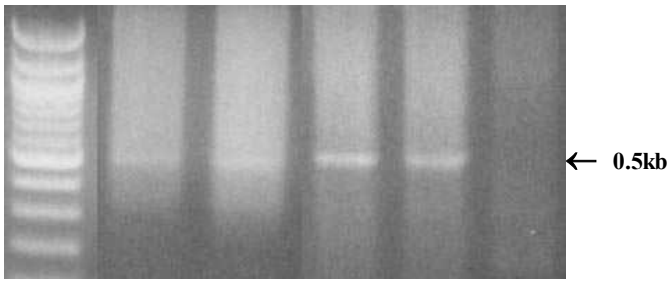

Fig. 2. Amplification of korean rockfish IGF-I cDNA by RACE.
5' and 3' RACE amplification of korean rockfish liver IGF-I. Double strand IGF-I cDNA was synthesized from korean rockfish liver mRNA and ligated to cDNA adaptor AP1. M : molecular size marker (1 Kb ladder), lane 1, 2 : 5' RACE products, lane 3, 4 : 3' RACE products.

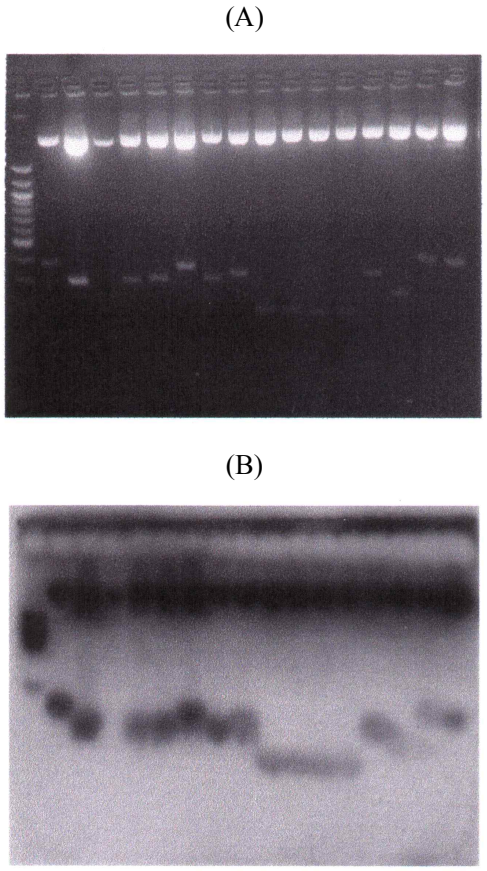

Fig. 3. Southern blot analysis of RACE products.
RACE products digested with *EcoR* I and *Pst* I from clones with insert DNA. RACE products were electrophoresed in a 1% agarose gel, transferred onto nitrocellulose membrane and hybridized with 108 bp korean rockfish IGF-I cDNA. Panel (A) : 1% agarose gel electrophoresis patterns. Panel (B) : Southern hybridization.

Structure of KR IGF-I

The cDNA sequence corresponding to the coding region of the KR IGF-I preprohormone, as indicated in Fig. 4, is 558 bp long. The deduced IGF-I protein con-

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TACTCACTATAGGGTTCGAGCGGTCGCCCGGGCAG
GTTCTCACTTCTCCAAAACGACCCTGCGCAATGGA
ACAAAGTCGGAATATTGAGATGTGACCTTGTCCGA
CCGACCTCTCATCCTCTTTCTCCCCCTTTTTTAAT
GCCTTCAAACAAATTCCTTTTCGCCGGGCTTTGAC
TTGCCGAGACCCGTGGG

                                    33
ATGTCTAGCGCTCTTTCTTTTTCTTGGCATTTA
 M  S  S  A  L  S  F  S  W  H  L
                                    66
TGTGATGTCTTCAAGGCTGCGATGTGTTGTATC
 C  D  V  F  K  A  A  M  C  C  I
                                    99
TCCTGTAGCCACACCCTCTCCCTACTGCTGTGT
 S  C  S  H  T  L  S  L  L  L  C
                                   132
GTCCTCACCCTCACTCCGACCGCAACAGGAGCG
 V  L  T  L  T  P  T  A  T  G  A
                                   165
GGACCGGAGACCCTGTGCGGAGCGGAGCTGGTC
 G  P  E  T  L  C  G  A  E  L  V
                                   198
GACACGCTCCAATTTGTGTGCGGGGAGAGAGGC
 D  T  L  Q  F  V  C  G  E  R  G
                                   231
TTTTATTTCAGTAAACCAGGTTATGGCCCCAAC
 F  Y  F  S  K  P  G  Y  G  P  N
                                   264
GCACGGCGGTCACGTGGTATAGTCGACGAATGC
 A  R  R  S  R  G  I  V  D  E  C
                                   297
TGTTTCCAGAGCTGTGAGCTGCGGCGCCTGGAG
 C  F  Q  S  C  E  L  R  R  L  E
                                   330
ATGTACTGCGCACCTGCCAAGACTAGCAAAGCA
 M  Y  C  A  P  A  K  T  S  K  A
                                   363
GCTCGCACCGTGCGTGCACAGCGCCACACAGAC
 A  R  S  V  R  A  Q  R  H  T  D
                                   396
ATGCCAAGAGCACCTAAGGTTAGTACAGCAGGG
 M  P  R  A  P  K  V  S  T  A  G
                                   429
CACAAGGGCACCGAGCGTAGAACAGCACAGCAG
 H  K  G  T  E  R  R  T  A  Q  Q
                                   462
AAAGACAAGACAAAAAACAAGAAGAGAGCATTA
 K  D  K  T  K  N  K  K  R  A  L
                                   495
CCGCATACACATGCTTTGCTCTTCATGCGCCAA
 P  H  T  H  A  L  L  F  M  R  Q
                                   528
AGCCAGCTTCTTACATTTTGTGTAGGAATTGTA
 S  Q  L  L  T  F  C  V  G  I  V
                                   561
TGTGAAGAATTCCTTCATATGCCTGGACTT
 C  E

CTGTGATATGCTTAAAACTGGTTTTGGCCAATACG
AAATATCCTTCAATAAACATTGTCCCAAAAAAAAA
AA
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Fig. 4. Nucleotide and deduced amino acid sequence of the Korean rockfish IGF-I cDNA obtained from RACE. Double lined letters indicate amino residues of the domain expressed in *E. coli* host vector system. Underlined and bolded sequences show polyadenylation signal and poly(A) tail, respectively.

sists of 185 amino acids spanning the signal peptide, domains B, C, A, D and E. The B domain consisted of 29 amino acids (aa), the C domain of 9 aa, the A domain of 21 aa, the D domain of 8 aa, and E domain of 67 aa. The E domain of this IGF from korean rockfish is shorter than the other teleost IGF-I is. Amino acid sequence comparison also shows that this KR IGF-I has sequence identities of 93.6%, 90.7% and 85.4% in comparison with flounder, chinook salmon and human, respectively. It is concluded that the KR IGF-I cDNA encodes for a preproIGF-I sequence (Fig. 5).

Recombinant KR IGF-I (rKR IGF-I) protein expression and biological function

To investigate the biological properties of rKR IGF-I, the cDNA encoding, the KR IGF-I was subcloned downstream of the bacteriophage T7 promoter in an expression vector pET 22b(+). *E. coli* BL21 (DE3) was transformed with the resulting plasmid, pET 22b(+) / KR IGF-I, and the transformed bacteria were induced to produce the recombinant protein with a polyhistidine tag at its N-terminus. Extracts were prepared from *E. coli* and analyzed by the SDS-PAGE. As shown in Fig. 6, the whole-cell extracts contain the recombinant protein of about 7 kDa as one major protein band (Lane 2), which is not present in the bacteria carrying the control plasmid (Lane 1). This rKR IGF-I was found to localize in the insoluble fraction of cell extracts and was successfully purified under denaturing conditions with a Ni^{2+} column (Lane 3).

After purification of the rKR IGF-I, the rKR IGF-I revealed a single band of 7 kDa on the SDS-PAGE. To test biological function of rKR IGF-I polypeptides,

B domain **C domain**

Korean rockfish	GPETLCGAELVDTLQFVCGERGFYFSKP-G	YGPNARRS--R
Flounder	----------T-	----------
Chinook salmon	----------A----------N-----	----SS----HN-
Human	----------A----------N-----	---SSS---APQT

A domain **D domain**

Korean rockfish	GIVDECCFQSCELRRLEMYCA	PLKSSKAA
Flounder	----------	-A-------
Chinook salmon	----------	-V-SG----
Human	---------R---D----------	-L-PA--S-

Fig. 5. Comparison of deduced amino acid sequence between human and several fishes. Dot line(-) indicates identical amino acid sequence.

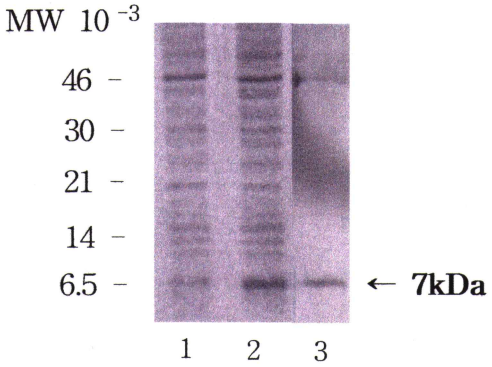

Fig. 6. Expression of korean rockfish IGF-I mature peptide in *E. coli* BL21(DE3).
Cells were cultured in LB medium with 100 μg/ml ampicillin at 37°C until OD_{600} reached 0.6. Then, 0.1 mM IPTG was added for induction of Korean rockfish mature protein synthesis. The cells were harvested after 180 min induction. The fusion proteins were purified by Ni^{2+} chelation resin column (Novagen). Lane 1 : the expression protein with the pET 22b(+) vector alone, lane 2 : the expression protein with the pET 22b(+) ligated IGF-I insert, lane 3: the expression protein after purification as a single band.

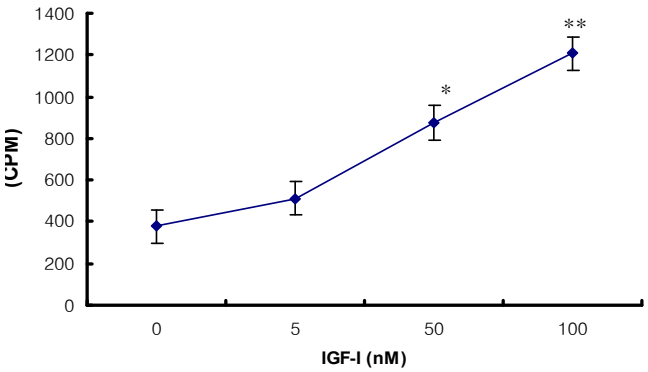

Fig. 7. Effects of recombinant korean rockfish IGF-I protein on stimulated CHES-214 cell proliferation. The following effects were measured for different concentrations of recombinant korean rockfish IGF-I. Asterisks indicate that the value is significantly different (*$p < 0.01$, **$p < 0.001$) from the control group (0 mM). The [^{3}H]-thymidine into DNA synthesis experiment was repected three times.

they were analyzed by in vitro assay of incorporation of [^{3}H]thymidine into DNA differentiation. The test quantities were between 0 and 100 nM and incorporation ability significantly increased over 50 ng (Fig. 7, *$p<0.01$ and **$p<0.001$).

Discussion

To isolate cDNA encoding for IGF-I in korean rock-

fish, a strategy based on regions of nucleotide conservation was used to generate the fragment of cDNA encoding the B-, C-, A-, D domains. A sequence nucleotide alignment was carried out on IGF-I sequences of several fish species to design primers for the RT-PCR and RACE. PCR products generated by 3' and 5' RACE procedures of about 500 bp were combined to generate the composite full cDNA sequence. A comparison of the nucleotide and deduced amino acid sequences of the korean rockfish IGF-I with that from other species indicated that the cDNA sequence obtained encompassed the entire mature peptide. The rigion of cDNA corresponding to the mature peptide was 558 bp in length. The conservation of the deduced amino acid sequence in the B and A domains clearly indicates the sequence obtained is a number of the IGFs gene family. At the amino acid level, the IGF-I exhibits highly identity to IGF-I of different vertebrates. These results suggest that IGF-I is well conserved among vertebrates.

Previously, we have shown that in korean rockfish in vivo administration of GH increases endocrine production of IGF-I in the liver (data not shown). It has been well established that in the teleost, hepatic IGF-I expression is regulated by growth hormone [4,7,13]. Recombinant bovine IGF-I resulted in hypoglycemia while mammalian IGF-I can stimulate growth rate of coho salmon [14]. Perez-Sanchez el al [16] found that the recombinant human IGF-I inhibited GH release after IGF-I stimulation, and GH release was stimulated in a dose dependent manner. In order to understand the biological function of IGF-I in fish, it is essential to obtain pure IGF-I. Many studies have used bovine and human proteins to examine the physiological role in fish [3,14-16]. Although these IGF-I proteins should produce highly active stimulation and regulation effects, it is not sufficient for a function to be associated with fish. In the present study, to characterize the IGF-I protein function of fish, we have developed the IGF-I / His tag fusion protein expression system. The rKR IGF-I protein was expressed in E. coli and appeared to significantly stimulate thymidine incorporation in dose-dependent manners. These data suggest that the rKR IGF-I protein may have a similar function in fish as compared with those in mammals.

Thus, it is reasonable that the rKR IGF-I protein produced by the E. coli can be used for large scale purification of biological functional protein through fusion protein expression. In turn, this is an important application potential in analyzing the function of intact fish IGF-I protein.

요 약

어류의 insulin-like growth factor-I (IGF-I)의 생화학적 작용기작을 연구하기 위하여 한국산 조피볼락의 IGF-I cDNA 유전자 cloning을 행하였다. 완전한 cDNA 유전자 염기서열은 PCR과 RACE 방법을 통하여 얻어진 DNA로부터 결과를 얻을수 있었다. 결정된 IGF-I의 염기서열은 flounder, chinook salmon, human IGF-I의 염기서열과 비교한 결과 각각 93.6%, 90.7%, 85.4%의 높은 상동성을 보였다. 생화학적으로 활성이 있는 재조합 IGF-I을 얻기 위하여 IGF-I의 B-C-A-D domain 부분을 PCR로 얻은 뒤 *E. coli* BL21(DE3)에 넣어 overexpression 시켰다. Ni-NTA columnn을 사용하여 순수한 재조합 단백질을 정제할수 있었다. 정제된 단백질은 SDS-PAGE 상에서 7 kDa의 단일 band를 보여 주었으며 [^{3}H]-thymidine 결합정도를 측정하는 방법으로 활성을 가지고 있음을 확인할수 있었다.

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