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研究报告

青鱼生长激素的重组表达及其多克隆抗体的制备

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摘 要:以含有的青鱼生长激素编码区 cDNA 的重组质粒 pbcGHc 为模板,高保真 PCR 扩增青鱼生长激素(GH)成熟肽 cDNA 序列,定向插入原核表达载体 pET-28a,构建青鱼 GH 原核表达质粒 pET-bcGH。将 pET-bcGH 转化大肠杆菌 BL21(DE3),IPTG 诱导青鱼 GH 基因在大肠杆菌中的融合表达,SDS-PAGE 凝胶电泳结果显示一条 23 kDa 的诱导表达重组青鱼 GH 带。以草鱼 GH 多克隆抗体为一抗,Western blot 证明,该重组青鱼 GH 具有免疫学活性。将经过亲和层析、透析纯化后的重组青鱼 GH 作为抗原,采用改进的方法对家兔进行皮下免疫注射,获得青鱼 GH 多克隆抗血清。以该多抗为一抗,Western blot 可以检测出 4 ng 的抗原量;并且在青鱼垂体组织抽提液中和血清中检测到一种能与该抗血清作用的大小为 21 kDa 的蛋白质。结果表明:得到的青鱼 GH 多克隆抗血清具有较好的免疫特性。

关键词:青鱼;生长激素;重组表达;多克隆抗体

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In vitro Expression and Antibody Preparation of Black Carp (*Mylopharyngodon piceus*) GH

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Abstract: The cDNA fragment encoding the mature polypeptide of growth hormone (GH) for the black carp (*Mylopharyngodon piceus*) was PCR amplified and subcloned into pET-28a. The recombinant expression plasmid pET-bcGH was transformed into *E. coli* BL21(DE3) and fusion polypeptide containing a 6xHis-tag at the N-terminus was expressed after IPTG induction. The fusion protein band of 23 kDa or so showed immunoreactivity to the polyclonal antibody aganist grass carp GH. The recombinant GH for black carp was purified by affinity chromatography and dialysis. Using the fusion protein as an antigen, through the modified immunization method, the polyclonal antiserum to black carp GH was obtained. Immunochemistry results showed that the antiserum could detect the antigen as low as 4 ng. The protein of 21 kDa in black carp pituitary protein extracts and blood serum could be detected by western blot analysis in which polyclonal antiserum to black carp GH was used as the primary antibody. All these results showed that the polyclonal

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antiserum against black carp GH was not only effective but also highly specific.

Key words: black carp; growth hormone; recombinant expression; polyclonal antibody

Fish growth hormone (GH) is a kind of single strand polypeptide secreted by anterior pituitary. Fish GH is composed of amino acids usually with the numbers varying from 173 to 188, and thus its molecular weight varies from 20 kDa to 22 kDa. It can improve the speed of individual growth and development; accelerate the process of protein formation and lipid degradation. More than 40 kinds of fish GH genes had been cloned and many of them had been used for the research of recombinant expression or gene transferring[1~7]. Study on the fish GH is very important in both basic research of fish biology and aquaculture. Black carp (*Mylopharyngodon piceus*) is one of "the Four Chinese Carps", which possesses many features, such as outstanding growth rate, large body size, good flavor, etc. All these made it becoming one of the major species of fresh water aquaculture in China. There were few reports about the GH for black carp. In this study, GH for black carp was expressed as a fusion protein and the polyclonal antibody against it was prepared, which made a good foundation for the further study on black carp GH.

1 Materials and Methods

1.1 Materials

Recombinant plasmid DNA pbcGHc containing the open reading frame (ORF) of the GH cDNA for black carp[8], *E. coli* BL21(DE3) and pET-28a were stored in the lab, *Pfu Taq* DNA polymerase, pUCM-T vector, UniQ-10 DNA gel extraction Kit were purchased from Sangon; T4 DNA ligase, *Nde* Ⅰ, *Bam*H Ⅰ from Promega, polyclonal antibody against GH for grass carp was a gift from Porfessor Wang Ya-Ping in Institute of Hydrobiology, CAS; Ni-NTA Magnetic Agarose Beads from Qiagen, protein molecular marker from MBI. Secondary antibody (anti-rabbit) from Amersham, enhanced chemilluminescence detection kit from ECL, Amersham; New Zealand white rabbit were purchased from the animal center in the Center South University.

1.2 Methods

1.2.1 Constructing the expression plasmid for black carp GH

Pfu Taq DNA polymerase was used to amplify cDNA fragment encoding the mature polypeptide of GH for black carp. P1 (forward primer) was 5′-GAGCATCCATATGTCAGASAACCAGCGGCTCTT-3′, and *Nde* Ⅰ site was underlined; P2 (reverse primer) was 5′- TGTCGCTGGATCCTTACAGGGTG-CAGTTGGAAT-3′ with *Bam*H Ⅰ site underlined. Reaction condition: 94℃ 5min, (94℃ 30 s、55℃ 30 s、72℃ 1 min) × 25, 72℃ 7 min. DNA fragment of interest was exercised from agarose gel and purified using UniQ-10 kit. T/A cloned the DNA fragment into pUCM-T and transformed the recombinant plasmid (pUCM-bcGH) into *E. coli* DH5α. After screened, the positive clone was sent to Sangon for sequencing.

pET-28a was chosen as the expression vector and *E. coli* BL21(DE3) as the host strain. When pUCM-bcGH and pET-28a were digested with *Nde* Ⅰ and *Bam*H Ⅰ and then ligated the DNA fragment of interest with the vector, the recombinant expression plasmid pET-bcGH and pET-28a were transformed into *E. coli* BL21(DE3), respectively.

1.2.2 Expression of recombinant GH for black carp

Incubated *E. coli* BL21 (DE3) transformed by pET-bcGH and the control transformed by pET-28a in 3 mL LB with kanamycin (50 μg/mL) at 37℃ separately till the OD_{600} up to 0.5, then, the BL21 (DE3) transformed by pET-bcGH was transferred into 250 mL LB containing kanamycin (50 μg/mL) for middle scale culture till its OD_{600} up to 0.8. Pipetted 3 mL cell culture into another tube for PAGE analysis. Added IPTG into the cell culture transformed by pET-bcGH and the control to the final concentration of 0.8 mmol/L to induce the expression of black

carp GH according to the pET system manual (Novagen). Pipetted 1 mL cell culture of pET-bcGH (without induce), 1 mL cell culture of pET-bcGH (induced), and 1 mL control, span to collect the cells, added 100 μL PBS and resuspended the pellets; added 100 μL 2 × sample buffer into it and treated the mixture with super sonication for 15 min, the protein bands were resolved by 10% SDS-PAGE and the gel was stained with Coomassie brilliant blue to detect the expression of the black carp GH. The gel was then scanned to measure the percentage of the induced expressed fusion protein in the whole cell proteins.

1.2.3 Western blot analysis

The whole proteins of the cells transformed by pET-bcGH (induced) and the control (transformed by pET-28a) were resolved by 10% SDS-PAGE electrophoresis and the protein bands were transferred into the supported nitrocellulose membrane. By using polyclonal antibody against grass carp GH with different dilutions (50x, 200x, 1 000x, 2 000x) as the primary antibody, and goat anti-rabbit IgG as the secondary antibody (1 : 4 000), Western blot was carried out to detect if the induced fusion protein was immunocompetent to the polyantibody against grass carp GH[9]. The blot was visualized with DAB.

1.2.4 Purification of GH for black carp

The induced cell culture transformed by pET-bcGH was harvested by centrifugation at 6 500 g for 15 min at 4℃. The collected cells was washed and lysed according to the manual of Qiagen. The fusion polypeptide of black carp GH was collected through affinity chromatography by using Ni-NTA Magnetic Agarose Beads according to the manual book; the elute from affinity chromatography was dialysed against 3 mol/L urea/PBS for two changes (each for about 2～3 h) at 4℃ to purify the black carp GH.

1.2.5 Preparation of the antiserum against black carp GH

Using the purified recombinant black carp GH as the antigen, the rabbit was immunized to prepare the antisera against it according to the modified immunization method[10,11]: on the first day and third day, 1 mL sample A (300 μg antigen with the same volume of the Freund's complete adjuvant) was injected intradermally on the back and proximal limbs of the rabbit; on the 28 th day, 1 mL sample B (300 μg antigen with the same volume of the Freund's incomplete adjuvant) was injected in the same way. On the 35 th day, harvested the blood from the arteriae carotis, 37℃ for 2 h and 4℃ over night, then collected the antisera. The antigen aliquots of 0.5 ng, 1 ng, 2 ng, 4 ng and 10 ng were sampled and resolved by 10% SDS-PAGE electrophoresis. Black carp GH polyclonal antiserum was used as the primary antibody (1 : 500), goat anti-rabbit IgG as the secondary antibody (1 : 10 000), immunoreactivity of the antiserum was detected with an enhanced chemilluminescence detection kit according to the company's instruction.

The whole proteins were extracted from pituitary of black carp according to the method of references[12～14]. Blood was collected from the caudal artery of black carp and span at 6 500 g for 15 min at 4℃ to separate the blood cells from the serum. Sampled pituitary extracts of 2 μg and 15 μg serum of black carp, 10% PAGE was run to separate the protein bands, and Western blot analysis was performed as above.

2 Results

2.1 Construction of the expression plasmid of black carp GH

Pfu Taq DNA polymerase was used to amplify the cDNA fragment encoding the mature peptide of black carp GH. Sequencing result of the recombinant plasmid pUCM-bcGH showed that, compared with the black carp GH cDNA sequence (AF389238), only one nonsense mutation existed at 385 site (gag − gaa). The plasmids pUCM-bcGH and pET28a were digested with *Nde* I and *Bam*H I, and the DNA fragment for black carp GH mature peptide was subcloned into pET-28a to construct the recombinant plasmid pET-bcGH and the construct was transformed into *E. coli* BL21 (DE3)(Fig. 1).

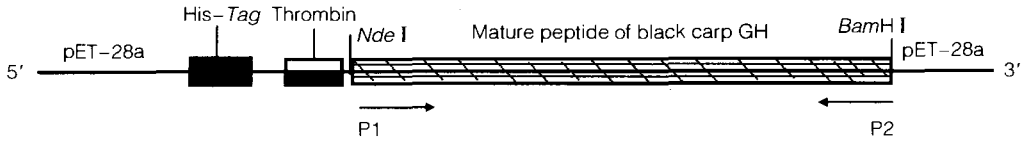

Fig.1 The structure of pET-bcGH

2.2 Expression of GH for black carp

10% SDS-PAGE analysis result showed that there was a protein band with the molecular weight of 23 kDa or so in the total proteins of *E. coli* BL21 (DE3) transformed by pET-bcGH (induced). However, there was no protein band of this weight in the cell culture transformed by pET-bcGH (without induce) and the control (transformed by pET-28a). According to the theoretic translation product of pET-bcGH, the induced band was the recombinant black carp GH. Scanning result indicated that the induced expressed fusion protein was up to 30.4% of the total proteins, see the details in the Fig. 2,A.

2.3 Western blot analysis

The polyclonal antibody against grass carp GH was used as the primary antibody, the total proteins of the cells transformed by pET-bcGH (induced) and the control (transformed by pET-28a) were detected through western blotting. The results showed that the induced protein band of 23 kDa had the strong immunoreactivity to the polyclonal antibody. When the primary antibody was at higher dilutions, the hybridization background was clearer (Fig. 2, B).

2.4 Purification of GH for black carp

Ni-NTA Magnetic Agarose Bead was used to bind the recombinant black carp GH polypeptide with the 6xHis-tag. After affinity chromatography, only the induced expressed fusion protein of black carp GH existed, see the details in Fig.2, C). The collected fusion protein was dialysed against 3 mol/L urea/PBS for two changes (each for about 2～3 h) at 4℃. The concentration of purified recombinant black carp GH was measured through Bradford method and adjusted to 500 μg/mL.

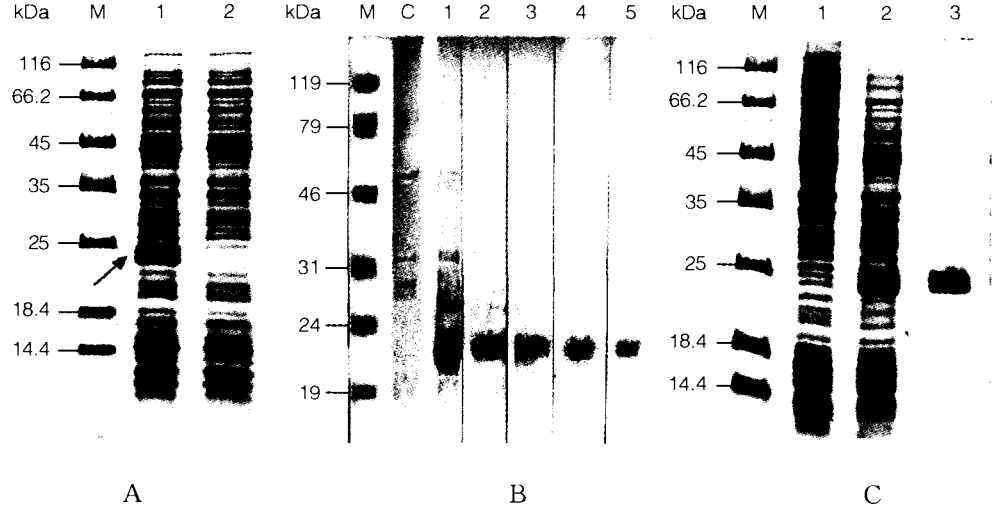

Fig.2 The expression, purification and Western blot of GH gene for black carp

A: Expression (the arrow indicats the induced fusion protein). M: Protein molecular marker (MBI); 1: Total cell protein analysis of BL21 transformed by pET-bcGH (induced); 2: Total cell protein analysis of BL21 transformed by pET-bcGH (without induce). B: Western blot. M: Prestained protein molecular marker (MBI); C: Control (BL21 transformed by pET-28a); 1～5: Serial dilution of primary antibody (50x, 200x, 500x, 1 000x, 2 000x) was used to detect the BL21 transformed by pET-bcGH (induced). C: Purification. M: Protein molecular marker (MBI). 1: Total cell protein of BL21 transformed by pET-bcGH (without induce); 2: Total cell protein of BL21 transformed by pET-bcGH (induced); 3: Collected recombinant black carp GH after affinity chromatography.

2.5 Preparation of the antibody against black carp GH

Using the purified recombinant black carp GH as the antigen, the polyclonal antiserum against black carp GH was prepared successfully on the 35th day through the modified method. Western blot analysis showed that the polyclonal antibody was able to detect the antigen (23 kDa or so) as low as 4ng (Fig. 3, A). Using the polyclonal antiserum against black carp GH as the primary antibody, from the Western blot results of black carp extract and blood serum, a protein band of about 21 kDa was detected by the antibody, which was consistent with the molecular weight of black carp GH mature peptide. (Fig. 3, B). This indicated that the polyclonal antiserum had the specific immunoreactivity to black carp GH.

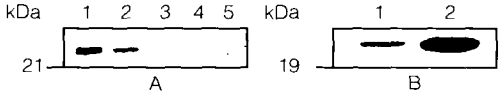

Fig. 3 Western blot analysis using the antiserum to black carp GH as the primary antibody

A: Western blot of antigen aliquots with different amount 1~5: 10 ng, 4 ng, 2 ng, 1 ng, and 0.5 ng. B: Western blot of the tissues of black carp. 1: Black carp serum; 2: Black carp pituitary extract.

3 Discussion

GH is very conservative among fishes and many research works were carried out to look for new data for the evolution of fish through molecular genetic analysis of fish GH genes. What's more, GH is one of the most important hormones for fish. It can regulate the growth and development of fish. So, aiming at enhancing fish's growth rate, most studies on fish GH were carried out. For example: treating the fish with exogenous fish GH protein can improve the fish growth speed; or, transferring alien gene containing fish GH gene into fertilized eggs to produce transgenic fish with faster growing rate. Fish GH is a kind of single strand peptide without subunit, which does not need the modification such as glycosylation. So, it is a rather good way to express fish GH in prokaryotic system such as *E. coli*. Black carp is one of "the Four Chinese carps"; good flavor and large size made it becoming an important economical species of fresh water aquaculture. There were many papers about GH of grass carp, silver carp and bighead carp, but the report about black carp GH was very few before. The successful expression of GH for black carp and preparation of polyclonal antibody against it had built a good foundation for the further study on the GH gene for black carp and enriched the fish molecular biology study.

Research on transgenic tetraploid fish had been carried out based on the feature that both the female tetraploid fish and the male tetraploid fish were fertile. It implied a promising future to produce infertile triploid transgenic fish in a large scale by hybriding the transgenic tetraploid fish with normal diploid fish[15~18]. Through microinjection, we transferred the "all-fish" transgene containing black carp GH gene cDNA into the eggs of the tetraploid fish, and the contrast culture results showed that the transgenic tetraploid fish had faster growth rate than that of their non-transgenic siblings (data not shown). Determination of the GH level in the tissues of transgenic fish and the control is very important to verify the over-expression of the exogenous transgene in the host fish. Though it is very hard to distinguish the endogenous GH from the exogenous GH produced by the transgene through immunochemistry method, the comparison of total GH level in the tissues between the transgenic fish and the control could still provide us a strong data. Using the modified method, we obtained the polyclonal antibody against the black carp GH successfully 35 days upon immunization injection. And the results of Western blot showed the polyclonal antibody against the black carp GH could detect the antigen as low as 4ng. Furthermore, its immunoreactivity to the GH in the pituitary extracts and serum of black carp implied that further modification and purification of the antibody could be used for transgenic study.

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