Cloning and Higher Expression of Recombinant Human Insulin-like Growth Factor-1

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Abstract

Insulin-like Growth Factor (IGF) is a type of important growth factor, of which chemical structure is similar to proinsulin. There are two relative polypeptides - IGF-1 and IGF-2. This study aims to produce high yield viable IGF-1 using genetic modification and various other methods. [Life Science Journal. 2009; 6(3): 32– 36] (ISSN: 1097 – 8135)

Key words: IGF-1; fusion protein; higher expression; serum free culture medium

1. Introduction

IGF-1and IGF-2 which belongs to the IGF family play a significant role in the proliferation, differentiation, apoptosis, and growth of tissues, generated and developed of tumor cells. IGF-1 in particular has a special role in influencing cell growth. It correlates with the occurring of diseases such as cardiovascular disease, metabolic syndrome, diabetes, insulin antagonistic. Due to the fact that IGF has properties that low protein expression and difficulty to isolated.

In 1957, Salmon and Daughaday[1] first found that IGF-1 and IGF-2 could promote the cartilage to absorb 35s in sulphate. They named them as sulphation factors [2]. In 1963, Froesch[3] described them as NSILA1 and 2.

In 1972, they were named as Somatomedin [4]. In 1976, Rinderknecht and Humbel [5] isolated two active factors. They shared high degree of structural homology with insulin. They renamed them as insulin-like growth factor-1 and insulin-like growth factor-2 (IGF-1 and IGF-2) [6]. In 1978, Rinderknecht and Humbel identified the structure and characteristic of IGF-1 and IGF-2[7].

In this research we isolate the total RNA from healthy human placenta tissues. Based on the records of IGF-1 from GenBank, reference number A29117, the primers were designed and a 227bp IGF-1 segment was gotten through Reverse Transcription PCR methods. After sequence identification, the homologous was 99% compare with A29117 coden region of IGF-1 polypeptide. The experiment constructed pET-32a-IGF-1 Fusion Protein Expression Vector, and uses Ampicillin resistance selection, PCR amplification and enzyme cutting to confirm linkage. IPTG was then used to induce the expression of target protein. Analysis of the sequence showed that the target protein didn't express well was due to rare codons interference. Hence, using contig PCR methods to get a gene and constructed a pET-32a-rIGF-1, inducing higher expression after transformation, expression level up to 38%. The protein was purified using his-tag affinity column chromatography, then putted it into CHO cells culture and confirmed its bioactivity in cells. It has an activity to stimulate cell proliferation.

2. Materials and Reagents

E.coli JM109, *E.coli* BL21 bought from TAKARA biotechnology (Dalian) Co. Ltd. Plasmid: pMD18-T form TAKARA biotechnology (Dalian) Co. Ltd. pET-32a (+) bought from Novagen Company. Cell: CHO cell system supplied by Company: Harbin Pharmaceutical Group Biological Engineering Co., Ltd.

Cloning and expression of hIGF-1

We isolated the total RNA from healthy human placenta tissues. The primers were designed and a

227bp IGF-1 segment was gotten through Reverse Transcription PCR methods.Primers:

P1:CCATGG GGACCGGAGACGCTCTGCGGGGCTG
Nco Ⅰ
P2: CTCGAG CTAAGCTGACTTGGCAGGCTTGAGG
Xho Ⅰ

Reaction conditions:

10×ExBuffer	2.5μl
dNTPs （2.5mmol/L）	2.0μl
P1 （10μM）	1.0μl
P2 （10μM）	1.0μl
Ex Taq （5U/μl）	0.5μl
Template（ < 1μg/μl）	1.0μl
ddH_2O	17μl
total volume	25μl

94℃	5min	
94℃	30s	30 circles
60℃	30s	
72℃	30s	
72℃	10min	
4℃	∞	

Linked the gene with pMD18-T vector, then transformed into *E.coli*JM109.

Constructed pET-32a-IGF-1 Fusion Protein Expression Vector used Ampicillin resistance selection, PCR amplification and enzyme cutting to confirm linkage.

SDS-Polyacrylamide Gel Electrophoresis

Preparation of the separate solution and condensable solution

Separate solution: Table 1-1 component of the 12% polyacrylamid separate solution.

Condensable solution: Table 1-2 component of the polyacrylamid condensable solution.

Electrophoresis buffer:

5 x Tris- Glycin electrophoresis buffer:

Tris-base	15.1g
Glycine	94g
10%SDS	50ml
pH 8.3	
Water	till 1000ml

Modification, clone and expression of IGF-1 gene

After small amount of the gene expression, it was found that the low expression of the target gene even changed the express condition. Through the rare codons analyses, we designed and synthesized 3 DNA single strands and called IGFa, IGFb, IGFc and 2 PCR primers: Pup, Plow. Used contig PCR methods to get a gene and called it rIGF-1.

IGFa：
5’-GGCCCGGAAACCCTGTGCGGCGCAGAACTGG TGGATGCACTGCAGTTTGTGTGCGGCGATCGCG GCTTTTATTTCAACAAACC-3’

IGFb：
5’-AGCTGCGAAAGCAGCATTCATCCACAATGCC GGTCTGCGGCGCGCGGCGACTGCTGGAGCCATA GCCGGTCGGTTTGTTGAAAT-3’

IGFc：
5’-AATGCTGCTTTCGCAGCTGTGATCTGCGCCGC CTGGAAATGTATTGCGCGCCGCTGAAACCGGCG AAGTCAGCA-3’

Pup: CCATGG GCCCGGAAACCCTGTGCGGCGCAG
Plow:CTCGAGCTATGCTGACTTCGCCGGTTTCAG

Dissolved the synthetic gene segment with the sterile water to run PCR reaction, then after enzyme cutting identification, we constructed the expression vector to express the gene.

Bioactivity Test of rIGF-1

Purified the target protein

Processing the cell break, inclusion body washing, dissolving, renaturation and then purified the fusion protein. Take out the renaturation solution from 4℃. Centrifuge in 30mins with 5000rpm and removal the hybridprotein. Put the supernatant into a clean triangular flask to process the His-tag column purification.

Balanced solution: 20mM sodium phosphate, 0.5M NaCl, 5 mM imidazole (pH 7.4).

Elutriant solution: 20mM sodium phosphate, 0.5M NaCl, 0.5 mM imidazole (pH 7.4).

Filtrating with 0.45μm filter membrane after prepare of the solution. Obtain the target protein through enzyme cutting and process the serum-free cell culture.

Bioactivity test of IGF-1

The concentration of the IGF-1 is 0.76mg/ml through the testing of UV spectrophotometer. Prepared the serum-free medium with the final concentration as 100μg/ml、50μg/ml、25μg/ml to test the activity of insulin.

Put the serum-free DMEM as negative control, and the DMEM with 25μg/ml IGF-1 as the positive control.

Table 1-1 component of the 12% polyacrylamid separate solution

Content	water	30% acrylamide	1.5M Tris-HCl (pH8.8)	10%SDS	10% Ammonium Persulfate	TEMED
（ml）	1.28	1.6	1.04	0.04	0.04	0.004

Table 1-2 component of the polyacrylamid condensable solution

Content	water	30% acrylamide	1.0M Tris-HCl (Ph6.8)	10%SDS	10% Ammonium Persulfate	TEMED
（ml）	1.4	0.33	0.25	0.02	0.02	0.002

Preparation of the medium:

DMEM basic medium

DMEM+100μg/mlIGF-1

DMEM+50μg/mlIGF-1

DMEM+25μg/mlIGF-1

DMEM+25μg/ml insulin.

Adherent culturing the CHO cell with 3-4 generation with good cell shape and stable condition, then digest the cell from 24 pores plate, and removal the digestive juice. Culturing the cell with the five different medium, observe the cell with micro. Use the blood cell counting chamber to calculate the cell amount per ml and the motility rate of the cell.

3. Result and Analysis

Cloning and Expression of hIGF-1

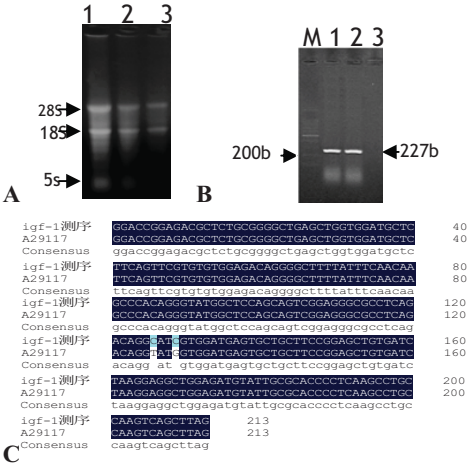

Figure 1-A. Isolation of total RNA from placenta. B. PCR Agarose gel electrophoresis analysis of IGF-1 gene. M: 100 ladder, 1-2: DNA strip, 3: Blank. C. Comparison of nucleotide sequence of IGF-1.

SDS-PAGE of the IGF-1

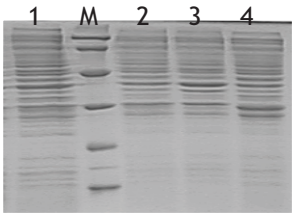

Figure 2. pET-32a-IGF-1SDS-PAGE.
1: Un-induced, M: LMAP Maker, 2: Induced for 2h, 3: Induced for 3h, 4:Induced for 4h.

Modification, Clone and Expression of IGF-1

Modification of the gene sequence

Sequence after the modification:

GGC CCG GAA ACC CTG TGG GGC GCA GAA CTG GTG GAT GCA CTG CAG TTT GTG TGG GGC GAT CGC GGC TTT TAT TTC AAC AAA CCG ACC GGC TAT GGC TCC AGC AGT CGC CGC GCG CCG CAG ACC GGC ATT GTG GAT GAA TGG TGG TTT CGC AGC TGG GAT CTG CGC CGC CTG GAA ATG TAT TGG GCG CCG CTG AAA CCG GCG AAG TCA GCA TAG

Figure 3-A. Comparison the nucleotide sequence of rIGF-1 and IGF-1. 3-B. Comparison of the amino acid sequence of rIGF-1 and IGF-1.

Cloning and Expression of the Modified Gene

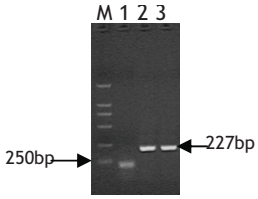

Figure 4.Slipcing PCR of E.coli preference. M: DL2000, 1: Blank, 2 and 3: PCR result.

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rigf-1基因  GGCCCGGAAACCCTGTGGGGCGCAGAACTGGTGGATGCAC   40
测序结果    GGCCCGGAAACCCTGTGGGGCGCAGAACTGGTGGATGCAC   40
Consensus   ggcccggaaaccctgtggggcgcagaactggtggatgcac
rigf-1基因  TGCAGTTTGTGTGGGGCGATCGCGGCTTTTATTTCAACAA   80
测序结果    TGCAGTTTGTGTGGGGCGATCGCGGCTTTTATTTCAACAA   80
Consensus   tgcagtttgtgtggggcgatcgcggcttttatttcaacaa
rigf-1基因  ACCGACCGGCTATGGCTCCAGCAGTCGCCGCGCGCCGCAG   120
测序结果    ACCGACCGGCTATGGCTCCAGCAGTCGCCGCGCGCCGCAG   120
Consensus   accgaccggctatggctccagcagtcgccgcgcgccgcag
测序结果    ACCGGCATTGTGGATGAATGCTGCTTCGCCAGCTGTGATC   160
rigf-1基因  ACCGGCATTGTGGATGAATGCTGCTTTCGCAGCTGTGATC   160
Consensus   accggcattgtggatgaatgctgctt cgcagctgtgatc
测序结果    TGCGCCGCCTGGAAATGTATTGCGCGCCGCTGAAACCGGC   200
rigf-1基因  TGCGCCGCCTGGAAATGTATTGCGCGCCGCTGAAACCGGC   200
Consensus   tgcgccgcctggaaatgtattgcgcgccgctgaaaccggc
rigf-1基因  GAAGTCAGCATAG   213
测序结果    GAAGTCAGCATAG   213
Consensus   gaagtcagcatag
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Figure 5. Comparison of the sequence of rIGF-1

SDS-PAGE of the Expressed Modified Gene

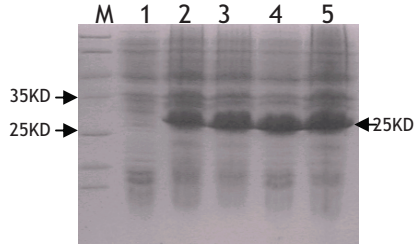

Figure 6. pET-32a-rIGF-1 SDS-PAGE. M: LMWP Maker, 1: Un-induced, 2-5: Expressed protein.

Bioactivity Test of rIGF-1

Purification of the fusion protein.

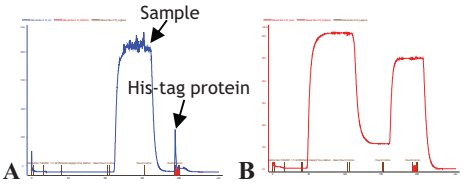

Figure 7-A. Optical absorption. B. Conduction.

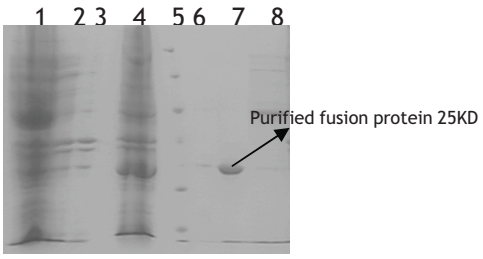

Figure 8. Product detection of purification of fusion protein by SDS-PAGE.

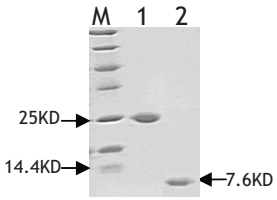

Figure 9. SDS-PAGE after purification. M: LMWP Marker, 1: Fusion protein before cutting, 2: Target IGF-1.

Bioactivity test

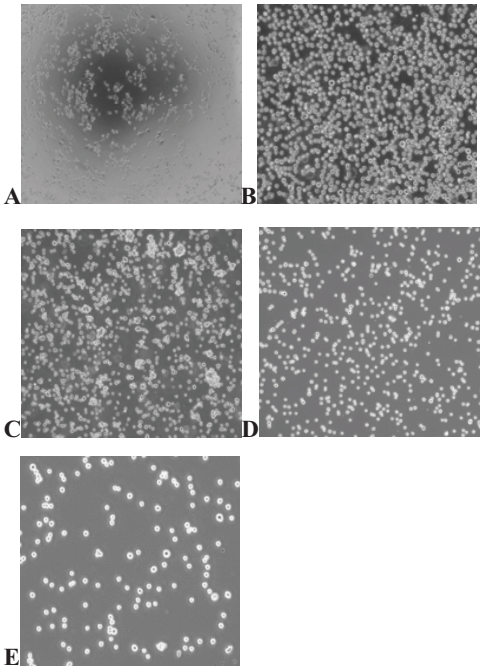

Figure 10-A. Negative control:Dead cell.B.Positive control.

C.DMEM+100μg/mlIGF-1.

D.DMEM+50μg/mlIGF-1.

E. DMEM+ 25μg/mlIGF-1.

4. Discussion

With the rapid develop of biotechnology, the shortage of the prokaryotic expression is coming out. For instance, most of the expressed protein is inclusion body of which has the difficult renaturation and purification. The target cannot process the glycosylation modifications which will influent the function of the protein. More and more researches start to use the eukaryotic cell. And the serum-free medium is the trend of the cell culture.

In this study we obtained the recombinant and high expressed IGF-1 polypeptide which has given the contribution to the serum-free culture.

References:

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