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Production and functional characterization of human insulin-like growth factor 1

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ABSTRACT

Insulin-like growth factor 1 (IGF-1) is a polypeptide hormone produced mainly by the liver in response to the endocrine growth hormone (GH) stimulus. This protein is involved in a wide range of cellular functions, including cellular differentiation, transformation, apoptosis suppression, migration and cell-cycle progression and other metabolic processes. In the current study, human heart cDNA was employed to isolate IGF-1 encoding fragment using reverse transcriptase (RT) PCR. The isolated fragment was cloned into pET32a expression vector and then transformed into the competent *Escherichia coli* Origami 2. After selecting the correct colony with the highest expression level, the colony was cultured and induced with IPTG. Recombinant IGF-1 expression was detected by SDS-PAGE and His-tagged protein purification was performed with the affinity chromatography. In order to confirm the activity of the resultant protein, biological activity of the recombinant IGF-1 was assayed through inducing proliferation of MCF-7 cells. Molecular techniques, including PCR, restriction digestion, mass spectrometry analyses, SDS-PAGE and biological activity analyses of this protein confirmed the correct cloning, expression, and function of IGF-1 in this study. Overall, we provided a rapid and cost effective production and purification method for IGF-1 protein, which is biologically active and functional.

Keywords: Insulin like Growth Factor 1; Recombinant protein; Over-expression; *Escherichia coli*.

Introduction

Insulin-like growth factor-1 (IGF-1) also known as somatomedin C, is a small single polypeptide from the insulin-related peptides family. IGF-1 is a polypeptide composed of 70 amino acids with a molecular weight

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of 7649 Dalton (1). Structurally the mature IGF-1 consists of A and B domains which are homologous to the insulin chains but they connect to each other by C domain and also by disulfide bonds (1, 2). This protein has three disulfide bonds that help stabilize its conformation (Fig. 1). The structural similarity between IGF-1 and insulin allows this protein to bind to the insulin receptor, but with a lower affinity (2).

Figure 1. The three disulfide bonds (Cys 6 and Cys 48, Cys 18 and Cys 61, and Cys 47 and Cys 52) are shown in as magenta (10).

IGF-1 plays a very important role in fetal growth and differentiation and also it is involved in a wide range of cellular functions, including cellular differentiation, transformation, apoptosis suppression, migration and cell-cycle progression and other metabolic processes (1). IGF-1 also has several metabolic functions which are mediated through insulin receptor due to high homology between insulin and IGF-1 (3). Recombinant human IGF-1 (rhIGF-1) was first used for an experimental therapy in the late l980s and it was approved by US Food and Drug Administration (FDA) for the treatment of patients with some kinds of severe primary IGF-1 deficiency in 2005 (3). Recently, rhIGF-1 is considered as a suitable candidate for the treatment of chronic liver disease, insulin resistance/hyperinsulinemia, diabetes, some neurological disorders, stroke, cystic fibrosis, wound healing and some other pathological conditions (3). In this study, we report the production of recombinant human IGF-1 in *E. coli*, which provides a rapid and cost-effective purification method for the IGF-1 recombinant protein with the required biological activity.

Materials and Methods

Isolation of human IGF-1 cDNA

Total RNA was isolated from human heart cells using the RNeasy mini kit, as per the manufacturer's instructions (Qiagen, Valencia, CA, USA). The first strand cDNA synthesis was performed using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), oligo dT primers and 2µg of purified total RNA. Primers were designed to recognize the human *IGF-1* cDNA sequence (Gene bank accession no: NM_000618.3), the coding sequence of the mature peptide was amplified by PCR using the following primers: IGF-F (5' TTCATGATATCGGACCGG AGACGCTCTGC 3') which introduced an EcoRV restriction enzyme site at the 5' end (underlined sequence) and IGF-R (5' ACATTTCTCGAGTTAAG CTGACTTGGCAGGTTG 3') that included an XhoI restriction enzyme site at the 5' end (underlined sequence). Human *IGF-1* gene was amplified in 20 µl reaction containing 20 ng of template cDNA, 0.5 µM of each primer, 2 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 1X PCR buffer and 2.5 units of Taq polymerase. Amplification steps included pre-incubation at 94˚C for 4 min, 30 cycles at 94˚C for 40 seconds, 64˚C for 40 seconds, and 72˚C for 40 seconds, followed by one incubation step at 72˚C for 10 min. Next, the PCR product was analyzed by electrophoresis on a 1% agarose gel.

Construction of pET32a/IGF-1 expression vector

The PCR product was digested with EcoRV and XhoI restriction enzymes (Fermentas, Lithuania), purified using high pure PCR product purification kit (Roche, Mannheim Germany) and cloned into the pET32a vector (Novagen, Madison, WI, USA) which was digested with the same restriction enzymes. The recombinant construct that carried the *IGF-1*

(pET32a/*IGF-1*) was transformed into the competent *E. coli* (DH5α strain) (Novagen, Madison, WI, USA) by the heat-shock method, as described by the manufacturer (User Protocol TB 009 Rev. F 0104). The transformed bacteria were selected by screening the colonies in the presence of Ampicillin (100µg/ml). Colony PCR-screening was performed to identify colonies with the correct engineered plasmid. The transgene nucleotide sequence of selected colonies was analyzed by DNA sequencing.

Expression and purification of recombinant IGF-1

The expression host *E. coli* Origami 2 (DE3) pLysS strain (Novagen, Merck KGaA, Darmstadt, Germany) was used as the expression host for pET32a/*IGF-1* vector. This strain contained a T7 RNA polymerase sequence under the control of the *lacUV*5 promoter and has specific mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, which greatly enhance disulfide-bond formation in the *E. coli* cytoplasm. The pET32a expression vector also contains a 6His-Tag, which facilitates affinity purification. A single colony of transformed *E. coli* Origami 2 with pET32a/*IGF-1* was incubated overnight in a shaking incubator in 2 ml of LB medium that contained 100 µg/ml ampicillin at 37˚C and shaken at 180 rpm. The next day, the culture was diluted 1:100 in fresh LB that contained 100 µg/ml ampicillin and 2% glucose, and then cultivated at 37˚C until the OD_{600} of the media reached 0.6. Recombinant fusion protein expression was then induced by the addition of 0.2 mM isopropyl-d-thiogalactopyranoside (IPTG; Fermentas, Lithuania). Cells were grown for 6 hours under an induction temperature of 37˚C. Induced cells were harvested by centrifugation at 6000×g for 20 min; cell pellets were then frozen at -80˚C until being used for protein purification. Prior to purification, cell pellets were thawed and resuspended in a 10 ml lysis/binding buffer of cells and incubated on ice for 30 min. The lysate was further disrupted by sonication on ice for 10 min, with 45 sec pulses and a 15 sec rest period between pulses. The cell debris was precipitated by centrifugation at 14000×g for 30 min and the supernatant was used for purification. The recombinant IGF-1 fusion protein, which contained the His-Tag, was purified under native conditions by Ni-NTA kit, as described previously by Rassouli *et al.* (4).

SDS-PAGE and mass spectrometry analysis

The concentration of the purified protein was determined by the Bradford method (5). Identical volumes of different elution fractions were mixed with a 1/5 volume of 5X loading buffer [1 M Tris-HCl (pH= 6.8), 10% w/v SDS, 0.05% w/v bromophenol blue, 50% glycerol, and 200 mM β-mercaptoethanol] and heated at 95˚C for 5 min prior to SDS-PAGE using a 12% (w/v) separating gel, followed by staining with 0.1% coomassie brilliant blue R-250. Bands of interest were excised from the SDS-PAGE gel and samples were analyzed by liquid chromatography, coupled with tandem mass spectrometry (LC-MS/MS) at York University.

Biological activity of human IGF-1

The biological activity of IGF-1 was measured by its ability to promote the proliferation of human Michigan Cancer Foundation-7 (MCF-7) cell line (6). The MCF-7 cell line was obtained from the Iranian Biological Center (IRBC Code: C10062). Activation of tyrosine kinase type-1 insulin growth factor-1 receptor (IGF-1R) with IGF-1 or IGF-2 has been implicated in the progression of breast cancers, such as the MCF-7 cells (7).

Cell culture

MCF-7 cells were cultured in Dulbecco's modified eagle's medium DMEM-F12; (Invitrogen, 21331-020) supplemented with 10% fetal bovine serum (FBS; Gibco Inc. Canada) 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco Inc. Canada).

Assay medium

Serum Free Medium (SFM) was used for IGF-1 bioassay according to Ogasawara method (8) with some modifications. To examine the effect of IGF-1 on proliferation rates of MCF-7 cells, they were cultured in DMEM-F12 supplemented with 200 µg/ml BSA (Bovine Serum Albumin) & 10 µg/ml transferrin (Gibco Inc. Canada).

Bacterial Endotoxin Test and Proliferation assay

Limulus amebocyte lysate (LAL) assay was performed to assess Endotoxin content. The rates of cell proliferation were assayed using the Cell Titer 96 Non-Radioactive Cell Proliferation MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS [(a)]] Assay Kit (Promega). Briefly, serial dilutions of recombinant IGF-1 and commercial R&D IGF-1 (I-1271) (0–20 ng/ml) were provided in 96-well flat-bottom plate in SFM. Then MCF-7 cells were added to the aforementioned plate and incubated for 5 days at 37˚C in a humidified, 5% CO_2 atmosphere. Subsequently, cell viability was measured by adding 20 µl of the MTS reagent into each well and cells were incubated at 37˚C for 3 hours. After this time, the absorbance was detected at 490 nm with a microplate reader. Concomitantly, the products of two different batches of IGF-1 were tested three times.

Results

Construction of IGF-1 fusion protein expression vector

The 209 bp IGF-1 was amplified from total RNA harvested from human heart cells. The PCR product was analyzed by gel electrophoresis on 1% agarose gel and the expected 209 bp product of IGF-1 was detected (Fig. 2). In addition, we performed PCR for another five colonies and the presence of the gene was confirmed in these colonies as illustrated in all colonies (Fig. 3). Extracted plasmids were then sequenced and the sequence identity was confirmed by comparing data deposited in databases, using the Basic Local Alignment Search Tool (BLAST) software (Fig. 4).

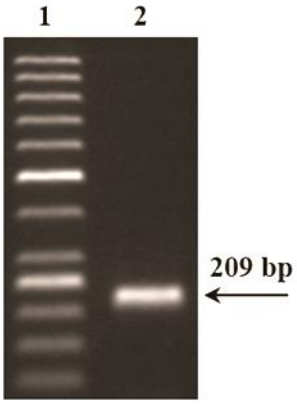

Figure 2. Electrophoresis of PCR product on agarose gel (1%). Lane 1; DNA ladder (50 bp, Thermo, NYSE: TMO), lane 2; IGF-1 (209 bp).

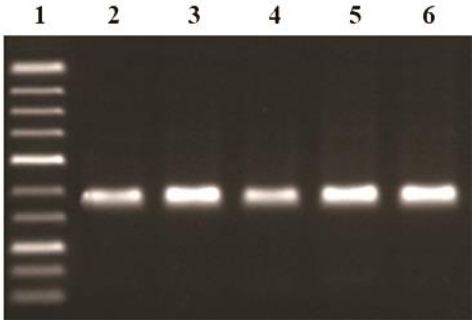

Figure 3. Electrophoresis of colony PCR products on agarose gel (1%). Lane 1; DNA ladder (1 kb plus, Thermo), lane 2-6; IGF-1 (209 bp).

Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA

Sequence ID: ref|NM_000618.3| Length: 7321 Number of Matches: 1

Range 1: 364 to 573 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
388 bits(210)	2e-106	210/210(100%)	0/210(0%)	Plus/Plus

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Query  552  GGACCGGAGACGCTCTGCGGGGCTGAGCTGGTGGATGCTCTTCAGTTCGTGTGTGGAGAC  611
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364  GGACCGGAGACGCTCTGCGGGGCTGAGCTGGTGGATGCTCTTCAGTTCGTGTGTGGAGAC  423

Query  612  AGGGGCTTTTATTTCAACAAGCCCACAGGGTATGGCTCCAGCAGTCGGAGGGCGCCTCAG  671
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Sbjct  424  AGGGGCTTTTATTTCAACAAGCCCACAGGGTATGGCTCCAGCAGTCGGAGGGCGCCTCAG  483

Query  672  ACAGGCATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTAAGGAGGCTGGAGATGTAT  731
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484  ACAGGCATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTAAGGAGGCTGGAGATGTAT  543

Query  732  TGCGCACCCCTCAAGCCTGCCAAGTCAGCT  761
            |||||||||||||||||||||||||||||
Sbjct  544  TGCGCACCCCTCAAGCCTGCCAAGTCAGCT  573
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Figure 4. Confirmation of the DNA sequencing result by comparing to NCBI database using BLAST software.

Expression and purification of recombinant IGF-1

The pET32a/IGF-1 plasmid, with confirmed sequence identity, was transformed into *E. coli* Origami 2 (DE3) pLysS, which served as an expression host. The addition of IPTG induced the over-expression of the recombinant protein (with an approximately 26 kDa molecular-weight) (Fig. 5). The expressed protein was purified successfully via affinity chromatography using Ni-NTA kit and the target protein was eluted with 250 mM imidazole. The purified IGF-1 fusion protein identity was confirmed by trypsin digest and LC/MS/MS analysis of the protein band excised from the SDS-PAGE gel (Fig. 5). The results indicated that our fusion protein matched with human IGF-1 (Accession no: NM_000618.3) (data not shown).

Bacterial Endotoxin Test and Proliferation Assay

LAL assay revealed that all samples of each IGF-1 batch used for the toxicity detection were endotoxin-free. The biological activity of the recombinant Royan-IGF-1 was assessed by MTS assay according to its ability to induce the proliferation of MCF-7 cells, a breast cancer cell line which needs IGF-1 protein as a critical growth factor. The results presented in Fig. 6, indicated that the Royan-IGF-1 can promote the proliferation of MCF-7 cells in a dose-dependent manner, up to 4 ng/ml and its activity range is almost identical to the commercial IGF-1.

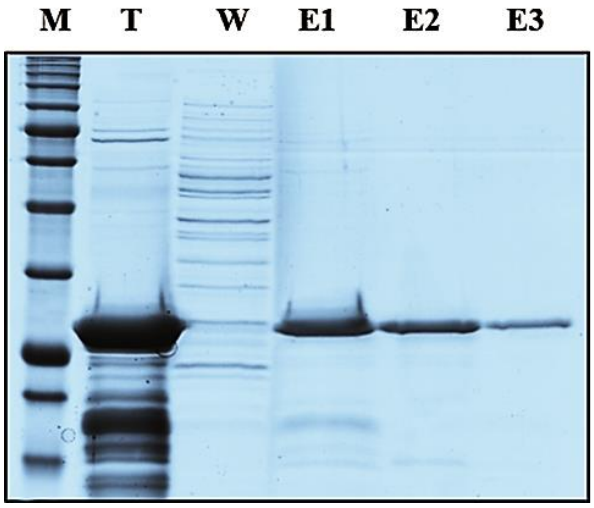

Figure 5. SDS-PAGE analysis of the fusion protein induced by IPTG. Proteins were separated by SDS-PAGE and stained with coomassie brilliant blue R-250. M: Protein size marker; CO: Cut Off; W: Wash; E1: Elution 1; E2: Elution 2; E3: Elution 3. The purified proteins showed the band corresponding to the expected size (26 kDa), which represents the target proteins and His-tag.

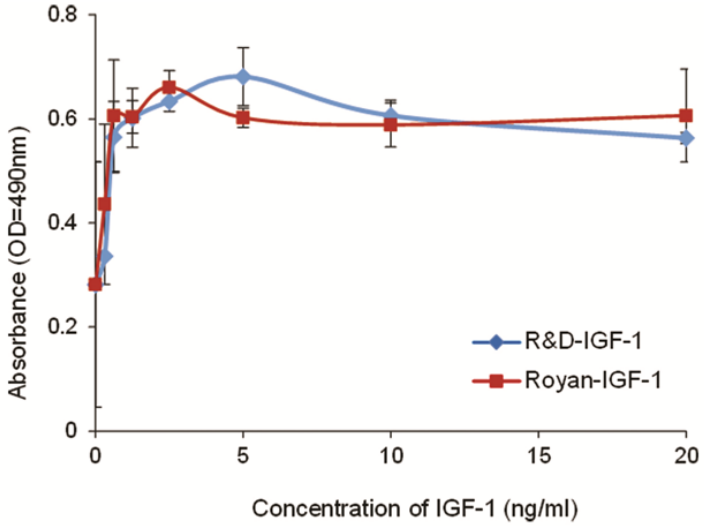

Figure 6. The biological activity of Royan-IGF-1 and R&D IGF-1 on the MCF-7 cells analyzed by MTS assay. Comparison of cell proliferation in responses to different doses (0.37–20 ng/ml) of recombinant IGF-1 (■) and commercial R&D IGF-1 (□) for 5 days incubation.

Discussion

IGF-1, as a mitogenic polypeptide growth factor, induces the proliferation and survival of various cell types including muscle, bone and cartilage tissue in vitro (9). In this study, the human IGF-1 recombinant protein coding sequence was cloned and expressed under the control of the T7 promoter. Ni-NTA kit was used for affinity purification. The recombinant human IGF-1 protein contains 70 amino acids and 3 intra-molecular disulfide bonds (Cys 6 and Cys 48, Cys 18 and Cys 61, and Cys 47 and Cys 52) that help stabilize its conformation (Fig. 1). The formation of native disulfide bonds is an important event in the folding of many proteins. The *E. coli* strains lack most of the necessary components for the formation of disulfide bonds. In this study, we have resorted to the use of the Origami 2 (DE3) pLysS strain as an expression host, which harbors mutations in both the trxB and gor genes. These mutations greatly enhance disulfide bonds formation in the *E. coli* cytoplasm. Endotoxins can lead to major problems in cell cultures and our results have shown that Royan-IGF-1 recombinant protein was endotoxin-free which means this home-made protein could be used with no concern of endotoxin presence. To validate the functionality of the purified IGF-1, we applied the MTS assay. Our results demonstrated that there was no significant difference between the Royan-IGF-1 and the commercial IGF-1 in terms of cell proliferation. Taken together, these findings suggest that commercial IGF-1 can be replaced with Royan-IGF-1, which results in a substantial reduction in the cell culture costs. It should be noted that we were successful to establish the Quality Control (QC) method to produce recombinant IGF-1 for the first time in Iran and there are no such reports from another company. Although the generation of recombinant IGF-1 has been reported before (11), their production methods are still limited and there is no evidence describing QC to produce this protein.

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