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Gene construction, expression, and characterization of double-copy truncated form of human insulin-like growth factor Ⅰ[1]

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KEY WORDS gene expression; insulin-like growth factor Ⅰ; polymerase chain reaction; ultrafiltration; electrophoresis

ABSTRACT

AIM: To increase the production of recombinant truncated form of insulin-like growth factor Ⅰ [des(1-3) IGF-Ⅰ], purify the expressed product, and compare its bioactivity with that of standard IGF-Ⅰ. **METHODS**: The second copy of des(1-3) IGF-Ⅰ gene was inserted into the previously constructed pExSec1/IGF-Ⅰ to form a pExSec1/2(IGF-Ⅰ) expression plasmid, then the plasmid was transformed into a protease-deficient *E coli* strain BL21(DE3). The engineered bacteria were cultured and induced by IPTG at 12 ℃. The expressed product was purified through ultrafiltration and Sephadex G-50 gelfiltration. The bioactivity of the preliminarily purified protein was tested by MTT method and compared with standard IGF-Ⅰ. **RESULTS**: The amount of des(1-3)IGF-Ⅰ expressed by pExSec1/2(IGF-Ⅰ) reached up to 20 % of the total soluble bacterial protein, which was higher than the amount (12 %) expressed by a single copy of pExSec1/IGF-Ⅰ gene. The purity of recombinant des(1-3) IGF-Ⅰ reached 49 % and 82 % after ultrafiltration and gelfiltration. The bioactivity of des(1-3) IGF-Ⅰ after gelfiltration was about 77 % of standard IGF-Ⅰ at the same concentration. **CONCLUSION**: The yield of recombinant des(1-3) IGF-Ⅰ was increased about 8 % by construction of expression plasmid with two copies of des(1-3)IGF-Ⅰ gene compared with only one copy of gene, and preliminarily purified des(1-3) IGF-Ⅰ showed about 77 % bioactivity compared with standard IGF-Ⅰ.

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INTRODUCTION

Insulin-like growth factor Ⅰ (IGF-Ⅰ) is also known as somatomedin C. It is a single-chain polypeptide of 70 amino acid residues (M_r 7500). IGF-Ⅰ is believed to be the major anabolic mediator of growth hormone. It plays a fundamental role in promoting cell growth, proliferation, and differentiation both *in vivo* and *in vitro*. It may be useful in the treatment of many diseases such as dwarfism, retardation of growth, osteoporosis, and diabetes by promoting the developments of muscle and skeleton[1]. In the therapy of insulin-dependent diabetes mellitus and non-insulin-dependent diabetes mellitus, IGF-Ⅰ is irreplaceable[2]. For the treatment of motor neuronal disorders, it is being used in the first and second clinical phases[3,4].

Truncated form of IGF-Ⅰ [des(1-3) IGF-Ⅰ], lacking the first three residues in the N-terminus of IGF-Ⅰ, has been found in the human fetus, adult brain, bovine colostrums, and porcine uterus. The biological potency of des(1-3) IGF-Ⅰ has been reported to be *1.4-10 times higher than that of full length form*[1,5].

In 1985, the first recombinant human IGF-Ⅰ was expressed as a fusion protein in *E coli*, which was cleaved by cyanogen bromide[6]. Li *et al*[7,8] have reported the expression of recombinant human IGF-Ⅰ in *E coli*. But the products were also fusion proteins in the form of inclusion body, for which more expensive and time-consuming protein solubilization and refolding procedures were needed in the process of extraction and purification of recombinant IGF-Ⅰ.

We have studied des(1-3) IGF-Ⅰ gene expression previously[9]. To further increase the production of des(1-3)IGF-Ⅰ, an expression vector pExSec1/2(IGF-Ⅰ) with double-copies of des(1-3)IGF-Ⅰ gene was constructed and expressed. Then the bioactivity of the expressed product was tested and compared with standard IGF-Ⅰ.

MATERIALS AND METHODS

Materials *Taq* DNA polymerase, restriction endonucleases *Nde* Ⅰ, *Bam*H Ⅰ, *Bgl* Ⅱ, *Xba* Ⅰ, T_4DNA ligase, alkaline phosphatase (AP), dNTPs, Isopropyl-β-*D*-thiogalactopy-ranoside (IPTG), and Wizard™ PCR Preps-DNA purification system were purchased from Promega (USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) from Sigma; ultrafiltration membranes (NMWL: 30000) from Amicon INC (USA); Sephadex G-50 from Pharmacia; recombinant human IGF-Ⅰ from Boehringer Mannheim Biochemica(German); pExSec1/IGF-Ⅰ was constructed by Department of Life Science, Shanxi University.

Amplification of des(1-3) IGF-Ⅰ gene by polymerase chain reaction(PCR) The construction of pExSec1/2(IGF-Ⅰ) was shown in Fig 1. The second structure gene of des(1-3) IGF-Ⅰ was amplified by PCR from pExSec1/IGF-Ⅰ with the primer Ⅰ 5′-CGAGATCTATGACTCTGTGCGGTGCTGAGCTGGT-3′ as upstream primer, and primer Ⅱ 5′-CTGGATCCTTAAGCAGACTTGAAAGGCTT-3′ as downstream primer. PCR was performed with 30 cycles at melting, annealing, and extension temperatures of 94 ℃ (1 min), 55 ℃ (1 min), and 72 ℃ (1 min), respectively, with a final elongation at 72 ℃ for 5 min. The PCR product (220 bp) was purified by Wizard™ PCR Preps-DNA purification system, and digested with *Bgl* Ⅱ and *Bam*H Ⅰ.

Construction of pExSec1/2(IGF-Ⅰ) plasmid vector Plasmid pExSec1/IGF-Ⅰ DNA was extracted and purified by Wizard™ DNA purification system, cleaved with *Bam*H Ⅰ, and dephosphorylated with AP.

The PCR product was ligated by T_4DNA ligase into expression plasmid pExSec1/IGF-Ⅰ.

Characterization and expression of double-copy genes of des(1-3)IGF-Ⅰ The expression plasmid constructed above were used to transform *E coli* BL21(DE3). The positive colony of bacteria cultured in LB medium was chosen on agar plate with kanamycin. Then plasmid was extracted from cultured bacteria of this colony, and digested with restriction endonucleases *Nde* Ⅰ-*Bam*H Ⅰ or *Xba* Ⅰ-*Bam*H Ⅰ. Agarose gel electrophoresis was conducted and the sequence of this plasmid DNA was analyzed to characterize whether the insert in the plasmid was double-copy genes of des(1-3) IGF-Ⅰ and in correct reading frame(Bao Biochemistry of Dalian Co Ltd, China).

The bacteria containing pExSec1/2(IGF-Ⅰ) were grown in LB medium with kanamycin 100 mg/L at 37 ℃ to A_{600} 0.8−1.0, then induced with IPTG 0.4 mmol/L at 12 ℃ for 16 h. The bacteria were harvested by centrifugation and broken up by sonication, then centrifuged at 12 000 × *g*. The supernatant was collected and tested by 16.5 % SDS-PAGE electrophoresis, dyed with commassise bright blue R250, and scanned with GDS-8000 Gel-photography system (Ultra-Violet product, Science, USA) to determine the percentage of expressed des(1-3) IGF-Ⅰ in total soluble protein of the bacteria.

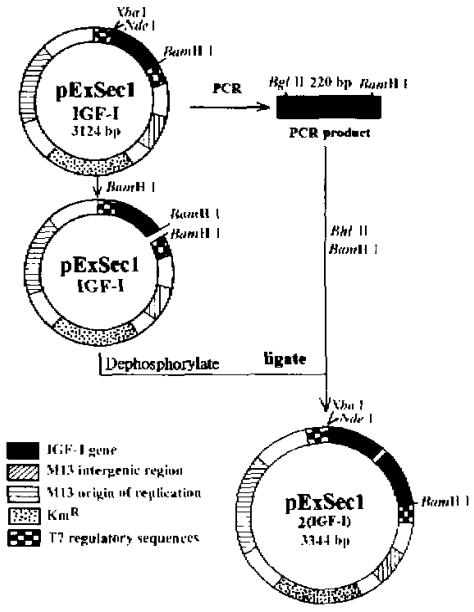

Fig 1. Scheme for construction of expression plasmid pExSec1/2 (IGF-Ⅰ).

Preliminarily purification of expressed des(1-3)IGF-Ⅰ

Ultrafiltration The supernatant obtained from sonication was ultrafiltrated by ultrafiltration membranes (Molecular weight cut-off point 30 000). Then, the filtrated parts were collected.

Chromatography Next steps were done at 4 ℃. Sephadex G-50 poured into a column (2 cm × 75 cm) was washed with Tris-HCl 40 mmol/L (pH 7.4) containing NaCl 36 mmol/L. The water above the gel bed was removed and the sample protein was diluted into 12.5 g/L, and eluted by Tris-HCl 40 mmo/L (pH 7.4) containing NaCl 36 mmol/L. UA-5 absorbency/fluorescence detector recorded the exclusion peaks. Each exclusion peak was collected and identified by 16.5 % SDS-PAGE electrophoresis.

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Bioactivity assay by MTT method MTT method reported by Chen *et al*[10] was used to determine the effects of des(1-3) IGF-Ⅰ with different purities and standard recombinant human IGF-Ⅰ on promoting proliferation of Balb-3T3 cells. For this assay, equal numbers of Balt/3T3 cells were plated on 96-well plates (50 000 cells per well) and maintained in regular growth medium for 48 h after adding various concentrations of des(1-3) IGF-Ⅰ with different purities and standard IGF-Ⅰ. MTT reagents 15 μL were added to each well (final concentration was 0.625 g/L) and incubated at 37 ℃ for 5 h. Then cells were lysed with Me_2SO 100 μL. After incubation at 37 ℃ for 1 h, the plates were then analyzed with a multi-well ELISA reader (Bio-Rad) at 595 nm and 655 nm ($A_{595-655\ nm}$).

Test of protein concentration Test of protein concentration was performed using commassise bright blue G-250[13] with crystalline bovine serum albumin as standard.

Statistical analysis Statistical analysis was carried out by SPSS software. One-way ANOVA and Newman Keuls were used to compare different test groups.

RESULTS

Amplification of des (1-3) IGF-Ⅰ gene by PCR The 1.5 % agarose gel electrophoresis showed that purified PCR product was about 220 bp, which was coincided with the prediction.

Purification and cut of plasmid pExSec1/IGF-Ⅰ Plasmid pExSec1/IGF-Ⅰ as the vector of double-copy IGF-Ⅰ was extracted and purificated by Wizard™ DNA purification system, cleaved with *Bam*H Ⅰ, and dephosphorylated with AP. Agarose 1 % was used to characterize the vector.

Construction and characterization of pExSec1/2 (IGF-Ⅰ) plasmid Agarose gel electrophoresis of plasmid pExSec1/2(IGF-Ⅰ) showed that the insert fragment was about 425 bp cleaved by *Nde* Ⅰ *Bam*H Ⅰ, and about 465 bp cleaved by *Xba* Ⅰ *Bam*H Ⅰ respectively (Fig 2). DNA fragment equals to the length predicted. The sequence analysis of the plasmid DNA indicated the existence of double-copy genes of des(1-3)IGF-Ⅰ in correct frame.

pExSec1/2 (IGF-Ⅰ) expression in *E coli* SDS-PAGE gel(16.5 %) electrophoresis of total soluble protein of *E coli* BL21 (DE3)/pExSec1/2 (IGF-Ⅰ) showed that there was an increased protein band of M_r 7400 demonstrated by GDS-8000 scanning, which was about 20 % of total soluble protein of the bacteria. It was about 8 % higher than that of des(1-3)IGF-Ⅰ expressed by pExSec1/IGF Ⅰ with only one copy of des (1-3)IGF Ⅰ gene (Fig 3).

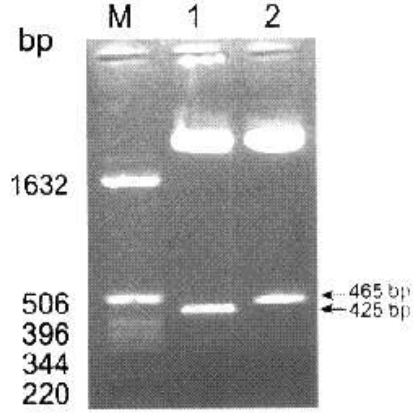

Fig 2. Agarose gel electrophoresis showed DNA fragmentation of plasmid Exc1/2 (IGF-Ⅰ) digested by restriction endonucleases. Lane M: pBR322/*Hinf* Ⅰ; Lane 1: pExSec1/2(IGF-Ⅰ)/*Nde* Ⅰ *Bam*H Ⅰ; Lane 2: pExSec1/2(IGF-Ⅰ)/*Xba* Ⅰ *Bam*H Ⅰ.

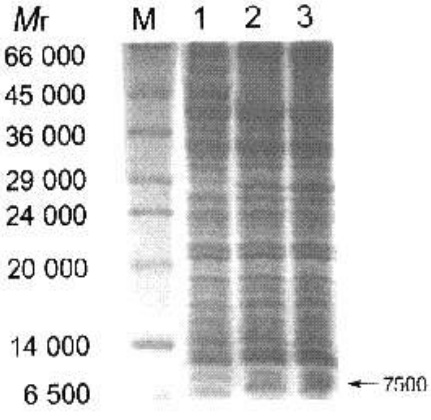

Fig 3. SDS-PAGE analysis of des(1-3) IGF-Ⅰ expression in *E coli* BL21(DE3). Lane M: molecular weight marker; Lane 1: supernatant of BL21(DE3)/ pExSec1; Lane 2: supernatant of BL21(DE3) /pExSec1/IGF-Ⅰ; Lane 3: supernatant of BL21(DE3)/ pExSec1/2(IGF-Ⅰ).

Preliminarily purification by ultrafiltration and gelfiltration The purity of expressed des(1-3) IGF-Ⅰ reached 49 % after ultrafiltration and 82 % after

gelfiltration on Sephadex G-50 reached (Fig 4).

Bioactivity assay With the elevation of purity and concentration, 3T3 cell proliferation by the expressed proteins showed enhancement and concentration-dependence in certain range of concentration (Tab 1).

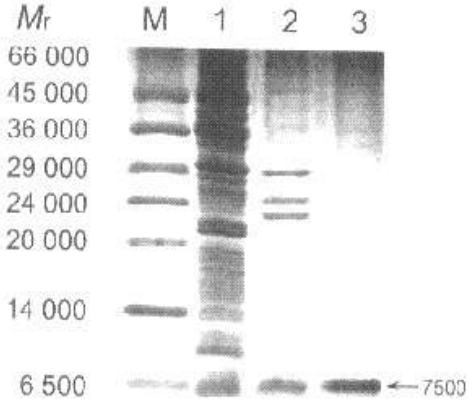

Fig 4. SDS-PAGE analysis of des(1-3) IGF-Ⅰ purity. Lane M: molecular weight marker; Lane 1: supernatant of BL21(DE3)/ pExSec1/2(IGF-Ⅰ); Lane 2: des(1-3 IGF-Ⅰ) purified by ultrafiltration; Lane 3: des (1-3) IGF-Ⅰ purified by ultrafiltration and gelfiltration.

At the same time, a standard bioactivity curve of recombinant IGF-Ⅰ was first established. The bioactivity of des (1-3) IGF-Ⅰ, preliminarily purified by ultrafiltration and gelfiltration was about 77 % of standard IGF-Ⅰ at the same concentration. The bioactivity increased with the elevation of purity and showed concentration-dependence to some extent (Fig 5).

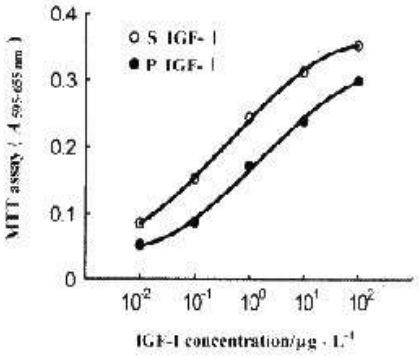

Fig 5. Bioactivity curve of standard recombinant IGF-Ⅰ (○) and des(1-3) IGF-Ⅰ purified by ultrafiltration and chromatography gelfiltration (●).

DISCUSSION

Generally, the level of gene expression is proportional to the number of copies of the transcribed gene in the host cells. The method ordinarily used to increase the copy number of target gene is to use the multi-copy of plasmid as an expression vector. However, increasing the plasmid copy number is not always effective in maximizing the yield of cloned gene product, because more of the energy of the cell is consumed to produce other plasmid-encoded proteins as the increase of plasmid number.

Another approach is cloning multiple copies of the target gene into an expression plasmid. Shen *et al*[11,12] have reported the use of constructing double and multiple copy of target gene in genetic engineering to increase the amount of aim protein expression. Prokaryotic expression frame is a kind of polycistronic mRNA, in which a group of structural protein genes are located in the same operon and controlled by the same promoter. This genetic arrangement of the structural genes and their regulatory genes allows for the coordinate expression of the concerned proteins. All of these linked genes are transcribed into one large mRNA molecule that contains multiple structure genes with independent start and stop codons respectively[13]. The expression product of double

Tab 1. Effects of four proteins on the proliferation of 3T3 cells. The results were obtained by MTT assay ($A_{595-655\ nm}$). $n=6$ wells. $\bar{x} \pm s$. $^bP<0.05$ *vs* control protein. $^cP<0.05$ *vs* coarse protein. $^hP<0.05$ *vs* ultrafiltration.

Groups	Concentration/μg·L^{-1} 30	100	200	300	600
Control protein	0.067 ± 0.001	0.072 ± 0.009	0.055 ± 0.007	0.053 ± 0.006	0.069 ± 0.010
Coarse protein	0.113 ± 0.015^b	0.135 ± 0.012^b	0.156 ± 0.017^h	0.192 ± 0.021^b	0.178 ± 0.020^b
Ultrafiltration	0.139 ± 0.014^{bc}	0.188 ± 0.020^{bc}	0.216 ± 0.030^{bc}	0.209 ± 0.029^b	0.189 ± 0.023^b
Gelfiltration	0.190 ± 0.020^{beh}	0.210 ± 0.021^{beh}	0.330 ± 0.035^{beh}	0.275 ± 0.030^{beh}	0.241 ± 0.031^{beh}

Control protein: whole protein of BL21(DE3)/pExSec1; Coarse protein: whole soluble protein of BL21 (DE3)/pExSec1/2(IGF-Ⅰ); Ultrafiltration: protein using ultrafiltration; Gelfiltration: protein using ultrafiltration and Sephadex G-50 gelfiltration.

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copies of des(1-3)IGF-Ⅰ gene was elevated about 8 % compared with only one copy of gene. The benefit of this method is the lower cost and easier operation, and hence is a feasible way to increase the amount of protein expression in genetic engineering.

IGF-Ⅰ is a small molecular protein. In the purification, the purity of recombinant des (1-3) IGF-Ⅰ reached 49 % after ultrafiltration by Millipore ultrafiltration membranes, while it could reach 82 % after chromatography gelfiltration with Sephadex G-50. In MTT assay, bioactivity of IGF-Ⅰ increased at certain extent with its purity and concentration increase.

At the same time, a standard bioactivity curve using recombinant human IGF-Ⅰ from Germany Boehringer Mannheim Biochmica was established. By comparison, the bioactivity of our preliminarily purified IGF-Ⅰ product could be about 77 % of standard recombinant IGF-Ⅰ. This was lower than that expected. It suggested that there were some inhibitors in non-all-pure IGF-Ⅰ and influenced the IGF-Ⅰ bioactivity.

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双拷贝截短型人胰岛素样生长因子Ⅰ基因的构建、表达和鉴定[1]

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关键词 基因表达; 胰岛素样生长因子Ⅰ; 聚合酶链反应; 超滤; 电泳

目的: 增加重组截短型胰岛素样生长因子Ⅰ[des(1-3)IGF-Ⅰ]的表达产量, 初步纯化表达产物后并与标准 IGF-Ⅰ比较其生物活性. **方法**: 将第二个 des(1-3)IGF-Ⅰ基因插入先前构建好的 pExSec1/IGF-Ⅰ表达质粒中, 形成 pExSec1/2(IGF-Ⅰ)的表达质粒. 将质粒转化入蛋白酶缺陷的大肠杆菌 BL21(DE3)中, 培养工程菌并用 IPTG 于 12 ℃低温诱导 des(1-3)IGF-表达. 超滤膜过滤和 Sephadex G-50 凝胶过滤纯化表达产物. 用 MTT 法测定纯化的 IGF-Ⅰ的生物活性, 并与标准 IGF-Ⅰ比较. **结果**: 双拷贝 IGF-Ⅰ的表达量达可溶性菌体蛋白的20 %, 高于单拷贝 IGF-Ⅰ的表达产量(12 %); 超滤和凝胶过滤后 des(1-3)IGF-Ⅰ纯度分别达 49 %和82 %; 经凝胶过滤后的 IGF-Ⅰ相对生物活性达标准 IGF-Ⅰ的 77 %. **结论**: pExSec1/2(IGF-Ⅰ)可增加 IGF-Ⅰ表达产量约 8 %; 纯化后的 des(1-3)IGF-Ⅰ生物活性为标准 IGF-Ⅰ的 77 %.

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