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Effect of chitosan on production of insulin-like growth factor i protein in *Escherichia coli*

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Abstract

Human insulin-like growth factor 1 (hIGF-1) is a kind of growth factor with clinical significance in medicine. Up to now, *E. coli* expression system has been widely used as a host to produce rhIGF-1 with high yields. Batch cultures as non-continuous fermentations were carried out to overproduce rhIGF-I in *E. coli*. Chitosan and its derivatives, which are known to possess multiple functional properties, have attracted considerable interest due to their biological activities and potential applications in the pharmaceutical, food, agricultural and environmental industries. Many researchers have focused on Chitosan as a potential source of bioactive material in the past few decades. This study focuses on the Chitosan activity on increasing the cell growth and rh-IGF-1concentration in batch culture by affecting on food uptake. The major objective of this study is over- production of recombinant human insulin-like growth factor 1(rhIGF-1) through a developed process by Chitosan as aneffective factor, in order to achieve a higher recombinant protein. According to our previous study analysis of experimental data showed that maximum production of rhIGF-I (1.26 g/l) was occurred in 32y culture medium at 32°C with 0.05 Mm IPTG as inducer and 10 g/l glucose concentration. Under this condition, we have also optimized the amount of Chitosan. Regarding to Minimum Inhibitory Concentration (MIC) of Chitosan (2 µg/ml) on *E.coli* (B/DE3), three concentrations of Chitosan (0.5, 1 and 1.5 µg/ml) were selected. Finally, 1.5µg/ml was selected as the optimum point of Chitosan amount and we reached to a concentration of 1.51 g/l rhIGF-1 at this point.

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Introduction

Human insulin-like growth factor 1 (hIGF-1) composes of 70amino acids and three disulfide bonds, which mainly occurs inhuman liver and serum. With the function of regulating proliferation and differentiation of a wide variety of cell types (Rajapaksha, Alvino *et al.*, 2012).hIGF-1isessential for normal fetal growth and development. It has 50% similarity to insulin and can increase insulin sensitivity and decrease the amount of glucose levels. Also, it is currently being developed as a therapeutic agent in cancer therapy, tissue reconstruction and insulin-resistant diabetes remedy (Jung , Bu *et al.*, 2013; Zhang, Wei *et al.*, 2010).This product has been mostly noticed in pharmaceutical industry because of the effective therapeutic outcomes in treatment of growth failure, diabetes, cancers, amyotrophic lateral sclerosis and also wound healing (De Sanctis, Skordis *et al.*, 2011).The potency of rhIGF-1 as an efficacious therapeutic agent has encouraged many researchers to produce it in various expression systems.

Recombinant human IGF-1 has been produced via recombinant DNA technology using a variety of host systems. So far, a variety of expression systems have been established to produce hIGF-1, including *Escherichia coli*, yeast, cell-free system (Reitz , Li *et al.*, 2013;Schlegel S, Rujas E *et al.*, 2013), transgenic plants (Walmsley, Doran *et al.*, 2012; Dugdale, Mortimer *et al.*, 2013). *E. coli* has some advantages rather than other hosts such as easy handling and culture, and high yields. These characterizations make it most widely used as a host to produce IGF-1(Terpe, 2006; Chung, Choi *et al.*, 2000). In this project we used *Origami* strain. *Origami* has mutations in both thioredoxinreductase (trxB) and glutathione reductase (gor) genes which greatly enhance disulfide bond formation in the *E. coli* cytoplasm. Then, *Origami* is recommended only for the expression of proteins that require disulfide bond formation for proper folding (Brüsehaber, Schwiebs *et al.*,2010).The goal of enhancement the production of recombinant proteins is to produce higher amount of functional product per unit volume per unit time (Babaeipour, Shojaosadati *et al.*, 2013). For *E. coli*, or any other fermentation system, the level of intracellular accumulation of recombinant protein is dependent on final cell density. Several methods have been developed for increasing recombinant protein production. These methods can be categorized as four strategies, namely, choice of culture media, mode of cultivation, strain development, and expression system control (Babaeipour, Shojaosadati *et al.*, 2010; Stone and Veevers, 1994).

Chitosan is a natural nontoxic biopolymer produced by the deacetylation of Chitin, a major component of the shells of crustaceans such as crab, shrimp, and crawfish. Researchers have shown attention to it due to its functional properties such as film-forming capabilities, mineral-binding properties, hypolipidemic activity, biodegradability, antimicrobial activity, immune adjuvant activity, acceleration of wound healing, and eliciting of phytalexins. Currently, Chitosan has received extensive attention for its variable applications in the biomedical, food, and chemical industries. There are studies in which Chitosan is used as membrane permeation-controlled transdermal drug delivery systems. Chitosan is able to enhance the paracellular permeability of mucosal membranes by opening the tight junctions and thereby improving the penetration of drug compounds (Valenta, Auner *et al.*, 2004).

There are also studies in which chitosan is used as a biopolymer controlling the release rate of the drug in transdermal drug delivery systems (Kumar, 2000; Tanner and Marks, 2008; Sudarshan, Hoover *et al.*, 1992).

Also, in previous studies the chitosan is considered to be a bactericidal or bacteriostatic , often with no distinction between activities. Recent data in literature has the tendency to characterize chitosan as bacteriostatic rather than bactericidal. Various theories have been proposed to explain the mode of action leading to the activity of Chitosan. Though the exact mechanism has yet to be elucidated, the intracellular leakage hypothesis is widely accepted(Nan, Xi-Guang *et al.*,2006; Raafat, von

Bargen *et al.*, 2008; Sedef Can, Sedef Erdal *et al.*, 2013).In this mechanism, positively charged Chitosan bindsthe negatively charged bacterial surface leading to altered membrane permeability, which results in leakage of intracellular constituents. (Goy, Britto *et al.*, 2009; Islam, Masum *et al.*, 2011).

In this study we decided to investigate Chitosan effect on memberan penetration in *E.coli* that can lead to increasing of food uptake, growth rate and IGF-1 concentration.

Materials and methods

Strain and plasmid

E. coli strain *Origami* (B/DE3) (Novagen) was the chosen host. *Origami*(B/DE3)has mutations in both the thioredoxinreductase (trxB) and glutathione reductase (gor) genes, which greatly enhance disulfide bond formation in the E. coli cytoplasm. Then, Origami (B/DE3) is recommended only for the expression of proteins that require disulfide bond formation for proper folding. The Origami (B/DE3)strain is tetracycline resistant. PET15b plasmid (Novagen), an ampicillin resistant plasmid, was used as the vector in this work and synthetic IGF-1 gene (synthesis was performed by Gene Script Inc (USA)) was cloned in PET15b plasmid under control of strong bacteriophage T7 promoter(fig. 1).

Culture medium

Base on previous study (Ranjbari, Babaie *et al.*, 2014, unpublished) 32y culture medium was used for optimization process.

32y (Merck Company) composition was: peptone 0.8 %(W/V), yeast extract 3.2% (W/V), NaCl 0.58 (W/V) in Tris-HCl 10 mM (pH=7.6)

Transformation and expression of rhIGF-1

The expression vector pET15b-rhIGF-1 was transformed into *E. coli* strain *Origami* (B/DE3). The transformed colonies were grown in 5 ml LB containing 100 µg/ml ampicillin and 100 µg/ml tetracycline at 37°C. When OD_{600} reached 0.6–0.8, isopropyl-β-D-thiogalacto pyranoside (IPTG)(Sigma-Aldrich) was added to a final concentration of 0.1 mM. The culture was incubated at 37°C for 4 h. Then, expression of IGF-1 was analyzed through SDS-polyacrylamide gel electrophoresis (PAGE).

Western blot analysis of rhIGF-1

The expressed rhIGF-1 was also analyzed by Western blotting using anti-human IGF-1 antibody (Abcam-USA) for further identification. Briefly, 20- 30 µg of total protein from cell lysate was loaded into the well of the SDS-PAGE gel 17.5%, along with molecular weight markers (Sigma). Next, gel was run for 1 to 2 hours at 100 V. Protein was transferred to nitrocellulose membrane (Bio-Rad). Membrane was blocked for 1 hour at room temperature using 5% blocking solution (PBS containing 5% milk). Then, Membrane was incubated with mouse anti-human IGF-1 monoclonal antibody in 5% blocking solution overnight at 4°C. After washing with PBS for five times, the membrane was incubated with horse radish peroxidase conjugated secondary antibody in 5% blocking buffer for 1.5 h with shaking at room temperature. After washing with PBS for five times, the membrane was then visualized using an enhanced - chemiluminescence kit (FIVEphotonBiochemicals) by following the protocol as the manufacturer suggested.

Minimum inhibitory concentration (MIC) of Chitosan

A broth macrodilution method was performed in order to determine the minimal inhibitory concentration (MIC) of Chitosan. At first an antibiotic dilution range was prepared. Sterile8 x 100 mm test tubes were used to conduct the test. The final twofold (or other) dilutions were prepared of antimicrobial agent volumetrically in the broth. A minimum final volume of 1 mL of each dilution is needed for the test. In second step the inoculum was prepared by making a direct broth suspension of isolated colonies selected from an 18 to 24 hour agar plate (Muller Hinton). The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland turbidity standard. This results in a suspension containing approximately 1 to 2 x 10^8 colony forming units (CFU)/mL for

Escherichia coli.The inoculum and the 0.5 McFarland standard tubes were compared against a card with a white background and contrasting black lines.Optimally within 15 minutes of preparation, the adjusted inoculum suspension was diluted in broth so, after inoculation, each tube contained approximately 5 x 10^5 CFU/mL. This can be accomplished by diluting the 0.5 McFarland suspension 1:150, resulting in a tube containing approximately 1 x 10^6 CFU/mL. The subsequent 1:2 dilution in step 3 brought the final inoculum to 5 x 10^5 CFU/mL.Within 15 minutes after the inoculum had been standardized as described, 1 mL of the adjusted inoculums was added to each tube containing 1 mL of antimicrobial agent in the dilution series (and a positive control tube containing only broth), and was mixed. This resulted in a 1:2 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculums. Then the inoculated tubes were incubated at 35 ± 2 ºC for 16 to 20 hours in an ambient air incubator. Finally the amount of growth in the tubes containing the antimicrobial agent was compared with the amount of growth in the growth-control tubes (no antimicrobial agent) used in each set of tests when determining the growth end points. For a test to be considered valid, acceptable growth (≥ 2 mm button or definite turbidity) must occur in the growth-control well. The lowest concentration at which the isolate was completely inhibited (as evidenced by the absence of visible bacterial growth) was recorded as the minimal inhibitory concentration or MIC.

Fermentation process

Inocula were prepared by inoculating single colonies from a freshly spread plate into 10 ml of culture medium with100 µg/ml Amp and 100 µg/ml tetracycline. The seed cultures were grown at 37°C for about 8h on a shaker incubator at 120 rpm. The final A600 value was typically about 1. Flask cultures (10 %(v: v)) in 250 ml flasks were inoculated and shaken at 120 rpm; and prepare the temperature according to our previous study (32º C) in a shaker incubator. Then, each flask was induced with 0.05 mM IPTG.

Analytical procedures

Cell growth was monitored by measuring culture turbidity and dry cell weight (DCW). Turbidity was determined by measuring the optical density (OD) at 600 nm. Samples were diluted with NaCl solution (9 g/l) to obtain an OD between 0.2 and 0.5. In order to determine DCW, 5 ml of broth culture was centrifuged at 4,000×g for 10 min, washed twice with deionized water, and dried at 105°C to constant weight. Expressed recombinant protein was determined and quantified by SDS-PAGE, densitometry(image J software), and Bradford methods (Braunstein, Halwer *et al.*,1986).Densitometry is the quantitative measurement of optical density in light-sensitive materials, such as photographic paper or photographic film, due to exposure to light. Optical density is a result of the darkness of a developed picture and can be expressed absolutely as the number of dark spots (i.e., silver grains in developed films) in a given area, but usually it is a relative value, expressed in a scale. Densitometry is particularly useful due to its sensitivity, accuracy and versatility, and it can be applied to proteins in gels or on membranes. In addition to being accurate, sensitive and reproducible, the technique is cost-effective, simple, and does not require a high degree of specialized training, yet provides technical advantages over other available tool (Vincent ,Cunningham.,1997). The rhIGF-1expression level was determined by SDS-PAGE on Polyacrylamide 17% (w/v). Gels were stained with Coomassie brilliant blue R250 and then quantified by gel densitometer. Bradford protein assay was used for the quantitative analysis of total protein.

Results and discussion

Transformation and expression of rhIGF-1

DNA sequence encoding human IGF-1 was cloned in pET15b vector to construct the recombinant plasmid pET15b-hIGF-1containing the exact human IGF-1 gene sequence confirmed by automated DNA sequencing. The expression plasmid was then transformed into *E. coli* strain *Origami*(B/DE3). In the presence of 0.1 mM IPTG, the expression of the

protein was induced. After induction, transformed cells were analyzed for rhIGF-1 expression by SDS-PAGE. As shown in fig. 2transformed bacteria were produced rhIGF-1.

Western blot analysis of rhIGF-1

The expressed rhIGF-1 was also analyzed by Western blotting using anti-human IGF-1 antibody (Abcam-USA) for further identification. According to fig. 3, Western blotting analysis approved production of rhIGF-1.

Table 1. Minimum Inhibitory Concentration of Chitosan on *E. coli* (B/DE3).

Test organism	E.coli(Origami B/DE3)				
Chitosan concentration(µg/ml)	0.5	1	2	4	8
Organism growth	+	+	-	-	-

Table 2. Chitosaneffect on rh-IGF-1 Expression percent base of densitometery (%) with three time repeats.

Chitosan concentration	Process duration(h)	Total protein (g/l)	Expression percent base of densitometry (%)	IGF-1 concentration (g/l)	Final OD_{600}	DCW (g/l)
0.5µg/ml	16 h	3.35	37	1.25	11.6±0.1	5.45±0.04
1 µg/ml	16 h	3.48	39	1.32	12.3±0.3	5.95±0.01
1.5 µg/ml	16 h	3.7	44	1.51	14.1±0.2	6.74±0.01

Effect of Chitosan on IGF-1 expression

Chitosan has been reported as an antimicrobial material against a wide range of target organisms like algae, bacteria, yeasts and fungi (Chen,Liau *et al.*, 1998; .Papineau, Hoover *et al.*,1991; Shahidi, Arachchi *et al.*,1999).

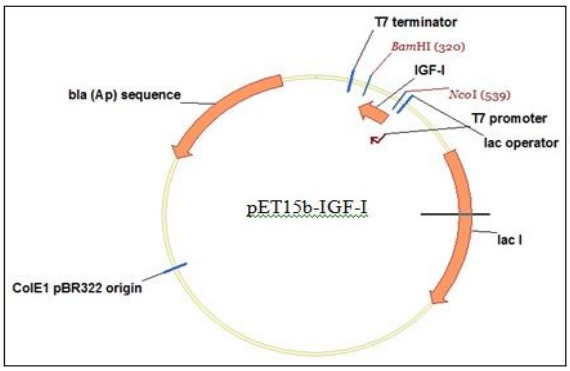

Fig. 1. Expression vector: pET15b-rhIGF-1, ampicillin resistant with strong bacteriophage T7 promoter

In our study, Chitosan was used as an enhancer that may increases membrane penetration of E. coli (B/DE3).Due to the above reason the concentration of chitosan was used lower than the minimum inhibitory concentrations (MIC).As shown in table (1), MIC value of Chitosan was achieved at the concentration of 2µg/ml. Therefore 0.5, 1 and 1.5 µg/ml of chitosan were selected. Transformed *E.coli*(B/DE3) by IGF-1 construct was treated for three time by each of the above concentration of Chitosan. At different amount of Chitosan, Dry Cell Weight(DCW) and the concentration of rhIGF-1 protein varied in a wide range from 5.45 to 6.74 g/l and 1.25 to 1.51 g/L consequently, the highest value observed when 1.50 µg/ml Chitosan was used (fig. 4).This result suggested that Chitosan might be an effective factor for the efficient production of recombinant protein. The optimal concentration of Chitosan was determined through analyzing induced cell samples. The results were also shown the expression level of hIGF-1 protein increased up to 1.51 g/ml (table 2).

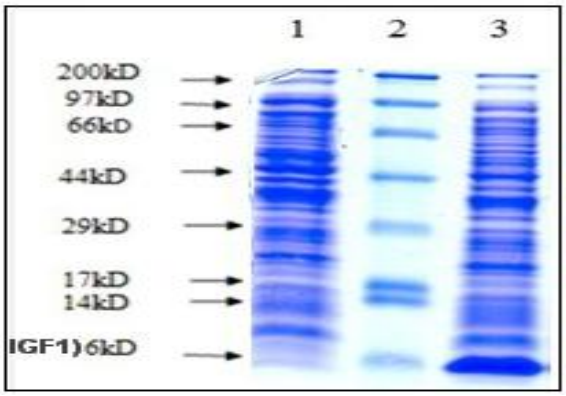

Fig. 2. Expression of rhIGF-1 in batch fermentation in LB mediumwith 0.1 Mm IPTG. Lane1: before induction. Lane 2: Marker, lane 3: after induction.

Researcher in previous studies showed that Chitosan is able to enhance the paracellular permeability of mucosal membranes in mammalian cells especially dermal cells by opening the tight junctions and thereby increasing the penetration of materials. (Valenta, Auner *et al.*, 2004).

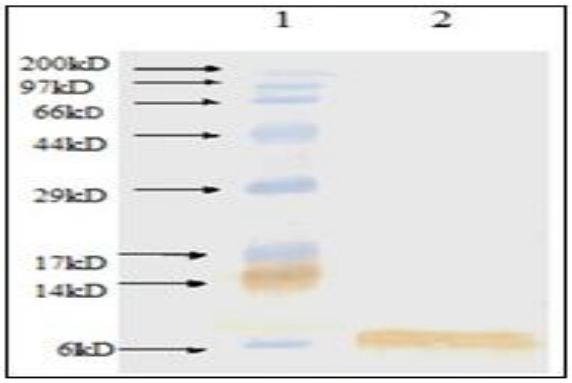

Fig. 3. Western blot analysis of produced rhIGF-1using anti-human IGF-1 antibody. Lane 1: Molecular weight marker, lane 2: produced rhIGF-1 in shaking flask experiment step (0.1 mM IPTG, 32 o C, TB medium).

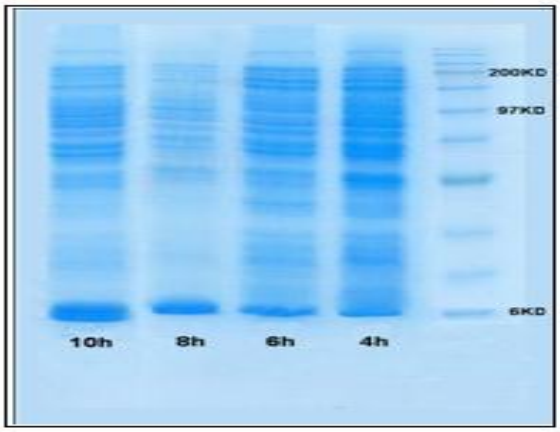

Fig. 4. SDS-PAGE gel of rhIGF-1 expression in shaking flasks experiment**:** 1.5 µg/ml Chitosan, 32y culture medium, temperature of induction 28 ° Cand 0.05 mM IPTG. (6 hours after induction).

Also, some studies showed that Chitosan has a positive charge and the outer membrane of bacteria consists essentially of lipopolysaccharides containing phosphate and pyrophosphate groups which render to the surface a density of negative charges. More adsorbed chitosan would evidently result in greater changes in the structure and in the permeability of the cell membrane.(Chung & Chen., 2008; Eaton, Fernandez *et al.*, 2008). Observations have confirmed that at higher concentrations, the chitosan tends to form a coating over the bacteria and properly result in intracellular leakage.

In current study we observed Chitosan can increase cell growth and final rhIGF-1 concentration that it can be due to the effect of it on membrane permeation with different mechanisms which explained.

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