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A Novel Signal Peptide Derived from *Bacillus Licheniformis* α-Amylase Efficiently Targets Recombinant Human Activin A to the Periplasm of *Escherichia coli*

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Abstract

Human activin A is a member of the transforming growth factor-β superfamily consists of two similar beta subunits. Activin A is expressed by different cells and displays numerous biological activities such as control of neuronal cell proliferation and differentiation, promotion of neuronal survival in the body. Therefore, recombinant production of activin A is beneficial because it can be used to treat many neurodegenerative diseases such as Alzheimer's and Parkinson diseases. In this study *E. coli* as a cheap and fast-growing host was selected to produce recombinant human activin A. As cytoplasmic expression of human activin A with complex structure and disulfide bonds produces inclusion bodies, so periplasmic expression of it can be beneficial. Therefore, we used modified Iranian *B. licheniformis* α-amylase signal peptide as a new signal peptide in order to translocate the recombinant activin A through the inner membrane. In this study human pro-activin A cDNA and signal sequence were cloned in pET21b vector and resulting vector transformed into the two strains of *E. coli* BL21. SDS-PAGE and western blot techniques were used to confirm recombinant activin A expression. Finally, our results indicated that the signal peptide used in this study was effective for secretion of activin A into the periplasmic space of *E. coli*.

***Keywords*:** Activin A, modified α-amylase signal peptide, periplasmic expression

Introduction

Activins are members of the transforming growth factor β (TGF) super family. They are biologically active as dimmers. In mammalians, four isoforms of the activin β-subunit have been identified: βA, βB, βC and βE. Activin A is dimmer of A subunits (βAβA) that are linked together by disulfide bond. (Walton et al., 2012; Weiss and Attisano, 2013). Activin A participates in numerous physiological processes in the body, including cellular differentiation, apoptosis, metabolism, wound repair, maintenance and survival of the neurons, immune response; so, it can be used as a therapeutic agent besides many other growth factors (Chen et al., 2006; Sulyok et al., 2004; Schubert et al., 1990). Because of its extensive biological roles, several mammalian cell line expression systems have been used in order to produce it as a recombinant protein (Cronin et al., 1998; Pangas and Woodruff, 2002). As these systems have several disadvantages, including cost, technical difficulties in maintenance of the transfected cell lines and relatively low yields, some have used other eukaryotic hosts such as *pichia pastoris* in order to produce activins (Papakonstantinou et al., 2009).

Escherichia coli (E. coli) is the most appropriate expression host for expression of many recombinant proteins because it has several advantages such as fast growth, cheap culture media, well-known genetics and many available commercial vectors. Therefore, in this work we used *E. coli* for expression of recombinant human activin A. The oxidative environment of periplasm of *E. coli* is suitable for expression of proteins with disulfide bonds because it contains chaperones and at least four enzymes called Dsb (disulfide bond formation) proteins which are involved in protein folding and disulfide bond formation (Choi and Lee, 2004; Berkmen, 2012). Generally, bacteria use different secretion systems to transport proteins to the periplasm. There are three main pathways for extracting protein from the cytoplasmic space in bacteria, Sec pathway, SRP dependent pathway and Tat pathway. Secretion in each of these pathways depends on the signal peptides, which are mainly located at the N-terminal of the protein (Natale et al., 2008). In this study, we investigated the efficiency

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of modified Iranian *Bacillus Licheniformis* α-amylase signal peptide (m.I.B.L.α-amylase signal p) to secrete recombinant human activin A protein into the periplasmic space of *E. coli*. To do this, we cloned the signal sequence and cDNA of pro-activin A into the pET21b (+) expression vector and investigated the expression level.

Materials and Methods

Unless otherwise specified, all reagents were purchased from Merck Company (Germany).

Bacterial strains, culture conditions and recombinant DNA technology

The BL21(DE3) and BL21(DE3) plysS strains were used as the expression hosts and pET21b (+) (Novagene-USA) plasmid was used as expression vector throughout the experiments. The cDNA of human pro-activin A β subunit (Accession No. NM_002192) and Iranian *B. licheniformis* α-amylase signal sequence (Accession No. AY842512) were obtained from NCBI gene bank. In order to increase the hydrophobicity of the signal peptide, one Met codon (ATG) was inserted between bases 39 and 40. The TopPred software was used to survey the hydrophobicity of the modified signal peptide. Then the fusion of modified signal sequence and pro-Activin A cDNA was synthesized by ShineGene company (China) and cloned using NdeI and EcoRI restriction enzymes (Fermentas-USA) into the pET21b (+) vector (Sambrook and Russel, 2001). The resulting vector was individually transformed in *E. coli* host strains using heat shock procedure (Sambrook and Russel, 2001). The bacterial cells were grown in Luria-Bertani (LB) medium supplemented with 100 $\mu g.ml^{-1}$ampicillin at 37°C.

In order to detect the colonies with h-pro-activin A coding sequence, after isolating recombinant clones on selective media, the plasmid DNA from each clone was extracted and analyzed by restriction mapping and sequence analysis. For the purification of plasmid DNA, alkaline lysate method was used (Sambrook and Russel, 2001). Double digestion of purified plasmid using appropriate restriction enzymes was used; in addition, recombinant clones were sequenced to confirm the insertion of the h-pro-activin A gene into the vectors without any base deletion or substitution.

Protein expression; Preparation of cytoplasmic and periplasmic proteins

For gene expression, 1% dilution of an overnight culture of transformants was transferred into fresh LB medium and incubated at 30°C to an OD_{600nm} of 0.6-0.8. Gene expression was induced by addition of 1 mM of isopropyl thio-β-D-galactoside (IPTG) to the bacterial media. The negative control test was carried out with the recombinant strain without adding inducer. The cells were grown for additional 4 hours and harvested by centrifugation at 5000 g. To obtain periplasmic proteins, osmotic shock procedure with some modifications was used (Libby et al., 1987). Cytoplasmic proteins were obtained using urea 8M (Hajihassan et al., 2016).

SDS-PAGE and immunoblot analysis

Proteins were separated by 15% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing and reducing conditions (Laemmli, 1970). For western blotting, the proteins from gel were transferred onto a nitrocellulose membrane (Millipore-USA) (Demaio, 1996) and membrane subsequently was treated with anti-human activin A monoclonal antibody (Abcam-UK; ab89307) with 1:1000 dilution in blocking buffer (PBS; 3% W/V skimmed milk). Peroxidase conjugated anti-mouse IgG (Sigma-USA; A9044) was added as a secondary antibody.

Results and Discussion

Construction of the pET21b::pro-Activin A expression vector

Several working groups produce activin A in eukaryotic hosts such as insect cell lines (Cronin et al., 1998), CHO (Pangas and Woodruff, 2002) and *Pichia pastoris* (Papakonstantinou et al., 2009). In this work two strains of *E. coli* were used in order to produce activin A. As production of complex eukaryotic proteins such as activin A with multiple disulfide bonds in the oxidative environment of the periplasmic space where suitable chaperons are present is beneficial (Berkmen, 2012; Rosano and Ceccarelli, 2014), we produced activin A in the periplasm of *E. coli*. For effective translocation of the protein across plasma membrane, appropriate signal peptide is usually located at the N-terminal of the target protein. In this study with the aim of addressing the recombinant human pro-Activin A in the periplasmic space of *E. coli* a novel signal peptide, Iranian *B. licheniformis* α-amylase signal peptide, was used. Usually, signal peptides contain three general domains: N-terminal domain with a net positive charge, a central hydrophobic region and a C-terminal region with the signal cleavage site. The data revealed that even natural signal peptides differ in production levels, depending on the type of recombinant protein fused to them (Low et al.,

2013). Even more efficient signal peptides need to be developed. As increasing the hydrophobicity of the center region of the signal peptide improve translocation of recombinant protein towards the membrane and into the periplasmic space, in this study a methionine residue was inserted between proline and leucine residues in the I.B.l.α-amylase signal peptide (Sahdev, 2008). Figure 1 shows the coding sequence and amino acid composition of I.B.l.α-amylase signal peptide and m.I.B.l.α-amylase signal peptide in comparison. The inserted Met residue is obvious in the figure.

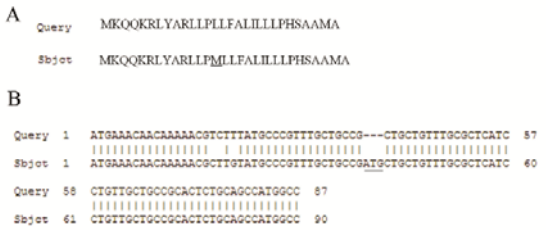

Figure 1. Amino acid composition (A) and sequence (B) of I.B.l.α-amylase signal peptide (Query) and m.I.B.l.α-amylase signal peptide (sbjct) in comparison. One inserted Met (ATG) is underlined. The alignment was done using NCBI BLAST.

After constructing the recombinant plasmid, it was moved into the BL21(DE3) and BL21(DE3) plysS strains of *E.coli* and a number of colonies in each case were isolated from selected media containing antibiotic. Plasmid DNA was isolated from the bacteria and restriction enzyme (RE) analysis was performed in order to release the signal p-pro-activin A insert with the size of 1320 bp (Figure 2). As shown in the figure (lane 2) double digestion with NdeI and EcoRI enzymes results two bands; one corresponds to the signal p-pro-activin A insert and the other corresponds to vector without insert. Also sequencing analysis using T7 terminator primer indicates the complete and correct insertion of the insert into the vector (data not shown here). The confirmed recombinant bacteria were subjected for subsequent expression analysis.

Effects of modified Iranian *B. licheniformis* α-amylase signal peptide on localization of Activin A

Total protein patterns from the bacteria carrying recombinant plasmid after induction with IPTG were analyzed by dot blotting using specific anti-human activin A monoclonal antibody to evaluate the production of rhpro-activin A. The results revealed that proteins obtained from both strains of bacteria were reactive to anti-human activin A antibody; a strong dark color dot indicates reactivity with antibody (Figure 3A). Uninduced bacterial extracts (protein expression without induction with IPTG) in DE3 strain was also reactive to the antibody because of basal transcription (promoter leakage) (Tegel et al., 2011).

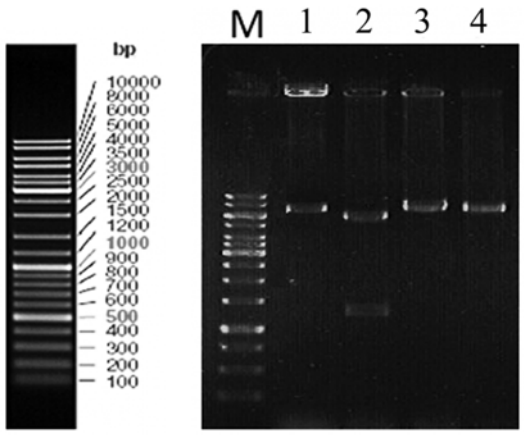

Figure 2. Restriction enzyme analysis of h-activin A construct by agarose gel (1% W/V) electrophoresis. M: DNA Ladder (Fermentas-USA). Lane 1: recombinant pET21b::h-pro-activin A DNA without digestion. Lane 2: digestion of recombinant pET21b::pro-activin A with NdeI and EcoRI restriction enzymes. Lane 3 and 4 are digestion of recombinant pET21b::pro-activin A with NdeI or EcoRI.

To analyze the efficiency of the signal peptide used in this study to secrete the pro-activin A into the periplasmic space, cellular fractionation (cytoplasm and periplasm) was carried out. SDS-PAGE and western blot experiments using anti-human activin A monoclonal antibody were done to analyze the protein patterns (Figure. 3B and C). Comparison of the periplasmic and cytoplasmic protein patterns in Figure 3 showed that both strains of bacteria harboring recombinant vectors secreted detectable amount of pro-activin A in their periplasms. It has to be noted that the two bands revealed in the western blot analysis are considered as processed and unprocessed forms of m.I.B.L.α-amylase signal p-hpro-activin A protein (48 and 45 KDa). Results obtained in this study indicated the periplasmic production of activin A in BL21(DE3) and BL21(DE3) plysS strains of *E. coli*. However western blot results showed that the detectable amount of activin A is yet present in the cytoplasm of *E. coli*; so to improve the secretary efficiency, it is suggested that some other modifications are necessary for developing a better signal peptide.

It is worth noting that the results of this study confirm the results presented by others about the

importance of the role of signal peptides in directing the target protein to the secretary machines in bacteria. Also, the results of this research and the results obtained by others indicated that the signal peptide optimization is usually necessary to achieve a high level of protein secretion (Han et al., 2017; Zamani et al., 2015; Low et al., 2013). There are many studies in this field. For example, Hajihassan and their coworkers showed that for periplasmic secretion of β.NGF in *E. coli*, DsbA signal peptide is the best choice (Hajihassan et al., 2016). In another study, Han and their coworkers used different natural and modified signal peptides to secrete the Alpha toxin$_{H35L}$ in *E. coli*. They showed that some modified signal peptide improved the yield of secreted Alpha toxin$_{H35L}$ by 3.5-fold (Han et al., 2017).

In conclusion, we introduce a novel and effective signal peptide to secrete the recombinant proteins to the periplasmic space of *E. coli*.

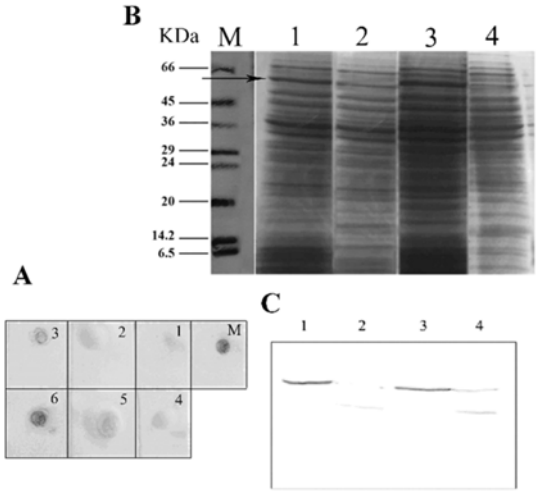

Figure 3. Dot blot assay of total proteins extracted from recombinant bacteria using anti-human activin A monoclonal antibody (A). Dots 1-3 are total proteins extracted from (DE3) plysS bacteria. 1-3 are proteins of bacteria lacking recombinant pET21b plasmid, proteins of bacteria carrying pET21b plasmid but without induction with IPTG, proteins of bacteria carrying pET21b plasmid after 4 h induction with 1 mM of IPTG respectively. Dots 4-6 are total proteins extracted from DE3 bacteria. 4-6 are proteins of bacteria lacking recombinant pET21b plasmid, proteins of bacteria carrying pET21b plasmid but without induction with IPTG, proteins of bacteria carrying pET21b plasmid after 4 h induction with 1 mM of IPTG respectively. M is commercial standard human activin A (Abcam- UK). SDS-PAGE (B) and western blot (C) analysis of the cytoplasmic and periplasmic protein patterns of recombinant bacteria carrying the pET21b::h-activin A plasmid. Lanes 1 and 2 are the cytoplasmic and periplasmic proteins of (DE3) plysS bacteria; lanes 3 and 4 are the cytoplasmic and periplasmic proteins of DE3 bacteria respectively. M shows the protein size marker (Sigma-USA). Recombinant activin A (48 KDa) has been shown with arrow.

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