Journal of Sciences, Islamic Republic of Iran 32(3): 205 - 211 (2021) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Improved Production of Recombinant Human Activin A in *Escherichia coli*

Z. Hajihassan*, N. Nazari, F. Armaghan

Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Islamic Republic of Iran

Received: 6 January 2021 / Revised: 15 May 2021 / Accepted: 26 June 2021

Abstract

Activin A is a member of transforming growth factor β (TGF-β) superfamily. It plays numerous roles in the body such as cell growth regulation and differentiation, wound repairing and modulation of inflammatory responses. More importantly, it can be used as a therapeutic agent; so recombinant production of it, especially in the periplasm of *E. coli* as an economical bacterium is of great value. The aim of this study is large- scale production of activin A with a correct structure. For this purpose, three strategies were used. First, an efficient and appropriate signal peptide, modified Iranian *Bacillus Licheniformis* α-amylase signal peptide, was selected to secrete activin A to the *E. coli* periplasm as a suitable environment for correct protein folding. Second, cytoplasmic chaperones, Dnak, DnaJ, GroEL/ GroES, TF (trigger factor) were expressed simultaneously with activin A. Finally, the agitation rate was optimized to achieve the highest production of Activin A at the bioreactor scale. Our results indicated that by the co-expression of TF with activin A and using agitation rate of 1000 rpm maximum expression of activin A in *E. coli* was obtained. More importantly, based on the CD spectroscopy results and bioassay test the produced activin A had the correct secondary structure as the commercial type and was fully active.

Keywords: Activin A; Trigger factor; Agitation rate.

Introduction

Activin A belongs to the superfamily of transforming growth factor β, and consists of two βA subunits connected by a single disulfide bond (1). Because this protein naturally has different functions, such as anti-inflammatory, and wound healing roles and also participation in the maintenance, and survival of the neurons, recombinant production of it is beneficial (2-5). Today this therapeutic protein is expressed in several eukaryotic and prokaryotic hosts (6-8). Production in prokaryotic hosts e.g. *E. coli* is affordable and simple, with the ability to scale up easily (9). Nevertheless, correct formation of disulfide bonds and proper folding are the main challenges of protein production in these hosts (10). Of course, periplasmic space of bacteria is a suitable environment in terms of oxidative conditions and the presence of molecular chaperones for production of proteins with disulfide bonds (11, 12). Thus, in this study, activin A was secreted to the periplasm of *E. coli* using efficient and modified signal peptide of *Bacillus licheniformis* α-

* Corresponding author: Tel: +982186093294; Fax: +982188497324; Email: hajihasan@ut.ac.ir

amylase enzyme (7).

It should be noted that in some cases, the co-expression of cytoplasmic chaperones not only has affected the protein solubility and folding but also has increased the periplasmic production of the interest protein (13-15). Therefore, in this study, in order to increase periplasmic production of activin A with the correct structure first, co-expression of cytoplasmic chaperones was used and then, the agitation rate, which is an effective factor in the success of protein expression in the bioreactor scale (16), was optimized.

Materials and Methods

Materials

Luria-Bertani medium (LB), Ampicillin, Chloramphenicol, anti polyhistidine peroxidase-conjugated monoclonal antibody (Cat No. SAB5300168), commercial recombinant activin A and isopropyl thio-β-D-galactoside (IPTG) were purchased from Sigma Aldrich Company (USA). Ni^{+2}-NTA chromatography resin was purchased from ABT (Spain). DAB was purchased from Biobasic Inc. (Canada). Other reagents were prepared from Merck Company (Germany). Plasmids with inserted cytoplasmic chaperones genes were purchased from Takara Company (Japan). Table 1 shows the properties of plasmids and chaperones used in this study.

Bacterial strain and expression vector

The BL21 (DE3) strain (Novagene, USA), with the T7 RNA polymerase gene inserted in it, and pET21a(+) vector (Novagene, USA) with His-tag sequence as an affinity tag were selected respectively, as an expression host and vector in this experiment.

The cDNA of human activin A (Accession No in NCBI gene bank: NM_002192) and modified *Bacillus licheniformis* α-amylase signal sequence were synthesized and cloned into pET21a vector using NdeI and XhoI enzymes by Shinegene company (China) (7). Sequencing was used to verify cloning accuracy (data not shown here).

Transformation and culture conditions

$CaCl_2$ and heat shock procedure was used to transform the vector to BL21 (DE3) strain (17). Transformed cells were cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) with 100 mg/ml Ampicillin, at 37˚C and 180 rpm.

Culture conditions in the bioreactor

A 5-L bioreactor (Sabaferm110, Zist Farayand Sanat, Iran) filled with 2 L medium was used for batch fermentation. Throughout the experiment, the pH was kept constant at 7.0 by the automatic addition of 2*N* NaOH or 2*N* HCl solution. The compressed air was sterilized through a cellulose filter and delivered to the bioreactor tank. Also, different agitation speeds (rotation speed of the mixer) of 200, 400, 600, 800, 1000, and 12000 rpm and constant aeration speed of 1vvm were used.

Co-expression of cytoplasmic chaperones and recombinant vector

In order to express the cytoplasmic chaperones with the recombinant vector carrying the activin A cDNA, first desired chaperone plasmids were transformed to the DE3 strain (17). For selection of transformed cells, bacteria were grown in LB medium containing chloramphenicol antibiotic. In the next step, the recombinant vector carrying activin A sequence was transformed to the previous strains and then cultivation was done overnight in LB medium containing the Ampicillin and Chloramphenicol antibiotics. Then, 1% dilution of an overnight culture of transformed cells was added to fresh LB medium containing special inducer for each chaperone plasmid according to Table 1. The cells were cultured at 37ºC until the absorbance at 600nm reached 0.6 and then incubation continued for 15 minutes at 15ºC. Finally, 0.5 mmol/L of IPTG was added for induction of the promoter. The incubation continued for about 24 h at 15ºC and a centrifugation step was performed at 5000×g for 15 min at 4˚C to collect the cells. Also, periplasmic proteins were extracted with osmotic shock method (18).

Table 1. The properties of plasmids used in this study.

No.	Plasmid	Chaperone	Promoter	Resistant Marker	Inducer (final concentration)
1	pG-KJE8	dnaK-dnaJ-grpE-groES - groEL	araB Pzt-1	*Chloramphenicol*	*L-Arabinose* (0.5mg/ml) *Tetracycline* (1-5ng/ml)
2	pGro7	groES-groEL	araB	*Chloramphenicol*	*L-Arabinose* (0.5mg/ml)
3	pKJE7	dnaK-dnaJ-grpE	araB	*Chloramphenicol*	*L-Arabinose* (0.5mg/ml)
4	pG-Tf2	groES-groEL-tig	Pzt-1	*Chloramphenicol*	*Tetracycline* (1-5ng/ml)
5	pTf16	tig	araB	*Chloramphenicol*	*L-Arabinose* (0.5mg/ml)

Gel electrophoresis and immunoblot analysis

Laemmli method was selected for SDS-PAGE (15% w/v) (19). For western blotting, after separation of the proteins by SDS-PAGE, they were transferred to the nitrocellulose membrane using a transfer buffer (25 mM Tris-HCl, 192 mM glycine, 15% methanol). TBS buffer (pH= 7.4) with 5% fat-free milk was then used for blocking the nonspecific sites. Treatment of the membrane with anti-His-tag monoclonal antibody conjugated with horseradish peroxidase (1:1000 dilution) and subsequently with a solution of DAB and hydrogen peroxide as enzyme substrates was performed for detection of interest protein (20).

Protein purification

Since produced activin A has a poly-histidine tail at the C-terminal, Ni^{2+}-NTA affinity chromatography (IMAC) was used to purify. The sample was added to the pre-equilibrated column with binding buffer (50 mM NaH2PO4, 300 mM NaCl, pH= 8). Then washing (50 mM NaH2PO4, 300 mM NaCl, 20 mM Imidazole, pH=8) and elution buffers (50 mM NaH2PO4, 300 mM NaCl, 500 mM Imidazole, pH=7) were added respectively to elute the activin A attached to the resin from the column (21).

Protein concentration determination

The Bradford method was used to determine the concentration of interest protein (22).

Circular dichroism (CD) experiment

CD spectrophotometer model 215 (Aviv Instruments Inc.) was used to determine the secondary structure of purified activin A. Purified activin A was dissolved in 10 mM Tris-HCl (pH= 7.4) buffer and its CD spectrum was drawn in the range of 190–260 nm with a spectral resolution of 1 nm. The scan speed was 20 nm/min, and the response time was 0.3330 sec with a bandwidth of 1 nm. A Quartz cell with a path length of 10 mm was used. Also, all measurements were performed at room temperature.

In vitro activin A bioassay

K562 erythroleukemia cells were selected to study the activity of recombinant activin A. Differentiation of these cells in the presence of activin A and also accumulation of hemoglobin indicates the functionality of activin A (23). For this purpose, the RPMI-1640 culture medium contained 10% of inactivated fetal calf serum and the antibiotics penicillin and streptomycin was used for cell culture. 50 ng/ml recombinant activin A was added to the medium to induce erythroid differentiation. Also, commercial recombinant activin A was used as a control. Inductively coupled plasma optical emission spectrometry (Varian 730-ES Axial ICP-OES) was then used for measuring the count of Fe^{2+} ions accumulated in the medium (24).

Results

The effect of cytoplasmic chaperones co-expression on the periplasmic production of activin A

Cytoplasmic chaperones are one of the critical parameters affecting the recombinant protein expression

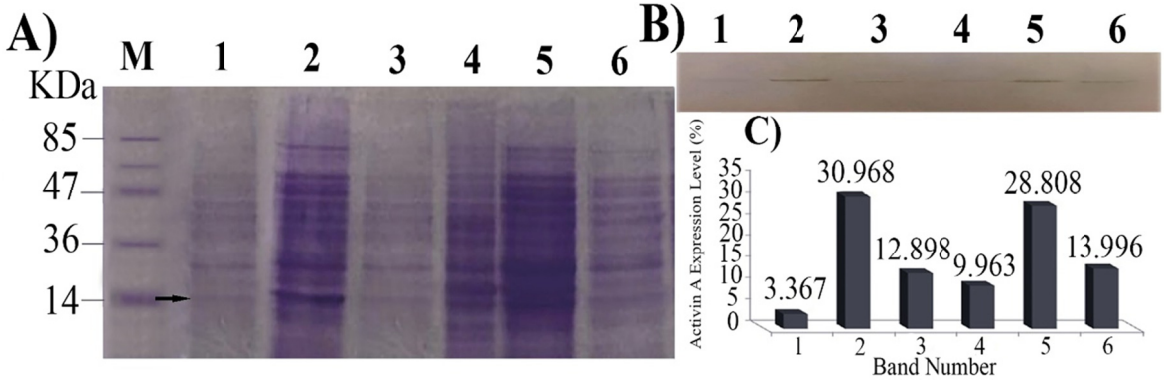

Figure 1. Analysis of the recombinant activin A expression-level. A and B are periplasmic proteins extracted from recombinant BL21 (DE3) strain analyzed by 12% SDS gel electrophoresis and Western blotting techniques, respectively. M is the molecular weight marker. Lanes 1 to 5 are periplasmic proteins with co-expression of pG-Tf2, pTf16, pGro7, pKJE7 and pG-KJE8 plasmids respectively; Lane 6 is extracted proteins without any chaperons. In lane number 1, 14.5 KDa band related to recombinant activin A is shown with a black arrow**.** C) Periplasmic expression level of activin A calculated by ImageJ software. All experiments were repeated three times under the same conditions.

level. Therefore, for co-expression of cytoplasmic chaperones with activin A, first transformation of cytoplasmic chaperones and recombinant vector containing activin A cDNA to *E. coli* (DE3 strain) was done and then, the expression of activin A and cytoplasmic chaperones was performed according to the methods section.

Figure 1 (A-C) shows the electrophoresis and western blotting analysis of the periplasmic proteins, respectively. Also Image J software was used to quantify the results (25). As can be seen, recombinant activin A was expressed in all samples, 14.5 KDa band, but the highest level of periplasmic activin A was obtained when co-expression of pTf16 chaperone (containing the Tigger factor) with recombinant vector was used.

Also Bradford method was used to determine the exact concentration of the periplasmic proteins. As shown in Table 2, the maximum amount of produced protein was related to the sample with co-expression of cytoplasmic chaperone pTf16, (calculated concentration = 1.963 ± 0.081 mg/ml) confirming SDS-PAGE results. The results also indicated that co-expression of cytoplasmic chaperones not only has affected the periplasmic production of activin A but also has affected the total level of protein expression.

Agitation rate optimization in the bioreactor scale

A 5-l bioreactor with a 2-l medium was used to produce recombinant activin A on an industrial scale. The agitation rate is a significant element in the overexpression of recombinant proteins in the bioreactor scale. Hence, to investigate the influence of agitation rate on activin A production, different agitation rates (200, 400, 600, 800, 1000, and 1200 rpm) were used. As illustrated in Figure 2, the highest production of activin A was obtained at 1000 rpm (43.009% measured by image J software (Fig. 2C)).

Protein purification and structural analysis

Recombinant activin A was purified successfully by IMAC chromatography and, the secondary structure of it was subsequently determined by CD spectroscopy to evaluate the quality of produced protein. Moreover, this structure was compared with commercial activin A (Fig. 3). The percentages of α-Helix, β-sheet, β-turn, and random coil for purified activin A were 22/2 %, 40/5%, 19%, and 25% respectively and with acceptable similarity to the activin A reported in UniProt (13% α-Helix, 46/5% β-sheet, 12% β-turn).

Table 2. Total protein concentration measured by Bradford assay.

Chaperone plasmid used	Total protein concentration (mg/ml)
No chaperone-Activin A	1.112 ± 0.096
pG-KJE8-Activin A	1.631 ± 0.073
pKJE7-Activin A	1.090 ± 0.0028
pGro7-Activin A	0.401 ± 0.005
pTf16-Activin A	1.963 ± 0.081
pG-Tf2-Activin A	0.450 ± 0.0012

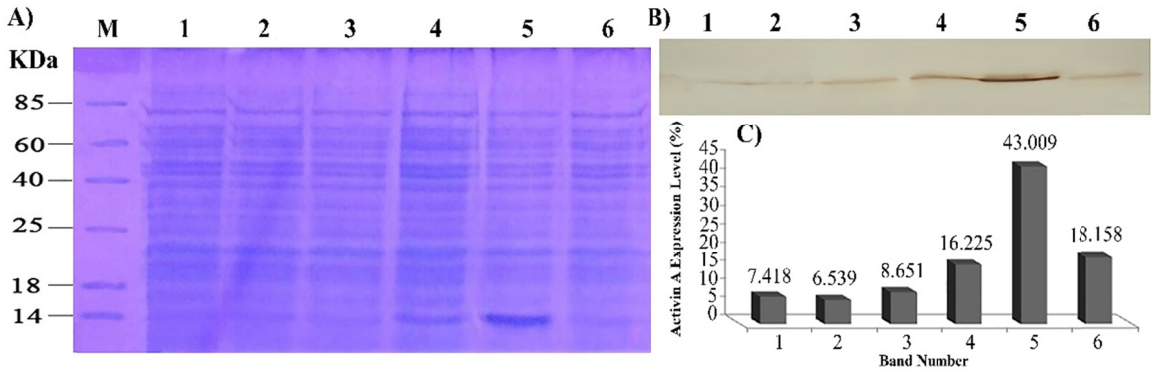

Figure 2. Analysis of the recombinant activin A expression level in different agitation rates in the bioreactor scale. The periplasmic proteins extracted from recombinant BL21 (DE3) strain analyzed by 12% SDS gel electrophoresis(A) and Western blotting(B) techniques, respectively. M is the molecular weight marker. Lanes 1 to 6 are extracted proteins in agitation rates of 200, 400, 600, 800, 1000 and 1200 rpm respectively. (C) Periplasmic expression level of activin A calculated by ImageJ software. All experiments were repeated three times under the same conditions.

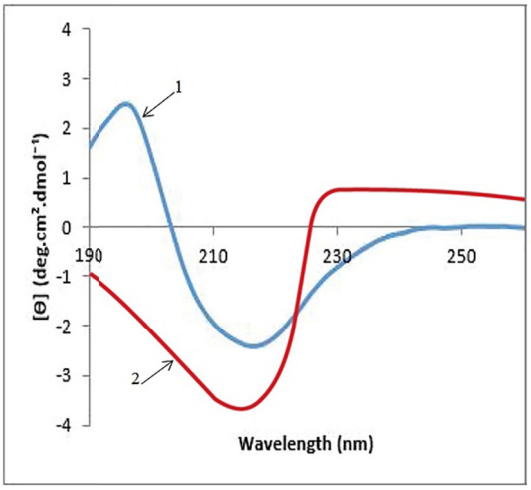

Figure 3. The CD spectra of commercial activin A (1) and recombinant activin A (2) recorded in the Far UV region, respectively. The experiments were performed at 25ºC.

In vitro activin A bioassay

Differentiation of K562 cell lines to red blood cells in the presence of activin A was evaluated to study the functionality of produced activin A. The results indicated that the amount of Fe^{+2} ions after 72 h in samples treated with recombinant activin A reached 4 ppm (2-fold increase) compared to the control. More importantly, these results were quite similar to the results obtained from the commercial activin A.

Discussion

E. coli is an economical and widely used host for recombinant protein production because it grows quickly in inexpensive and simple culture media and has well-characterized genetic. However, overexpression of recombinant proteins sometimes leads to accumulation of inclusion bodies (IBs) in the cell (10, 26). One of the most widely used strategies to increase the expression level of soluble protein in *E. coli* is the secretion of the protein of interest to the periplasmic space by fusion a suitable signal peptide to the protein (27). To date, many recombinant proteins have been secreted to the periplasmic space through secretion pathways using different signal peptides such as LamB, MalE, OmpA, OmpC, OmpF, OmpT, PelB, PhoA, PhoE, or SpA (27-31). Bacteria have many secretion strategies to pass proteins through the plasma membrane. Two common types of secretion pathways in *E. coli* are Sec pathway and Sec independent or Tat pathway (32, 33). Since the Sec route is the most widely used pathway to translocate proteins in an unfolded structure (33, 34), there are many studies on the secretion of proteins by it; for example, the secretion of human granulocyte colony-stimulating factor (hG-CSF) (35), hydrolytic enzymes such as alpha-amylase, mannanase and chitinase (36) and so on. Also, many efforts have been made to improve the efficiency of natural signal peptides in the secretion of target proteins by optimizing their sequences. For example, Jonet et al. (2012) illustrated that the optimization of signal peptide from *Bacillus sp.G1* increased the secretion of recombinant cyclodextrin glucanotransferase to the *E. coli* periplasm by 94% (37). Caspers et al. (2010) introduced many signal peptide variants for secretion of cutinase enzyme by performing mutagenesis on the signal sequence of α-amylase (AmyE) enzyme (38). Therefore, in this project to secrete activin A to the *E. coli* periplasm, modified signal peptide *of Bacillus licheniformis* α-amylase enzyme, which its secretory efficiency has already been proven, was used (7). Another approach to enhance the solubility of a recombinant protein is the co-expression of cytoplasmic chaperones with the target protein (39). Jhamb et al. (2012) confirmed that the co-expression of cytoplasmic chaperones, DnaKJE and GroEL-ES, not only has improved the solubility of xylanase protein but also has increased the yield of this protein (40).

In this study, co-expression of cytoplasmic chaperones GroEL, GroES, DnaK, DnaJ, and TF (Trigger Factor) was used to increase the periplasmic production of soluble human activin A. The results showed that among the chaperones used, the TF increased the periplasmic expression of activin A. TF is the first chaperone binds for a long time to the protein being synthesized and subsequently connects the target protein to SecB, a chaperone involved in the secretory pathway (13). Similar studies by Jia et al. and Sonada et al. in 2014 and 2011, respectively, demonstrated the positive effect of TF expression on the periplasmic production of human-like collagen and Single-Chain Fv fragments of antibodies (13, 41).

Finally, recombinant activin A was produced in a 5-L bioreactor to obtain lots of protein. Production on a semi-industrial and industrial scale requires special considerations; for example, agitation or shaking is one of the most critical factors during production in a bioreactor because stirring the culture medium prevents the formation of bacterial aggregates. More importantly, using an appropriate agitation speed directly affects the recombinant protein production (42, 43). Previous studies showed that the degree of oxygenation affected by the stirring or agitation speed has a direct effect on the growth of bacteria and protein production (44, 45). Therefore, in the present study different agitation speeds were used in order to achieve the highest production of

activin A in the fermenter. According to our results, the best and most suitable agitation speed was 1000 rpm. These results are consistent with data provided by Zaslona et al. in 2015 which showed that recombinant production of 1,3-β-glucanase in *E. coli* increased with moderate stirring and oxygenation speed (46); because at high agitation speeds cell disruption takes place (47).

Finally, as the correct structure of the recombinant protein and more importantly its functionality is important, the secondary structure of produced activin A was determined and its ability to induce differentiation of K562 cells to red blood cells was studied and compared to a commercial one. The results showed that recombinant activin A had correct secondary structure and was fully functional.

Conclusion

Briefly, in this study, in addition to using an appropriate signal peptide, two other approaches were used to achieve a high level of activin A production in the periplasmic space. One method was the use of different cytoplasmic chaperones, and the other was agitation rate optimization in the bioreactor. The results showed that by co-expression of TF with activin A and using agitation rate of 1000 rpm, maximum expression of activin A in *E. coli* was obtained.

Acknowledgment

The authors would like to acknowledge the financial support of University of Tehran for this research under Grant Number 28669/06/08.

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