Iraqi Journal of Biotechnology, April 2019, Vol. 18, No. 1, 83-89

Production of Recombinant IFN-α2b from Tobacco (*Nicotiana tobacum*) by Non-Chromatography Purification

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Received: January 27, 2019 / **Accepted:** April 10, 2019

Abstract: Developing genetically modified plants has been among wildly used strategies to produce recombinant proteins. Prior to have a large-scale production of a recombinant proteins, transient expression in a model plant is recommended as it could lead us to a proper insight of the subject. In this research, interferon alpha 2b gene (INF α2b) as a protein with antiviral and antitumor activities was subjected to be transiently expressed in tobacco and through *Agrobacterium tumefaciens* infiltration method. The optimized target sequence, IFN α2b, was designed to be constructed in such a way that it could be purified either by His-tag or Elastin-like peptide (ELP). Agro-infiltrated leaves were analyzed through real time PCR. In proteomics level, the ELP-tagged IFN was successfully purified through non-chromatography method and confirmed by SDS-PAGE electrophoresis. Considering the low level of recombinant protein production in the plants, one remarkable achievement of this research was application of ELP in combination with IFN α2b for purification of recombinant protein from total protein of the plant hosts.

Keywords: Non-chromatography purification, Plant transient expression, Interferon alpha 2b gene, SDS-PAGE.

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Introduction:

Plants provide an interesting platform for expression of important biological molecules. Recombinant protein production in plants offers several advantages over other production systems such as prokaryotes, yeasts or mammalian cells (1). The production of recombinant proteins in plants is considered to be safe and economical to the microbial and mammalian expression systems. Despite the fact that plant systems are recognized for producing recombinant proteins, there are some challenges including the need to enhance protein stability and recovery (2). One of the issue of plant protein expression is low yield of target protein. In many researches, the level of recombinant protein produced in plant system has been reported in negligible amount of total proteins (7,8). When the object of investigation is application of a recombinant protein following purification, using a feature enabling recombinant protein to be efficiently purified is valuable. Among purification strategies, an advantage of non-chromatography method is that they are not restricted to the equipment; therefore, they can be carried out simple and cheap, and also efficient. Elastin-like peptide (ELP) brings the mentioned features together. Application of ELP tag for purification of the interested protein has been known for successful separation of the target protein from the solution (9). This peptide is composed of 10-250 repeated motifs (10), each involves five amino acids (11). The length of ELP and the composition of each motif affect its ability for protein purification. Recently, many researches have been done to develop recombinant pharmaceutical

proteins based on plants as the expression system. One of the therapeutic proteins that has been known as antiviral and anticancer protein is interferon (12,13). The interferon alpha family displays antiviral and immunomodulatory activities on different type of cells (6). Human interferon alpha 2b (IFN α2b) has been used for the treatment of leukemia and about 86 countries have been using it in hepatitis and cancer treatments (7).

In this research we aimed to express interferon α2b gene in tobacco as a model for expression of the recombinant. In addition, examination the method based on ELP tag was considered for efficient purification.

Material and methods:

Bacterial cultures and plant materials:

In order to culture the bacterial strains LB medium supplemented with appropriate antibiotic was used. For cloning procedures *Escherichia coli* strain DH5a was grown at 37 °C. *Agrobacterium tumefaciens* strain C58 was used for transient transformation of the plant hosts. It was incubated at 28 °C with shaking at 180 rpm in LB medium supplemented with Rifampicin and Kanamycin with final concentration of 50 and 100 mg/L, respectively. For transient transformation of Tobacco (*Nicotiana tabacum*), tobacco plants grown under greenhouse conditions obtained from the farm.

Preparing gene constructs:

In this research, commercially available T-vector (p-TG19, Vivantis, Malaysia), an intermediate synthetic vector (pExt, provided by Dr. Alizadeh's Lab, University of Tehran, Iran) and the binary vector (pBI121, Clontech) were used for cloning purposes. The sequence of interferon α2b gene was adopted from NCBI data bank NG-029154. The optimized target sequence was designed as a 533 bp fragment containing required restriction sites and subjected to be synthesized via General Biosystem Company (USA). A specific pairs of primers designed by using Primer 3 online software (http://primer3.ut.ee/). Specific forward and reverse primers of IFN were as follow; IFN-Forward primer 5′ AACCATGGGCGGATCCTGTGATCTGCC and IFN-Reverse primer 5′ AAGAGCTCGAGTGCGGCCGCGAATTCC TTAC. During cloning procedures, PCR products, linear plasmids and fragments were gel purified to remove excess residuals using Glass milk method (8). Primary confirmation of TA-cloned IFN was carried out via *BamH*I, *BamH*I-*Xho*I and *Nco*I-*Xho*I restriction enzymes (Fig.1A). In the next step, the target sequence inserted into the intermediate vector to get the desired combination (IFN-His and IFN-ELP), subsequently it was located inside the binary vector pBI121 through *Xba*I-*Sac*I restriction sites. The vector harboring a designed ELP for high rate of purification (Patent numbe#) was provided by Dr. Alizadeh's Lab (University of Tehran, Iran). In this study, all cloning procedures were done according to the Sambrook and Russell protocols (9).

Agrobacterium-mediated transformation:

In order to transform plant, preparing competent cells of *Agrobacterium* C58 was carried out according to the protocol (10). Transformation of competent cells of *A. tumefaciens* were done according to the freeze and thaw method (13).

After primary confirmation of positive clones by colony PCR, a single clone was picked from selective LB agar medium and grown in 15 mL of liquid medium overnight at 28 °C with shaking. Then cultures were diluted in ratio 1:100, supplemented with 10 m*M* MES, 20 µ*M* Acetosyrongone, and appropriate

antibiotics. Next day, cultures were supplemented with 55 g/L sucrose and 150 µM AS, incubated 1-3h at RT, and then transformed to plant leaf samples by using vacuum pump. Infiltrated leaves were kept at 22 °C in a dark condition. After 3 days (72 hours), leaf samples were frozen in liquid nitrogen and were kept at -80 for further analysis (15).

Plant protein extraction:

Plant agro-infiltrated leaves were powdered in liquid nitrogen. For protein extraction, 200 µl of PBST buffer (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, pH=7.4 containing 0.05 % Tween-20) was added per 100 mg of leaf samples. The samples vortexed for 20 sec and were placed on ice for 15 min. Spin was done at 10000 ×g at 4 °C for 15 min. This step repeated twice for remove residuals. Finally, supernatant transferred to the new tubes and were stored at -20 °C for further analysis.

The investigated recombinant proteins were purified based on ELP tag. Plant extracts were used for purification based on non-chromatography method according to the protocol with some modification (5). In brief, ammonium sulfate with final concentration of 0.4 *M* added to each protein sample followed by incubation at 37 °C for 6 min. When a turbidity appeared in the samples containing ELP tag, centrifugation was done for 6 min at high speed (hot spin). Supernatant was transferred to new tube as control and pellet was resuspended in ice-cold PBS buffer. The samples were placed on ice for 10 min followed by centrifugation at 4 °C for 15 min at high speed (cold spin). Supernatant (*i.e.* purified recombinant proteins) was transferred to a new tube and stored until further analysis. Total proteins and purified recombinant protein tagged with ELP were separated through electrophoresis on 10 % SDS-PAGE.

Preparing samples for real time PCR:

Extraction of RNA from frozen tobacco leaf samplesd, 3 replicates, two gene constructs (*i.e.* pBI:IFN and pBI: IFN: ELP) carried out according to the manufacturer's instructions (Denazist Asia, Iran). Quantitation and qualification of extracted RNA were examined by using NanoDrop1000 Spectrophotometer and gel agarose electrophoresis. *DNase*I treatment was done to eliminate remaining DNA according to the protocol (Thermo Scientific). The *DNase*I treated-RNAs were converted into cDNA using PrimeScript RT reagent kit (Takara, Japan) according to the manufacturer's instruction. Specific forward and reverse primers designed to perform qRT-PCR (For qIFN CCTCTGATGAAGGAGGATTCCA and Rev qIFN GAATG AACGCATGATTTCTGCAC). The housekeeping gene beta-actin as an internal control was used to normalize the results. The reaction of PCR was composed of qRT-PCR master mix containing SYBR green fluorescent dye (Topaz gene company, Iran), specific IFN primers/housekeeping primers with final concentration of 0.2µ*M*, and diluted cDNAs as templates. The qPCR conditions were as follow; 94°C 20sec, 57°C 20sec, 72°C 20 sec. The melting curve obtained in the temperature range of 65-95 °C. Each run was included reactions with both specific and housekeeping primers. The results of relative expression of IFN gene was calculated by REST software.

Result and discussion:

In order to express interferon α2b gene in the plant host, the target sequence was inserted into the pBI121 binary vector while it was tagged with 6His tag or Elastin-like peptide (ELP). The gene construct pBI: IFN accuracy was confirmed primarily with colony PCR (Fig. 1B), followed by sequencing based on Sanger method via Microsynth Company (Switzerland) (Figure 2). The

sequencing of the plasmid of interest performed in two directions by using a forward universal primer and the reverse specific primer of interferon. In order to have an alternative for purification of target recombinant protein, the second gene construct (*i.e.* pBI: IFN: ELP) was made in which ELP was inserted at the 3´side of IFN α2b. The result of colony PCR on the candidate clones is shown in (Figure 1C). The map of both gene constructs is shown in Figure (3).

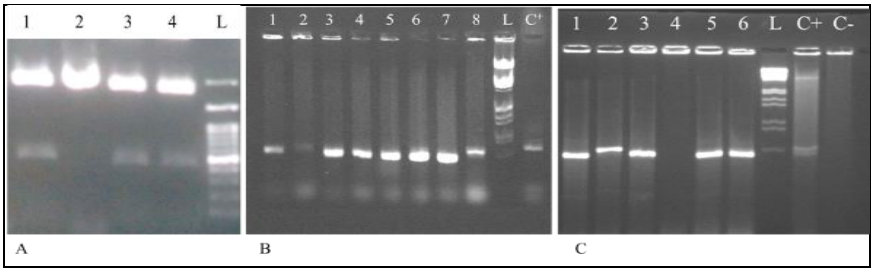

Figure (1): (A): Confirmation of resulting T-vector containing the optimized IFN α2b gene. Number 1, 3 and 4 indicate the same plasmid enzymatically digested by *Nco*I-*Eco*RI, *Nco*I-*Xho*I, and *Bam*HI-*Xho*I, respectively. The observed bands on the gel were according to the expectation. Number 2 shows uncut plasmid as control. Letter L indicates 100 bp plus DNA ladder (Sinaclon, Iran). (B): Electrophoresis of PCR products of colony PCR in order to select the candidate clones having pBI: IFN. Numbers 1-8 show individual clones analyzed for the presence of target gene. Letter C^+ indicates positive control. Letter L shows Lambda DNA/*Eco*RI+*Hind*III marker, 3 (Thermo scientific). (C): Result of colony PCR on candidate clones harboring pBI: IFN: ELP.

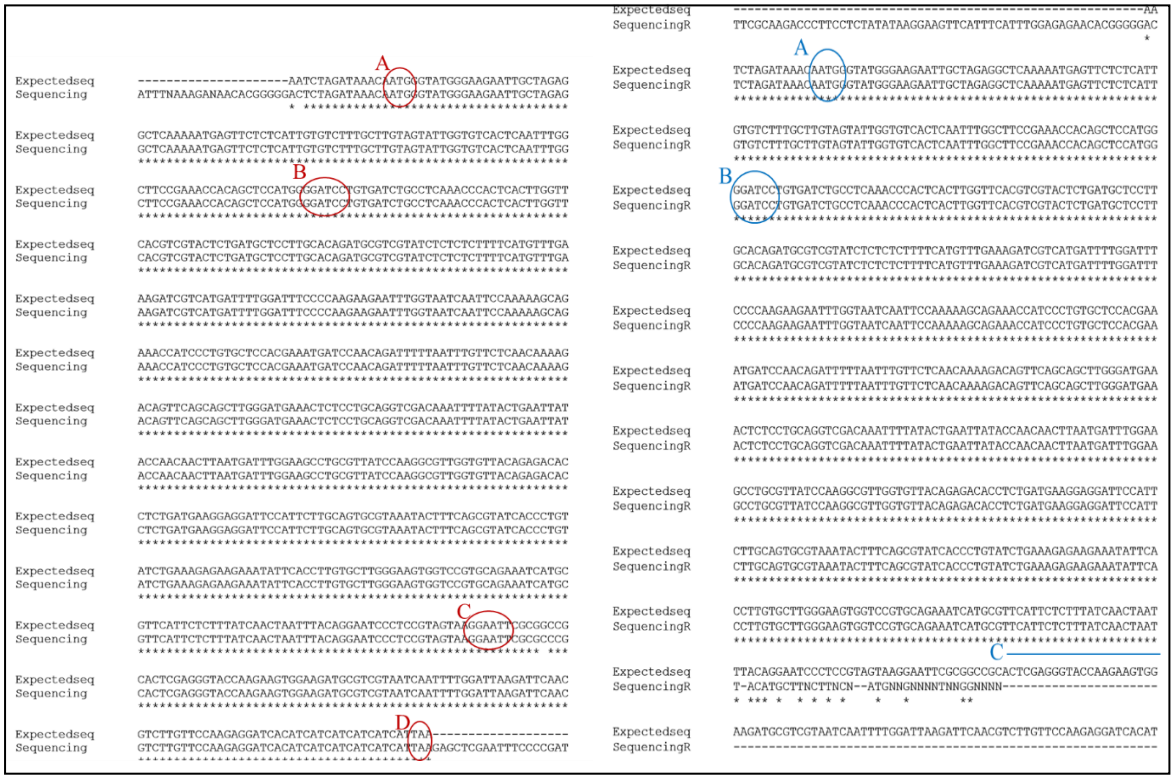

Figure (2): The results of sequencing for the gene construct pBI:IFN. In the pictures, Expectedseq is the name given to the original sequence and Sequencing is the name given to sequencing result. Pair wise alignment was done using Clustal Omega online software. The picture on the left indicates the sequencing result obtained via forward universal primer. Letter A in the picture indicates start codon ATG, letter B shows *Bam*HI restriction site in the initial of IFN. Letter C shows *Eco*RI restriction site at the end of IFN. Letter D is stop codon. The picture on the right indicate the sequencing result obtained via reverse specific primer. Letter A in the picture indicates start codon ATG, letter B shows *Bam*HI restriction site in the initial of IFN. Letter C shows the sequence at the end of IFN.

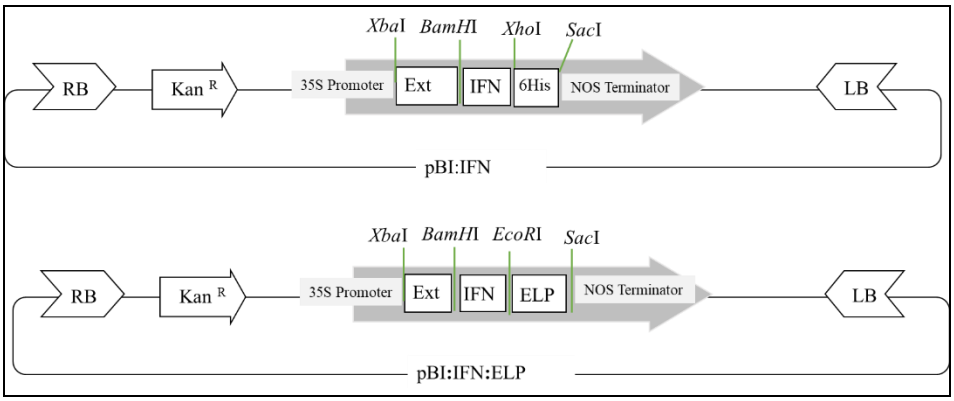

Figure (3): Schematic map of the gene constructs for plant transformation.

In the plant expression system, one issue is low level of expression besides the challenge for purification of recombinant proteins (2,5,8). For expression the investigated recombinant protein in plant, two plasmids pBI: IFN and pBI: IFN: ELP were introduced into the tobacco (*Nicotiana tabacum* cv. samsun), via *Agrobacterium tumefaciens*. In order to estimate relative expression of interested gene, transient expression of IFN α2b in tobacco was analyzed in transcriptome level.

The melting curve obtained through real-time PCR analysis indicates a specific amplification of investigated genes (17). According to the Fig. 4A, the obtained melting curve indicates that primers were able to anneal to the proper sites leading to the specific amplification of target gene and also similar situation required for amplification of both genes (*i.e.* IFN α2b and the internal control beta actin). As it is shown in the Fig. 4B, the level of transient gene expression of IFN in tobacco was detectable. These observations were similar to the results obtained by other researchers (14). In the research done by Rafiee and colleagues, the relative expression of ELP-tagged gene in tobacco and the bacterial expression of the investigated protein tagged with ELP was higher than the protein without ELP (12).

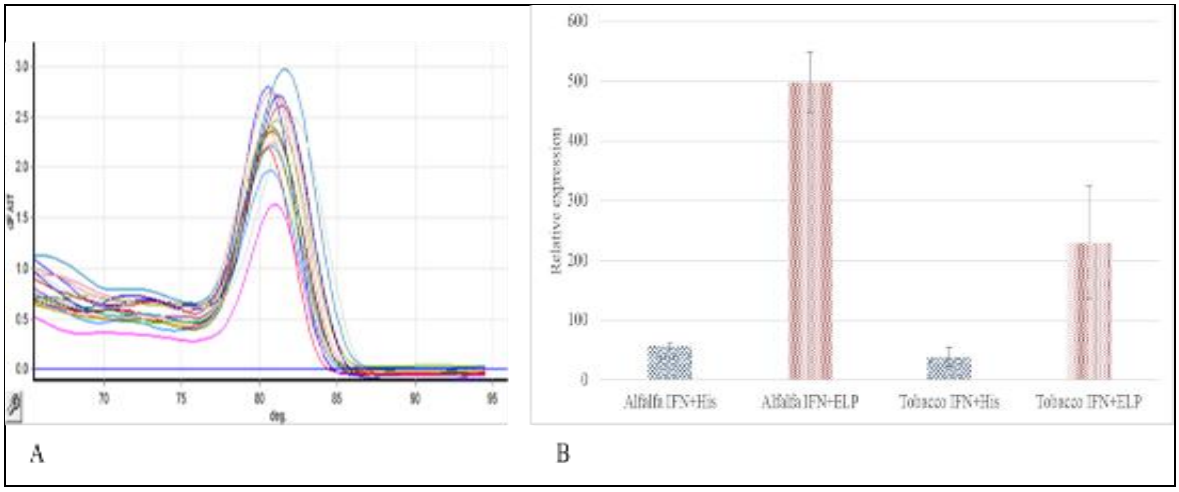

Figure (4): The expression result of investigated gene in tobacco. (A): An example of the melting curve obtained from one of the amplifications. (B): The relative expression of the target gene in tobacco. The expression is calculated and normalized relative to the internal gene control.

Since the low level of protein production has been reported in plants, the protein of interest cannot be clearly observed in SDS-PAGE before purification. In the (Figure 5A) separation of plant total proteins containing IFN:His is shown. As it was expected, total proteins of tobacco on the SDS-PAGE didn't show any differences compared to the control, however, it indicated expression when analyzed by qRT-PCR. With regard to the results of gene expression at RNA level (Figure 4B), the gene construct harboring ELP was more probable to lead to the higher level of target protein. With considering higher expression of IFN:ELP and also the ease of specific purification of recombinant protein based on ELP, it was decided to complete this part of the study with agro-infiltrated samples having IFN:ELP. Acrylamide gel electrophoresis was done for separation of total proteins containing IFN:ELP (Figure 5B). Considering the presence of ELP at the C-terminal side of IFN α2b recombinant protein, it was purified based on the ELP tag. With respect to the molecular weight of IFN:ELP, a band around 51 kDa was expected to be seen, however with considering the molecular weight of the ext signal peptide (Ext) (13), the target protein might be appeared in smaller size when enter to the appoplast spaces of the cells and losing the signal peptide. According to the results, a weak band of the purified protein from tobacco was observed by the SDS-PAGE. It appears that both the type of host and ELP characteristics affect the yield of recombinant protein production.

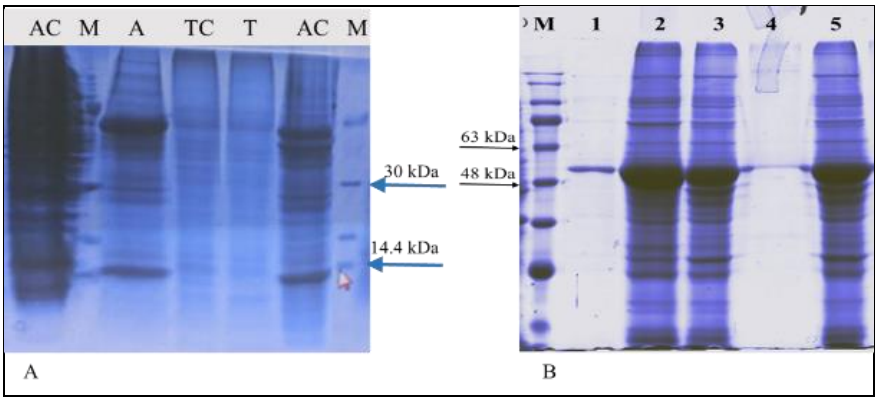

Figure (5): Electrophoresis of plant total proteins by 10% SDS-PAGE. (A): Plant total proteins containing IFN:His. AC and TC indicate control total protein extracted from tobacco. Letter A and T show total protein containing target protein from tobacco. Letter M shows protein ladder (Parstous, Iran). (B): Plant total proteins containing IFN:ELP and their purification. Number 1 indicates purified protein IFN:ELP and number 2 shows the respect total protein obtained. Number 3 shows total protein obtained from non-agro-infiltrated leaves. Number 4 indicates a band of purified protein from tobacco and number 5 is its respect total protein. Letter M shows protein ladder (Sinaclon Iran).

Conclusion:

In the current study, IFN α2b gene was located at the C-terminal side of ELP tag purification. Transformation of tobacco with *Agrobacterium*-mediated system led to express the target gene in plants. Therefore, purification was restricted to the agro-infiltrated leaves producing IFN:ELP. Purification of the recombinant protein from tobacco was led to the higher level of the protein. Paying attention to the low yield of recombinant protein in plant expression system, application of a purification tag with high ability to separate the target protein from the rest of total protein is an important improvement.

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