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# Expression of human interferon α-1 with enhanced stability *via* the tagging system of a stabilizing peptide

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ABSTRACT

Human interferon α-1 (hIFNA1) is one of several interferon α subtypes that have been studied and commercialized to treat various viral diseases including hepatitis B and C as well as malignant melanoma. Protein aggregation has been problematic for every step in commercial production, from purification to the packaging and delivery of pharmaceutical proteins. In a previous study, we demonstrated that a stabilizing peptide from the C-terminal acidic tail of α-synuclein (ATS) could be used as an effective fusion tag to increase the stability of target proteins such as human growth hormone (hGH) and granulocyte colony-stimulating factor (G-CSF). In this study, we applied this ATS fusion system to hIFNA1 in order to protect against the aggregation of hIFNA1 by environmental stresses, since hIFNA1 aggregates elicit an undesirable immune response in humans. As expected, ATS-fused hIFNA1 (hIFNA1-ATS) protein showed enhanced stability against thermal stress, agitation stress, and repetitive freeze/thawing stress in comparison with native hIFNA1. More importantly, hIFNA1-ATS fusion protein appeared to be 1.6 times more active than hIFNA1 in a cell anti-proliferation assay. Furthermore, the solubility of hIFNA1-ATS appeared to be 1.7 times higher than that of native protein. Our results suggest that the ATS-tag system could be a useful means for protecting hIFNA1 protein from aggregation by various external stresses and could be used to increase the solubility of protein.


## Introduction

Human interferon α-1 (hIFNA1)[1], a type I interferon, also known as B-cell interferon, leukocyte interferon and interferon α-D, consists of 166 amino acids [1] and is one of at least 22 subtypes of interferon α [2]. Human interferon α (hIFNA) proteins are currently used worldwide to treat a variety of diseases including malignant melanoma, hairy-cell leukemia, non-Hodgkin's lymphoma as well as hepatitis B and C [3,4]. However, interferon α proteins like other protein pharmaceuticals has the instability structurally and it remains a great barrier for pharmaceutical scientists because this poor stability often increases the probability of protein aggregation and degradation during the pharmaceutical processes.

The stability of pharmaceutical proteins against environmental stresses is an important factor regarding their efficacy during production, processing, storage, and delivery [5,6]. Various stresses on the proteins can cause structural alterations and induce protein aggregation, which can thereby lead to unexpected immune responses, cytotoxicity, and even anaphylactoid reactions during the clinical application of protein pharmaceuticals [7,8]. The mechanism of protein aggregation is still not fully understood, although various factors that induce protein aggregation have been reported, such as temperature, pH, ionic strength, shaking, sharing, protein concentration, metal ion concentration, freeze-thawing, and freeze-drying [9–12]. A number of improvements have been reported to prevent or minimize protein aggregation in the pharmaceutical industry such as adding effective stabilizers like sugars, polyols, polyethylene glycol (PEG) and other surfactants [13,14]. For example, PEG-GCSF maintained more than 80% of its initial biological activity after 72 h of incubation at 37 °C while native GCSF protein maintained less than 60% of its biological activity under the same conditions [15]. More recently, a fusion system with human serum albumin (HSA) has been proposed as an alternative to pegylation in order to promote protein stability and pharmacokinetics [16,17].

Our previous studies showed that fusing the C-terminal acidic tail of α-synuclein (119–140 residues of α-synuclein, ATS hereafter) could offer an effective means for preventing target proteins from aggregation by heat treatment, agitation, and repetitive freeze-thawing cycles [18,19]. In this study, we also explore this ATS fusion system to be used as an acceptable method to protect hIFNA1 from protein aggregation by environmental stresses.

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[1] *Abbreviations used:* hIFNA1, human interferon α-1; ATS, acidic tail of synuclein; hIFNA1-ATS, hIFNA1 fused with ATS; $EC_{50}$, the half maximal effective concentration.

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## Materials and methods

### *Construction of expression vectors of hIFNA1 and hIFNA1-ATS*

The human interferon α-1 (hIFNA1, NCBI Accession No.NM024013) gene was amplified by polymerase chain reaction (PCR) with forward primer, 5′-*ACGACTTGC*CATATG*TGTGATTCTCCCT*-3′ and reverse primer, 5′-*GTTTCA*GGATCC*TTATTCCTTCCT*-3′ containing an NdeI and BamHI restriction site, respectively, which are indicated in bold and underlined. The amplified PCR product was digested with NdeI and BamHI, gel-purified and cloned into pRSETA vector (Invitrogen, San Diego, CA, USA).

The hIFNA1-ATS fusion construct was produced by consecutively subcloning the PCR product of hIFNA1 which was amplified with primers, 5′-*ACGACTTG*CATATG*TGTGATCTCCT*-3′ and 5′-*ACAGTC*GGATCC*TTCCTTCCTCCTTAA*-3′ containing NdeI and BamHI restriction site (underlined), respectively into the pRSETA vector including gene encoding C-terminal acidic tail of α-synuclein (amino acids 119–140, ATS), as previously described [18].

### *Expression and purification of hIFNA1 and hIFNA1-ATS*

The expression vectors were transformed into the *Escherichia coli* BL21(DE3), (DE3)pLysS, and CodonPlus strains (Stratagene, USA) for expression of the proteins. To produce protein, a seed culture (10 ml) was transferred to 1000 ml of LB broth supplemented with 100 μg/ml ampicillin, and cultivation was carried out at 200 rpm at 37 °C until the optical density (O.D.) at 600 nm reached approximately 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added into the culture broth to be a final concentration of 1 mM for protein induction, and incubation was continued at 37 °C for 6 h. Cells were then harvested and resuspended with 20 mM Tris–HCl buffer, pH 8.0 containing 0.2 mM PMSF, 1 μg/ml leupeptin, and 2 mM EDTA. The suspended cells were disrupted by ultrasonication (ULSSO Hi-Tech Co., Ltd., Korea) on ice and centrifuged at 16,000*g* for 20 min at 37 °C to remove cell debris.

For purification of hIFNA1, the supernatant was applied onto DEAE-Sepharose (GE Healthcare Bio-Sciences AB) pre-equilibrated with 20 mM Tris–HCl, pH 8.0 containing 50 mM NaCl, 2 mM EDTA. After loading the supernatant, the column was washed with 20 mM Tris–HCl, pH 8.0 containing 80 mM NaCl and bound hIFNA1 proteins were eluted with a salt gradient of NaCl (90–200 mM) in 20 mM Tris–HCl pH 8.0. Several fractions containing hIFNA1 protein were collected and concentrated to 1 ml and this concentrated solution was then loaded and purified onto a Sephacryl™ S-200 gel column (1.5 cm/95 cm) with phosphate buffered saline (PBS), pH 7.4. The purification of hIFNA1-ATS protein was performed similarly to hIFNA1, with some minor alterations. Bound hIFNA1-ATS proteins were eluted with salt gradient (200–330 mM of NaCl), and applied to gel-filtration on Sephacryl™ S-200 column for further purification. Purified hIFNA1 and hIFNA1-ATS proteins were confirmed for their identity and purity by SDS–PAGE and MALDI-TOF MS (Matrix-assisted laser desorption ionization time of flight mass spectroscopy).

### *Biological activity of hIFNA1 and hIFNA1-ATS*

The Daudi cell line, a human Burkitt's B cell lymphoma line, was used to evaluate the biological activity of hIFNA1 and hIFNA1-ATS fusion protein, since Daudi cells are highly sensitive to interferon α and cell growth is inhibited by very small quantities of interferon α [4]. Antiproliferative activities of sample proteins were compared by measuring the [$^3$H]thymidine incorporation (cpm) according to the method described previously [16]. Daudi cells were seeded at $10^4$ cells in 100 μl per well in 96-well plates and hIFNA1 and hIFNA1-ATS were added in 100 μl volumes at a concentration of 20 μg/ml to the wells. After incubating the cells for 2 days, 0.5 μCi of [$^3$H]thymidine per well was added, and the cells were further incubated for 6 hours. Cells were harvested and incorporation of [$^3$H]thymidine was analyzed by scintillation counting.

### *Stability test against heat treatment*

The thermal stability of hINFA1 and hINFA1-ATS was investigated qualitatively in liquid by SDS–PAGE and spectrophotometer after heating the protein samples. Protein samples of 1 mg/ml in 20 mM MES (pH 6.0) were heated in a heat block (Seoulin Bioscience Inc., Korea) for 10 min at various temperatures (45 °C, 55 °C, 60 °C, 65 °C, 70 °C) and then cooled at room temperature. The level of heat-induced aggregation was monitored by measuring the apparent absorbance of samples at 405 nm, and the supernatant was analyzed on 15% SDS–PAGE after centrifugation at 15,000 rpm for 10 min.

### *Stability test against agitation*

Agitation was performed by continuous shaking of hIFNA1 and hIFNA1-ATS at 150 rpm on an orbital shaker (Superteck, Seourin Science Inc.) at room temperature as previously described [18]. One milliliter of each protein was prepared at 1 mg/ml in 20 mM MES (pH 6.0) after filtered with a 0.2-μm syringe filter to minimize the induction of aggregation by other impurities. The degree of aggregation was observed at each time point by measuring the turbidity at 405 nm. Each final sample was centrifuged and the supernatant was analyzed through a High Performance Liquid Chromatography (HPLC) column (Bio-Sil®SEC250, Bio-Rad) at 280 nm to examine the state of the solution.

### *Stability test against repeated freezing and thawing cycles*

The stability of hIFNA1 and hIFNA1-ATS against repeated freezing and thawing cycles was compared and investigated using the spectrophotometer and HPLC. Each protein sample at a concentration of 1 mg/ml in 20 mM MES (pH 6.0) was frozen in liquid nitrogen and transferred to a water bath maintained at 37 °C in order to thaw the samples. These freezing and thawing cycles were repeated for a total of 15 times, and aggregation was examined by monitoring at 405 nm every five cycles. Additionally, tested samples were centrifuged to remove aggregates, and the supernatants were investigated for the level of aggregation and the state of protein using an HPLC column at 280 nm.

### *Comparison of solubility for hIFNA1 and hIFNA1-ATS proteins*

The solubility of hIFNA1 and hIFNA1-ATS proteins was determined by concentration analysis. Protein samples were prepared at 1 mg/ml in 20 mM MES, pH 6.0, and the concentration of proteins was determined by the Bradford assay during centrifugation using Vivaspin 20 (10 kDa MWCO, Sartorius-Stedim Biotech, Germany). The centrifugation and quantification of proteins were continued until proteins were no longer concentrated.

## Results

### *Expression and purification of hIFNA1 and hIFNA1-ATS proteins*

Four different *E. coli* strains were used and compared to determine an adequate host for production of hIFNA1 and hIFNA1-ATS fusion protein. The hIFNA1 and ATS-fused proteins were not expressed in *E. coli* BL21 (DE3) and (DE3) plysS strains, while were over-expressed in *E. coli* BL21 CodonPlus (DE3)-RIL and RIPL strains (Fig. 1). The expression of heterologous proteins in *E. coli* is often