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Expression of soluble and active interferon consensus in SUMO fusion expression system in *E. coli*

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ABSTRACT

Protein production can be improved if methods for soluble protein expression are developed. Interferon consensus (IFN-con) is used to treat hepatitis C. IFN-con has superior activity compared to other clinically used interferon α subtypes. However IFN-con is a challenging protein to produce in a soluble form using an *Escherichia coli* expression system.

Here we describe the expression of soluble and active recombinant IFN-con in *E. coli*. The IFN-con gene sequence was optimised for expression in *E. coli*, which was then cloned into the Champion™ pET SUMO expression vector downstream of the SUMO fusion protein and under strong T7*lac* promoter. The SUMO-IFN-con fusion protein was efficiently expressed using the SHuffle™ *E. coli* strain and existed in soluble form as 86–88% of the total IFN-con. After removal of the SUMO fusion partner, approximately 50 mg of recombinant IFN-con of at least 98% purity (by RP-HPLC) was obtained from a 1 L fermentation culture. Using an A549/EMCV antiviral assay, the specific activity of the recombinant IFN-con was determined to be 960×10^6 IU/mg as calculated to NIBSC standard for IFN-con (3×10^5 pfu/mL virus titre). Comparison of the antiviral activity of the produced IFN-con to IFN α-2a showed that IFN-con displays 2.8 times greater activity, which is in good agreement with what has been reported in the literature for pure protein. IFN-con expression in a soluble form from *E. coli* allowed us to use a simple, two-step purification process to yield highly pure and active IFN-con which is more efficient than obtaining IFN-con from inclusion bodies.

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Introduction

Discovered more than 50 years ago, the interferons (IFNs)[1] are naturally occurring proteins that enhance innate and acquired immune responses [1,2]. The IFNs are an important class of therapeutic proteins that are effective against a wide range of conditions including viral infections, malignancies and multiple sclerosis [3]. The therapeutic potential of IFNs has resulted in the approval of numerous important medicines such as IFN α-2a (Roferon A®) and IFN α-2b (Intron A®) which have now been largely superseded by PEGylated forms of these proteins (Pegasys® and PEG-Intron®) [4,5]. These IFN α-2 based medicines are used as first line treatment of hepatitis C and also are important in treatment regimens for hepatitis B and leukemia [6,7].

In addition to endogenous IFNs, a non-natural recombinant interferon consensus (IFN-con) was designed by scanning the sequence of several natural IFN-α subtypes and assigning the most frequently observed amino acids in each corresponding position [8]. As with most IFN-α subtypes, IFN-con consists of 166 amino acids and contains two disulfide bonds at Cys 1-Cys 99 and Cys 29-Cys 139 [9]. The biological activities of IFN-con have been shown to be significantly greater than IFN α-2a and IFN α-2b in antiproliferative [5], antiviral [10] and NK cellular induction assays [11]. The greater potency and biological profile of IFN-con [12] led to the development of Infergen®, a marketed IFN-con that is used to treat a subgroups of hepatitis C patients who did not respond or relapsed to the standard IFN α-2 based treatments [13–15].

Despite the superior potency of IFN-con, production of this therapeutically promising protein remains challenging [16,17]. Infergen® is produced in *Escherichia coli* as an insoluble protein that must be refolded prior to final purification in a series of chromatography steps (Valeant Pharmaceuticals, Inc). Recently, other researchers have expressed IFN-con in *E. coli* as insoluble inclusion bodies, which required multiple steps to isolate, wash

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[1] *Abbreviations used:* IFNs, interferons; EMCV, encephalomyocarditis virus; DMEM, dubelcco's modified eagle's medium; IFN-con, interferon consensus; FBS, foetal bovine serum; IMAC, immobilised metal affinity chromatography.

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and solubilise the inclusion bodies before dilution refolding and chromatographic purification to yield active IFN-con [16,17]. Although a titre of 70 mg/L for the protein as inclusion bodies was obtained, there are losses associated with the processing necessary to obtain active IFN-con and the activity of the IFN-con was only 1.7 times that of IFN α-2a. Development of an effective method to express soluble and active IFN-con may significantly simplify its production and facilitate further investigation of the therapeutic potential of this protein.

Expression of protein via inclusion bodies is challenging as partially mis-folded protein increases the propensity for aggregation, which is considered a risk factor for increased immunogenicity [18,19]. Production of therapeutic proteins in a soluble and biologically functional form is desired for safety reasons and can significantly simplify downstream purification processes.

There has been much effort spent to develop methods to improve soluble protein production from *E. coli* [20–24]. Production of a desired protein as a fusion protein in *E. coli* is a common approach to increase the efficiency of expression of soluble protein and to simplify its purification. Widely used fusion partners include MBP [25], GST [24,26], NusA [27] and SUMO [28,29]. The genetic fusion of a target protein with another protein as a fusion partner may provide a more stable construct and significantly improve the solubility of the target protein, and has been demonstrated to be a useful strategy to advance research for otherwise difficult to produce proteins [26,30]. Judicious selection of a fusion protein is necessary because the cleavage step can leave residual amino acids fused with the protein of interest. In our study, it was essential to identify a system which would not leave any residual amino acids fused to the protein and would allow the production of only IFN-con.

Since IFN-con is more biologically active than other clinically used IFN-α subtypes, there is an opportunity to improve interferon-based therapies. To address this opportunity, we focused on developing a method to express IFN-con in a soluble and active form using the SUMO fusion expression system in *E. coli*. Herein we describe the optimisation of expression conditions and purification of the SUMO-IFN-con construct with N-terminal His-tag, enzymatic digestion to release SUMO fusion partner, purification of IFN-con and the biological evaluation of the purified IFN-con.

Materials and methods

Materials

Champion™ pET SUMO® Expression System (K30001), Platinum® *Taq* DNA Polymerase High Fidelity (10966018), One Shot® TOP10 Chemically Competent *E. coli* (C404003) and SUMO® protease (12588018) were purchased from Invitrogen. SYBR® Safe DNA gel stain (S33111) was obtained from Invitrogen. PCR Cleaning Kit (NA1020) was resourced from Sigma, QIAprep Spin Miniprep Kit (27104) and QIAquick Gel Extraction Kit (28704) was purchased from Qiagen. SHuffle® T7 Chemically competent *E. coli* (C3029H) was bought from New England Biolab. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Sigma–Aldrich (I6758-5G), Phosphate buffer saline (PBS), 10x solution, 20 L was purchased from Fisher (BP399-20). Lysosyme from chicken egg white (027K14051), DNAse 1 (DN25-100MG) and protease inhibitor-EDTA (P8849) were obtained from Sigma–Aldrich. Triton X-100 was obtained from Fisher (BP151-500). HisTrap™ FF (5 mL) column (17-5255-01) was purchased from GE Healthcare and operated on an ÄKTA Purifier™ system. PD-10 Desalting column (GZ17-0851-01) was purchased from GE Healthcare. Vivaspin 20 centrifugal concentrators with 10,000 MWCO (Polyethersulfone) membranes (VS1511) were purchased from Generon. LB broth (L3152) and Yeast Extract (92144) was ordered from Sigma–Aldrich. Tryptone (12787099) and kanamycin sulphate (BPE906-5) was ordered from Fisher BioReagents. Glass shake flask with 4 baffles and fluted neck borosilicate 2 L, (FB55907) and 250 mL (FB55901) were ordered from Fisher. Bacterial growth was conducted in CERTOMAT® BS-1 Incubation-Shaking Cabinet BBI-8865027 from Sartorius. SDS–PAGE was conducted with XCell Surelock™ Mini-Cell (EI0001), NuPAGE® 4–12% Bis-Tris gels MES running buffer (NP000202) were obtained from Invitrogen. Novex® Sharp protein markers (LC5800) from Invitrogen was used as molecular weight standards. The gels were stained with InstantBlue™ (ISB1LUK) from Expedeon. C8 column (5 μm, 15 cm × 4.6 mm) was purchased from Discovery®. A Jasco HPLC instrument running EZChrom software was used for analysis of purified protein. A549 cell lines were obtained from HPACC (86012804). Cells were incubated at 37 °C, 5% CO_2. Encephalomyocarditis virus (EMCV) (VR-129B) was obtained from LGC Standards. Methyl violet 2B was sourced from Sigma–Aldrich (198099). Microplate, 96F, sterile, pack of 50 from Fisher (TKT-180-070U). Foetal bovine serum (FBS) was purchased from Gibco (Fisher Scientific, VX25030024). Dubelcco's Modified Eagle's Medium (DMEM) was obtained from Gibco (Fisher Scientific, VX21969035). NIBSC IFN α-2a standard (95/650) and NIBSC IFN-con (94/786) standards were purchased from NIBSC. Opsys MR™ 96-well microplate reader (Dynex Technologies) was used for measuring absorbance in microtiter plates. ImageQuant™ LAS 4010 system was purchased from GE Healthcare. MS analysis was conducted on the Applied Biosystem Voyager System DE PRO MALDI-TOF mass spectrometer with nitrogen. The mass spectrometer was calibrated with the BSA calibration standard kit from AB Sciex.

Methods

IFN-con and IFN α-2a gene optimisation

The protein sequence of IFN-con [31] was recoded to optimise the DNA sequence for expression in an *E. coli* system. The DNA sequence was firstly optimised with GenePerfect software (Gene Oracle Inc.). The gene sequence was computationally designed by considering codon optimisation and GC content adaptation, elimination of mRNA destabilising motifs, secondary structures and secondary ORFs. The re-designed gene was synthesised at Gene Oracle Inc. and cloned in a pGOv4 vector and used for the pET SUMO expression vector construction. For the comparative biological studies, IFN α-2a was also produced using procedure identical to method described for production of IFN-con. The IFN α-2a sequence was retrieved from the Protein Data Bank (accession number P01563) and gene sequence was optimised as described for IFN-con.

Construction of recombinant pET SUMO-IFN-con and pET SUMO-IFN α-2a expression vectors

The IFN-con and IFN α-2a genes were cloned into the pET SUMO vector (Invitrogen). The IFN-con gene was amplified using a forward primer of 5′-TGCGACTTGCCTCAGACTCATAGTC-3′ and a reverse primer of 5′-TTATTATTCTTTACGGCGCAGACGCTCCTG-3′, while IFN α-2a gene was amplified using a forward primer of 5′-TGCGACCTGCCTCAAACACACAGTTTG-3′ and a reverse primer of 5′-TTATTACTCTTTCGAACGCAGAGATTCCTG-3′. All primers were designed using Translate tool accessed at the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/translate/) and synthetized at Invitrogen. Platinum® *Taq* DNA Polymerase High Fidelity was used for the PCR amplification. The PCR programme consisted of a DNA denaturation step at 95 °C for 3 min followed by 25 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1.5 min and a final elongation step at 72 °C for 10 min. The PCR reaction was analysed on 1% agarose gel stained with SYBR® Safe DNA gel stain and the electrophoresis was performed in 1× TAE buffer, at 190 V run for

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