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PRODUCTION OF FUNCTIONAL RECOMBINANT HUMAN INTERFERON-GAMMA BY RTX CPD-FUSION TECHNOLOGY

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Abstract

Human interferon-gamma (hIFNγ) is an important pleiotropic cytokine with therapeutic application. Recombinant hIFNγ has been shown to be an effective pharmaceutical against a wide range of viral, immuno-suppressive diseases with promising prospects in cancer immunotherapy resulting in a strong increase in demand and price. The aim of this study was to develop an efficient method for production of soluble and active hIFNγ. To this end a fusion gene encoding hIFNγ and a C-terminally located His_{10}-tagged RTX CPD was constructed by two-step PCR. The fusion gene was cloned in an expression vector under inducible promoter and expressed in different *E. coli* strains such as Rosetta (appropriate for expression of eukaryotic genes containing rare codons), and two chaperone containing strains – BL21(DE3)/pG-KJE8 and BL21(DE3)/pG-Tf2. The transformed bacteria were cultivated at 24 °C to favour proper folding of the recombinant protein. Under these conditions the strain BL21(DE3)/pG-KJE8 was chosen as more suitable for production of soluble hIFNγ. The latter was purified by Ni^{2+}-based affinity chromatography, the peak fractions were subjected to affinity chromatography combined with a direct on-column digestion to release hIFNγ, which was further purified by ion exchange chromatography. It is worth mentioning that this strategy, employing

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auto-cleaving and solubility-enhancing CPD tag, combined with on-column tag digestion was applied for the first time for production of hIFNγ.

Key words: human interferon-gamma, RTX CPD-fusion tag, recombinant expression

Introduction. Research and industrial needs for recombinant proteins continue to increase over time for their broad applications in structural and functional studies and as therapeutic agents. These applications often require large quantities of recombinant protein with desirable purity and biological activity. *E. coli* is the most widely used host for production of low cost recombinant proteins. Common pitfalls related to this expression system are protein stability, solubility, toxicity, etc. A novel approach aiming to overcome these obstacles is the use of affinity tags designed to facilitate protein purification by affinity chromatography ([1] and references therein). Since the tags may interfere with the properties of the target protein, various strategies have been developed for their removal during or after the purification step [2].

Among others, an efficient self-cleavage C-terminal fusion system was developed using the inositol hexakisphosphate-inducible *Vibrio cholera* MARTX toxin cysteine protease domain (CPD) [3,4]. Multifunctional-autoprocessing Repeats-in-Toxins (MARTX) toxins are large bacterial secretion proteins. The RTX CPD mediates autoproteolytic activation of the secreted protoxin upon entry into the eukaryotic cytosol. The autoprocessing is efficiently activated by the eukaryote specific small molecule inositol hexakisphosphate ($InsP_6$) [5]. Since bacteria do not utilize inositol signalling, the CPD is purified from *E. coli* as an uncleaved protein [6]. Subsequently, the CPD-tag is removed by autoprocessing induced by incubation with $InsP_6$ resulting in recovery of the protein of interest. Besides the advantage of the autoprocessing feature, the addition of the CPD-tag can in some cases function as a C-terminal solubility tag, improving recovery of soluble protein during overexpression [7]. Another advantage of CPD is that it is highly specific and attacks the fusion protein immediately after Leu, which is located before the first amino acid of the fusion tag [8].

Here we report the construction of a new expression vector and a new approach for soluble expression and purification of human interferon-gamma (hIFNγ) in *E. coli* BL21(DE3)/pG-KJE8 strain.

Materials and methods. ***Cloning of expression vectors.*** All enzymes and kits were purchased from Qiagen and New England Biolabs. Codon optimized synthetic genes encoding shortened version of hIFNγ (135 aa) was synthesized by Life Technologies. All primers were synthesized by Eurofins Genomics, Germany.

Expression vectors were obtained by two-step PCR. The first step aimed to generate a fragment *Nco*I-IFN(24-158)-CPD(GKIL) bearing *Nco*I restriction site, hIFNγ encoding sequence, and a sequence encoding the first 4 N-terminal amino acids of the CPD (GKIL). The fusion gene was amplified using a for-

ward primer IFN-MG-24-Nco (5′-atcgccatgggtcaggacccctacgtgaag-3′), a reverse primer IFN-158-CPD (3′-gcagaattttaccggatcccagcatctgggaacgcttgcgc-5′) and the vector pET28a(+) as a template. Amplified DNA was purified with PCR Cleaning Kit. The second step of the PCR aimed to generate the fragment *Nco*I-MG-IFN(135)-GS-CPD(207)-LE-H10-*Xho*I. In this step, the fragment *Nco*I-MG-IFN(24-158)-CPD(GSGKIL) (see above) was used as a forward primer and the primer CPD3650-H10 (5′-aattctcgagttagtgatgatgatg-3′) as a reverse. The PCR product was digested with *Xho*I and *Nco*I and ligated to the linearized vector pET28a(+). TOP10F' *E. coli* cells were used for transformation. Positive clones were selected by kanamycin resistance followed by Colony PCR. Plasmid DNA was isolated and sequenced (Eurofins Genomics, Germany).

Small-scale expression of C-terminal tagged recombinant hIFNγ. The expression vector pET28a/IFNγ-CPD-His_{10} was transformed into competent *E. coli* cells from 3 different strains: 1) BL21(DE3)/pG-KJE8, 2) BL21(DE3)/pG-Tf2 and 3) Rosetta. Aliquots of 2.5 ml from 5 ml night pre-cultures were used to inoculate flasks containing 100 ml of LB medium supplemented with kanamycin (70 µg/ml) and chloramphenicol (34 µg/ml) and appropriate chaperone inductors (5 ng/ml tetracycline for strain 1) and 1 mg/ml L-arabinose for both strains 1) and 2). Bacteria were cultured at 37 °C and at $OD_{600} = 0.6$ the recombinant gene expression was induced with 0.1 mM IPTG for 16 h at 18–24 °C. The cells were harvested by centrifugation and the pellet was resuspended in 10 ml lysis buffer for His-tag affinity purification supplemented with 1 mM PMSF and 1.5 mg/ml lysozyme and incubated on ice for 30 min. The lysed cells were sonicated and 4 ml of the lysate were centrifuged at 15 000 rpm for 20 min. The supernatants were stored on ice, and the pellet was resuspended in 6 M urea. The soluble protein was purified by Ni^{2+}-NTA Spin Column (IMAC). The eluate was treated with 100 µM inositol hexakisphosphate ($InsP_6$) for 30 min. The target protein expression level and the distribution of the protein between the soluble and insoluble fractions were analysed by SDS-PAGE. The protein concentration was determined by the method of Bradford.

Soluble expression and up-scale purification of IFNγ-CPD-His_{10} fusion protein. The recombinant plasmid pET28a/IFNγ-CPD-His_{10} was transformed into the bacterial strain BL21(DE3)/pG-KJE8. Batch cultures were prepared in LB medium supplemented with 70 µg/ml kanamycin, 34 µg/ml chloramphenicol, L-arabinose (1 mg/ml) and tetracycline (5 ng/ml). Bacteria were grown at 37 °C and the target protein expression was induced with IPTG (0.1 mM) at $OD_{600} = 0.6$–0.7. The cells were grown for 16 h at 24 °C. The biomass was collected by centrifugation. The bacterial pellet was washed with PBS and resuspended in 80 ml of buffer A (PBS, pH 7.4, 40 mM imidazole, pH 8.0, 5% glycerol and 25 mM ammonium acetate, pH 6.9) supplemented with lysozyme (1 mg/ml), DNase (10 µg/ml) and 4 "Complete, EDTA-free Protease Inhibitor Cocktail Tablets" (Roche Applied Science). After incubation on ice for 30 min

the cells were sonicated and the lysates were centrifuged at 15000 × g for 40 min at 4 °C. The supernatant was loaded on 10 ml prepacked Nickel Sepharose High Performance HisTrap column equilibrated with buffer A. The chromatography was performed on a ÄKTA™ purifier at flow rate of 4 ml/min. Proteins were eluted with a gradient of 40–500 mM imidazole, pH 8.0 in PBS, pH 7.4 containing 5% glycerol and 25 mM ammonium acetate, pH 6.9. Fractions were analysed by SDS-PAGE and those containing IFNγ-CPD-His were combined.

On-column digestion of CPD-His10 tag, up-scale purification and determination of hIFNγ biological activity. The pooled fraction after the first purification step was dialysed against buffer containing 20 mM HEPES, pH 8.0 and 40 mM NaCl and 40 mM imidazole at 4 °C for 12 h and loaded on 10 ml prepacked Nickel Sepharose High Performance HisTrap column equilibrated with the same buffer. In order to induce the auto processing of the CPD, 100 µM InsP6 was loaded on the column for 30 min. Using a slow flow rate the cleaved hIFNγ was collected in the flow-through using three column volumes of the loading buffer. The fraction was further purified by ion-exchange chromatography on Resource S column pre-equilibrated with buffer containing 40 mM NaCl in 20 mM HEPES, pH 8.0. The bound protein was eluted by 0.04–1 M NaCl gradient in 20 mM HEPES, pH 8.0. The complete removal of the CPD-His_{10} tag and the amino acid sequence of the obtained hIFNγ was verified (TOPLAB GmbH, Germany) and the protein concentration was determined by the method of Bradford.

The antiproliferative activity was determined by a modified kynurenine bioassay on WISH cells as described earlier [9].

Results. ***Cloning of hIFNγ having the C-terminal CPD protease in an inducible expression vector.*** The hIFNγ fusion protein having C-terminal CPD protease followed by affinity histidine decapeptide (His_{10}) was obtained by two-step PCR. After the first step, a fragment containing *Nco*I restriction site, the entire nucleotide sequence encoding hIFNγ and the N-terminus of the RTX protease was obtained. It was purified and used as a 5' primer in the second PCR, through which the *Xho*I restriction site, the nucleotide sequence encoding the RTX protease and the affinity histidine polypeptide (His_{10}) were introduced. The resulting expression vectors were transformed into competent *E. coli* TOP10F' cells and colony PCR was performed with 10 randomly selected colonies. Positive clones were further confirmed by hydrolysis with *Xho*I and *Nco*I.

The DNA fragments thus obtained encode the complete amino acid sequence of the fusion protein (352 amino acids with size of 41 kDa). They were cloned into an inducible expression vector pET28a(+) and their nucleotide sequence was verified by DNA sequencing. Figure 1 shows the amino acid sequence of hIFNγ-RTX-His_{10} fusion protein where hIFNγ is C-terminally shortened by 8 amino acids (comprises 135 amino acids instead of the full length of 143 amino acids), which is one of the most prevalent biologically active forms of the cytokine in the human blood [10].

MGQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKI
MQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDD
FEKLTNYSVTDLNVQRKAIHELIQVMAELSPAAKTGKRKRSQMLGS*G*
KILHNQNVNSWGPITVTPTTDGGETRFDGQIIVQMENDPVVAKAAANLAGKHA
ESSVVVQLDSDGNYRVVYGDPSKLDGKLRWQLVGHGRDHSETNNTRLSGYSAD
ELAVKLAKFQQSFNQAENINNKPDHISIVGCSLVSDDKQKGFGHQFINAMDANG
*LRVDVSVRSSELAVDEAGRKHTKDANGDWVQKAENNKVSLSWDAQG*LEHHHH
HHHHHH

Fig. 1. Amino acid sequence of the hybrid protein composed of **hIFNγ,** *CPD protease* and His_{10}-histidine decapeptide

Small-scale expression of the C-terminally tagged recombinant hIFNγ. Small-scale expressions were performed as an intermediate step to optimize the conditions for large scale expression of the soluble fusion protein. The expression vector pET28a/hIFNγ-CPD-His_{10} was transformed into three *E. coli* strains: BL21(DE3)/pG-KJE8, BL21(DE3)/pG-Tf2 (both chaperone expressing strains) and Rosetta (appropriate for expression of eukaryotic genes containing rare codons). After testing the efficiency of two different expression temperatures – 24 °C for the first two strains and 18 °C for Rosetta strain, the strain BL21(DE3)/pG-KJE8 was selected for up-scale expression (Fig. 2A). The BL21(DE3)/pG-Tf2 strain showed very low level of soluble expression (Fig. 2B). Although the expression in Rosetta strain led to higher yield of the fusion protein and more effective cleavage of the CPD-His_{10} tag, compared to the first strain (Fig. 2C), the removal of the fusion tag resulted in high propensity for aggregation of the target protein.

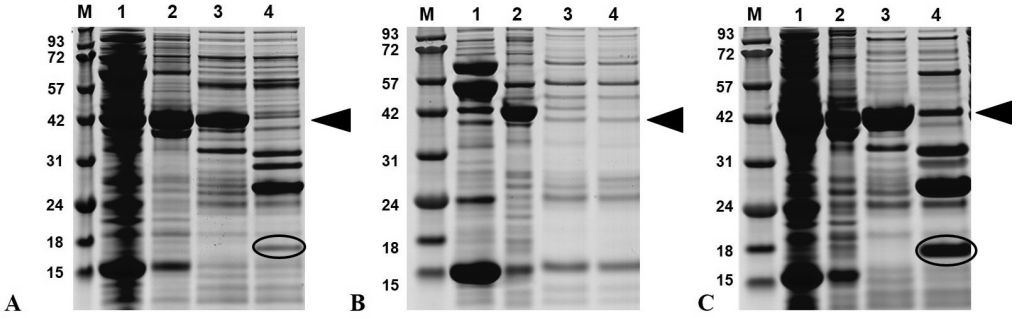

Fig. 2. **SDS-PAGE analysis of cell lysates after small-scale expression of hIFNγ-CPD-His10 in *E. coli* BL21(DE3)/ KJE8 (A), BL21(DE3)/pG-Tf2 (B) and RosettaTM (C) strains 1** – supernatant; **2** – pellet resuspended in 6 M urea; **3** – eluate; **4** – cleaved target protein, after autoprocessing of the CPD-His10. **M**: protein molecular weight markers in kDa. The arrow indicates the position of the fusion protein (∼ 41 kDa). The circled band shows the position of the cleaved target protein (∼ 17 kDa)

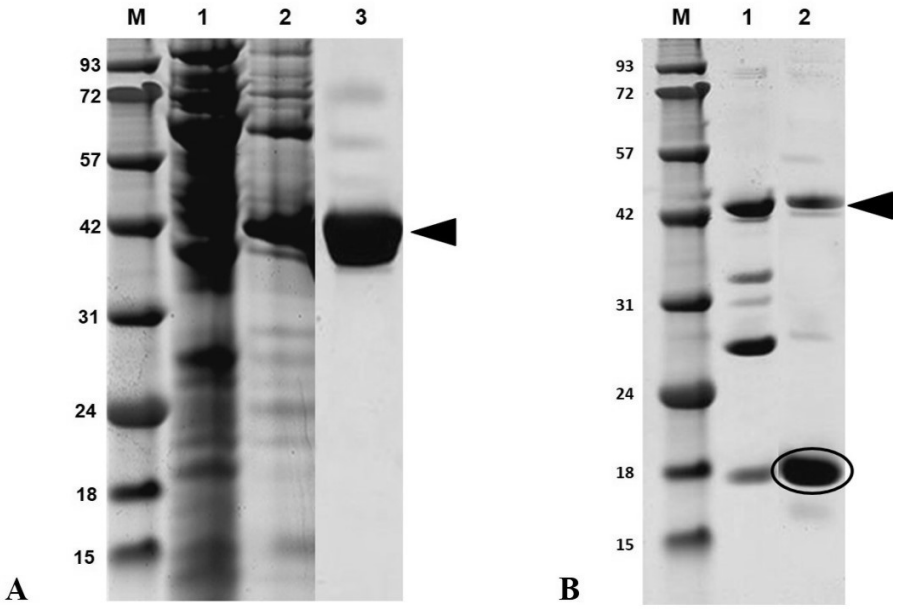

Fig. 3. **SDS-PAGE analysis of hIFNγ expression and purification. A) hIFNγ-CPD-His10 after IMAC chromatography. 1** – cell lysate before induction of gene expression; **2** – cell lysate after induction of the target protein expression; **3** – fraction after IMAC purification; **B) hIFNγ after activation of CDP-His10 autoprocessing. 1** – digested fusion protein loaded onto Resource S column; **2** – purified target protein after Resource S column. **M**: protein molecular weight markers in kDa. The arrow indicates the position of the target fusion protein ($\sim$ 41 kDa). The circled band shows the position of the cleaved target protein ($\sim$ 17 kDa)

Scaled up soluble expression and purification of hIFNγ-CPD-His$_{10}$ fusion protein. Cells from *E. coli* strain BL21(DE3)/pG-KJE8 (expressing both *E. coli* chaperon systems) were transformed and cultivated at 24 °C to favour proper folding of the recombinant fusion protein hIFNγ-CPD-His$_{10}$. The crude cell lysate was subjected to Ni^{2+}-NTA-affinity chromatography, the fusion protein was eluted with satisfactory purity (Fig. 3A) and was subjected to dialysis in order to reduce the imidazole concentration.

On-column digestion of CPD-His$_{10}$ tag and up-scale purification of hIFNγ. The dialyzed sample was loaded onto the pre-equilibrated His-Trap column, followed by loading of 100 µM $InsP_6$ to activate the autoprocessing of the CPD-His$_{10}$ tag. Following 30 min incubation, the cleaved target protein was collected with the flow-through fraction and further purified by cation exchange chromatography on Resource S column (Fig. 3B). The hIFNγ was identified by peptide MS fingerprinting, which showed that the C-terminus corresponded to that of its natural prototype. This indicated that the CPD-His$_{10}$ tag was removed completely. The yield of hIFNγ with a purity of 85% was 7 mg per gram wet cell mass.

Biological activity of hIFNγ. The antiproliferative activity of the hIFNγ produced and purified by the new methodology was measured by a modified kynurenine bioassay [9]. Our results showed that the hIFNγ specific activity was as high as 8×10^6 IU/mg protein. The specific biological activity of highly purified hIFNγ is $2–5\times10^7$ IU/mg [11]. The observed here slightly lower specific activity is due to the presence of protein impurities in the preparation that increase the mass of the total protein.

Discussion. The therapeutic significance of hIFNγ has challenged scientists for years to develop and optimize methods for its production and purification. When expressed in *E. coli*, hIFNγ tends to aggregate in inclusion bodies where the cytokine is insoluble and biologically inactive. Common shortcomings of the standard purification methods based on inclusion bodies includes low protein yield, necessity of denaturation and refolding of the target protein and insufficient stability in water solutions. Gene fusion technologies have been developed to prevent formation of inclusion bodies and therefore to improve purification of the recombinant proteins. We have previously developed a successful method for expression and purification of soluble and biologically active hIFNγ using SUMO fusion methodology with yield similar to the one reported here (6–7 mg per gram wet cell mass) [12]. Since the SUMO enzyme necessary for tag-removal is very costly, we developed a system in which the self-cleaving domain CPD of the RTX protease is used as fusion partner. This methodology has the advantage to be versatile, inexpensive and utilizes highly specific, inducible, self-cleaving protease tag that cleaves exclusively at the fusion protein junction. In addition, we have constructed the hIFNγ expression vector in a way that leaves the cytokine with native C-terminus (3'-end truncated gene encoding for 135 amino acids with Leu at the C-terminus) that bears no foreign amino acids after the tag removal. Besides the novelty in plasmid construction and optimization of the expression conditions, we were able also to improve the protease cleaving conditions.

The CPD fusion methodology was used for expression and purification of a variety of recombinant proteins [3, 7, 13] and antimicrobial peptides 14, 15 where the auto-cleavage was performed either in solution or on Ni^{2+}-beads that led to vastly lower yields varying from several micrograms to 35 mg per Litre culture broth. Tellez and Castaño-Osoriov [15] reported unsuccessful on-column digestion of Lucilin peptide most probably due to aggregation. In our case the on-column digestion resulted in minimal protein loss and high yield (7 mg per gram wet cell mass) of soluble and biologically active hIFNγ. Of course, there is further room for optimisation of the purification protocol in order to obtain a purity of 95–98% of the final product.

Conclusions. Here we report the development of a method for inducible expression and purification of soluble hIFNγ using auto-cleaving CPD-deca-histidine tag as a fusion partner. The self-cleaving protease RTX CPD is activated by incubation with InsP6 and cleaves exactly at the site of junction. The expression, pro-

tease cleaving conditions and purification protocol are optimised to achieve high purity and yield of the target protein. The method is inexpensive, straightforward and can be used for production of other recombinant proteins with therapeutic application.

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