Biochemical and in silico evaluation of recombinant *E. coli* aminopeptidase and in vitro processed human interferon α-2b

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Abstract: *Escherichia coli* is an extensively used host for the production of recombinant proteins, making its N-terminal methionine aminopeptidase (MAP) an attractive candidate for studies on posttranslational protein processing. The present study describes the recombinant production and properties of MAP from the DH5α strain of *E. coli*. The soluble and active enzyme was produced in *E. coli* BL21 (DE3) RIL - codon plus cells under a T7 promoter system and purified by anion-exchange chromatography. It exhibited a molecular weight of 29,200.94 Da by MALDI-TOF analysis. The purified enzyme showed specific activity of 1.64 U/mg with methionyl-p-nitroanilide and 1.51 U/mg with synthetic tetrapeptide substrate ‘MGMM’ in a discontinuous HPLC-based assay. In vitro studies showed the processing of up to 36% of Met-INFα-2b in 40 min. In silico studies revealed that the ES-complex formation between the enzyme and interferon has a ΔG –683.07 kJ/mol. Molecular docking results showed that the processed INFα-2b has greater binding affinity with IFNAR2 receptor as indicated by ΔG –784.53 kJ/mol, significantly lower than that of methionine containing INFα-2b (ΔG –717.63 kJ/mol). These findings emphasize the functional superiority or better efficacy of N-terminal methionine processed recombinant interferon.

Key words: Methionine aminopeptidase, *E. coli*, human interferon, in vitro, in silico

1. Introduction

The production of recombinant proteins and their therapeutic applications have brought a revolution in biomedical sciences. Recombinants of many proteins including lymphokines, interferons and interleukins (Rider et al., 2016; Shepelkova et al., 2016; Lagasse et al., 2017), enzymes (Seal, 2013; Vairo et al., 2013; Dolores and Ortiz, 2016), and hormones like insulin and human growth hormone (Urbano et al., 2012; Miljic et al., 2013) have been used in recent times. In the *E. coli* expression system, a proportion of recombinant proteins retain an unprocessed methionine at the N-terminus, which is contrary to their native forms. Attempts have been made to make replicas of native proteins for therapeutic applications (Goh et al., 2017; Wingfield, 2017). Methionine aminopeptidase (MAP) is a metalloprotease that catalyzes the hydrolytic removal of N-terminal methionine of an elongating polypeptide chain during the process of protein synthesis (Giglione et al., 2004; Arif et al., 2015). The hydrolysis of N-terminal methionine is mandatory in 50%–70% of nascent proteins (Bingel-Erlenmeyer et al., 2008; Kanodia et al., 2011) and usually occurs only when the penultimate amino acid, which is adjacent to the initiator methionine, has a radius of gyration of 1.29 Å or less. These amino acids are glycine, alanine, proline, serine, cysteine, threonine, and valine (Xiao et al., 2010). The methionine aminopeptidases belonging to different origins have similar active sites and mechanism of action but differ in individual active site residues, which may possibly alter the behavior of the enzyme towards different substrates and inhibitors (Lowther et al., 1999; Helgren et al., 2016). Recombinant therapeutic proteins are converted to their native form by removal of N-terminal methionine residue (Arif et al., 2015; Calcagno and Klein, 2016). Because of its easy manipulation, cultivation, high yield, and better economy, the *E. coli* strains include the most extensively used protein expression systems (Elleuche et al., 2015; Cantu-Bustos et al, 2016; Chen et al., 2016; Jia and Jeon, 2016). Once produced in *E. coli*, activity of aminopeptidase is one of many decisive factors for a recombinant protein to exhibit a replica of its native form (Tripathi, 2016).

In silico 3-D structure determination and molecular docking techniques are increasingly used tools and techniques to study the binding specificity of hormones.
and substrates against their corresponding receptors and enzymes (Venkatachalam et al., 2003). During the last two decades, about 60 docking software programs and tools have been developed for commercial and academic applications (Allen et al., 2015; Yang and Zhang, 2015). The present study describes the recombinant production, purification, and biochemical properties of E. coli N-terminal methionine aminopeptidase, and application of enzyme in the processing of recombinant interferon. We have also applied in silico tools to evaluate the interaction of met-INF with E. coli MAP, binding free energy changes of met-INF, and nonmet-INF molecules with the interferon receptors.

2. Materials and methods

2.1. Preparation of recombinant plasmid

The E. coli (DH5α) methionine aminopeptidase gene was PCR amplified, using primer sequences 5’-catatggctatccaatagccccc-3’ and 5’-tatttgcgtgcagaggatatgcc-3’ under optimized conditions (2 mM MgCl₂, 1.5 mM dNTPs, Taq buffer solution, 20 pM of each primer, 2.5 units Taq polymerase). The complete open reading frame encoding methionine aminopeptidase (MAP) was ligated into pTZ57R/T (Thermo Fisher Scientific catalogue number: K1213) by T/A cloning. The gene consisting of 795 base pairs was restricted out from the plasmid above by NdeI and EcoRI and ligated into pET21a (+) expression plasmid (Novagen catalogue number: 69770-3) using the procedure described by our previous studies with modifications (Arif et al., 2016).

2.2. Cloning and expression of the MAP gene

The recombinant plasmid pET21-MAP (expression plasmid ligated with gene encoding enzyme) was used for the transformation of E. coli BL21 (DE3) - RIL codon plus cells (Agilent Technologies catalogue number 230240). Clones confirmed for the presence of recombinant plasmid were used to grow the culture on a small scale; gene expression was induced by 0.4 mM IPTG when the OD of culture was 0.6 at 600 nm. The culture was incubated overnight at 20 °C and expression was analyzed on 15% SDS-PAGE (Laemmli, 1970).

2.3. Purification and molecular weight determination

The cell pellet (4.3 g) obtained from 1 L of E. coli culture induced for protein expression was suspended in 50 mL of 50 mM phosphate buffer pH 7.5 and sonicated for 1 min (6 pulses of 10 s at moderate power). The sonicated sample was centrifuged at 14,000 × g for 15 min at 4 °C and the supernatant was transferred to a sterile falcon tube followed by filtration using a 0.45 μm pore size filter (Millipore Inc.). The enzyme was purified by dialysis and anion-exchange chromatography using Q-Sepharose equilibrated with 40 mM HEPES buffer pH 7.5. The sample was applied to the column at a flow rate of 2 mL/min, unbound proteins were washed with the equilibration buffer, and bound proteins were eluted with a linear gradient of 0.5 M NaCl solution. Fractions of 5 mL were collected, quantified, and analyzed separately. The entire purification process was conducted at 4 °C and protein was quantified by Bradford's method (Bradford, 1976). A desalted purified enzyme sample (2.7 mg) was mixed with 4.5 mg of sinapinic acid in 1 mL of 30% acetonitrile containing 0.15% trifluoroacetic acid, and a small aliquot of the mixture was spotted on a mass spectrometric plate and allowed to air dry. The mass spectrum of the enzyme was recorded with a Bruker Autoflex MALDI-TOF (Bruker Daltonics Inc., Billerica, MA, USA).

2.4. Methionyl p-nitroanilide-based assay

A modified method adopted from the literature was applied for the enzyme assay (Zhou et al., 2000). Methionine aminopeptidase catalyzes the conversion of methionyl p-nitroanilide to p-nitroaniline and methionine. p-Nitroaniline absorbs light at 405 nm. To the experimental and reference cuvette were added 1440 µL of 40 mM HEPES buffer pH 7.5, 400 µL of 1 mM KCl, 60 µL of 50 mM CoCl₂, and 3 µL of substrate (100 mM methionine p-nitroanilide in ethanol). To the experimental cuvette was added 75 µL (97.5 µg) of enzyme and to the reference cuvette 75 µL of buffer. The change in absorbance at 405 nm was measured over 30 min. The extinction coefficient of absorbing species (p-nitroaniline) was considered as 9.96 mM⁻¹ cm⁻¹ for the calculation of enzyme activity. The assay was performed for the determination of optimum pH and temperature for enzyme.

2.5. HPLC-based enzyme assay using MGMM as substrate

The assay method described by Xiao et al. (2010) was applied with modifications to measure the enzyme activity. The retention time and volume for the synthetic tetrapeptide MGMM and its product glycine-methionine-methionine GMM were separately determined. Solutions containing 10 µg of MGMM and GMM were separately taken in Eppendorf tubes, diluted to 20 µL with filtered distilled water, and loaded on an analytical C8 chromatographic column. The retention volume was determined with 1.0 mL/min flow rate, 0% to 30% linear gradient of acetonitrile in 0.1% TFA water with 1% ramp per min. The tri- and tetrapeptide eluted at 16% and 23% of acetonitrile, respectively. The retention time of both MGMM and GMM provided a baseline for the enzyme assays. For the enzyme assay, 175 mM KCl, 50 mM CoCl₂, 20 µL of enzyme 1.3 µg/µL, and 25 mM HEPES buffer 60 µL were taken and incubated at 30 °C for 10 min so that the reaction mixture attained the temperature (solution A). In another Eppendorf tube, 1.9 mM substrate and 92 µL of 25 mM HEPES pH 7.5 were added (solution B). After 10 min of enzyme incubation with the KCl and CoCl₂ solution B was added to solution A.
at 30 °C. The reaction mixture (66 µL) was removed at time zero and added to an Eppendorf tube containing 5 µL of 10% TFA to quench the reaction. Similarly, the reaction of 66 µL of sample was quenched after 5 and 10 min intervals. The samples were separately loaded on the chromatographic C8 column and profile of substrate to product was observed under the optimized conditions. One unit was defined as the amount of enzyme required to eliminate N-terminal methionine from 1 µmol of tetrapeptide MGMM in 1 min.

2.6. Processing of N-terminal methionine from Met-INFα-2b
The recombinant interferon alpha 2b, with N-terminal methionine was obtained from our previous studies (Arif et al., 2015). In an Eppendorf tube (A), 400 µL of reaction mixture containing 175 mM KCl, 50 mM CoCl₂, 200 µg of enzyme, and 25 mM HEPES buffer pH 7.5 were incubated at 37 °C for 10 min. In another Eppendorf tube (B) 200 µg of Met-INFα-2b prepared in 25 mM HEPES buffer pH 7.5 was added to make 400 µL reaction. From tube A, 80 µL of solution was transferred into another Eppendorf tube containing 10 µL of 10% TFA; then 20 µL of solution from tube B was added and kept at –20 °C until analysis. To the remaining solution A, all the solution B was added and then 100 µL of the reaction mixture was taken at a time at an interval of 10 min, quenched. Timewise change in the HPLC elution peaks was monitored, and the product formation and substrate consumption were calculated (Arif et al., 2015).

3. Results
3.1. Production of recombinant E. coli methionine aminopeptidase
Maximum expression of recombinant enzyme in the soluble and functional form was obtained when the host bacterial cells were induced overnight under 0.4 mM IPTG at 20 °C and 100 rpm. Enzyme was purified by anion exchange chromatography; major enzyme fractions eluted from the Q-Sepharose Column between 0.15 and 0.25 M NaCl were analyzed on SDS-PAGE (Figure 1). The molecular weight determined by MALDI-TOF was 29200.941 Da (Figure 2). The purified enzyme has optimum pH 7.5 and maximum activity was measured at 37 °C in the presence of 2 mM cobalt ions.

3.2. Enzyme activity with different substrates
The specific activity of enzyme with methionyl-p-nitroanilide was 1.64 µmol/min/mg. At time 0, the HPLC profile indicated a single peak for the substrate elution. After 5 min, half of the substrate was converted to the

![Figure 1](https://example.com/figure1.png)  
**Figure 1.** SDS-PAGE photograph indicating the expressed and purified E. coli methionine aminopeptidase. Lane M - Protein Marker (Thermo Fisher catalogue No. SM 0671), Lane 1- pET21a containing induced cells, Lane 2 - pET21a-MAP containing induced cell lysate. Lane 3 - Purified enzyme.
product and after 10 min all the MGMM tetrapeptide was converted into the product GMM (Figure 3). Looking at the elution peak area of the substrate and assuming that half of the substrate was consumed in 5 min, the specific activity of the enzyme was calculated as 1.51 µmol of the substrate consumed/min/mg of enzyme (Table 1).

3.3. Processing of human interferon α-2b
The HPLC profile of recombinant human interferon α-2b showed three peaks representing three species, one without N-terminal methionine having molecular weight 19,265 Da, the second acetyl-interferon α-2b without N-terminal methionine but acetylated having molecular weight 19,307 Da, and the third Met-interferon α-2b with methionine having molecular weight 19,396 Da. For the purified recombinant *E. coli* methionine aminopeptidase, the HPLC profile indicating the progress of the reaction was demonstrated after 0, 10, 20, 30, and 40 min (Figure 4), and the reaction was stopped after 36% of the substrate was consumed (Supplementary Table ST1).

3.4. Protein 3-D modelling and validation
The I-TASSER-generated Met-IFNα-2b protein model was visualized by PyMOL Molecular Graphics System, Version 1.2r3pre (Figure 5). RAMPAGE, a protein structure validation server, and the Ramachandran plot collectively revealed that the template-based structure of IFNα-2b built was quite stable (Supplementary Table ST2; Supplementary Figure SF1). Despite having only one amino acid difference in the primary structure, the Met-INF and nonmet-INF have visible differences in their secondary structures and 3-D arrangements.

3.5. Molecular docking and comparative analysis
Molecular docking of IFNAR2/INFα-2b without methionine exhibited the lowest free energy change (ΔG = −784.53 kJ/mol), indicating higher binding affinity, followed by IFNAR2/met-INFα-2b (ΔG = −717.63 kJ/mol) (Figure 6). The formation of INF-enzyme complex was described. The interaction of amino acids with the cobalt ions in the active site pocket was elaborated. The free energy change for the ES-complex formation was calculated as ΔG = −683.07 kJ/mol (Figure 7).

4. Discussion
Generally the recombinant proteins expressed in *E. coli* represent the translation product comprising the
amino acid sequence encoded by the gene. From time to time, however, posttranslation modifications have been observed that modulate the activity of most eukaryote proteins (Mann and Jensen, 2003). One amongst these is the removal of N-terminal methionine catalyzed by methionine aminopeptidase and acetylation by acetyltransferase (Liao et al., 2004). These enzymes are found ubiquitously and show preference for the removal of N-terminal methionine if the next residue in the sequence has a small side chain (Xiao et al., 2004; Aksnes et al., 2016). In the case of E. coli expression, the recombinant protein is overexpressed; the native E. coli aminopeptidase being produced in limited amount fails to process all of the recombinant polypeptides. Hence, in the present study, in vitro processing of interferon α-2b at the N-terminus was the major part of the investigation. The in vitro removal of N-terminal methionine from the recombinant proteins is well established (Arfin et al., 1988; Mitra et al., 2006; Arif et al., 2015, 2016). Our study was meant to report the expression and purification of E. coli enzyme with emphasis on the in silico insights into the ES-complex formation by met-INFα-2b and MAP, the binding free energy change of ES-complex formation. We also investigated the binding free energy changes of INF molecules with and without methionine at the N-terminus and their corresponding receptor. The methionine aminopeptidase gene from E. coli DH5 α was expressed in E. coli BL21 (DE3) RIL codon plus cells. Gene expression in the bacterial culture was induced by 0.4 mM IPTG after the onset of the log phase. Induced bacterial culture was incubated overnight in a shaking incubator adjusted to 20 °C and 100 rpm (Ben-Bassat et al., 1987; Lowether et al., 2000). Recombinant E. coli methionine aminopeptidase was purified by anion exchange chromatography; it exhibited a molecular weight of 29,200 Da on MALDI-TOF, which is nearest to the theoretical molecular weight of enzyme without N-terminal methionine as determined by ProtParam (29,311.91 Da). Specific activity of enzyme was 1.64 U/mg in the methionyl p-nitroanilide-based assay. It exhibited 1.51 U/mg specific activity with synthetic tetrapeptide (MGMM) in an HPLC-based assay (Figure 3). Studies on MAP have shown different specific activity when different substrates were used (Vasina et al., 1996), indicating the effect of substrate structure on enzyme activity (Lowther

Figure 3. Reverse phase HPLC analysis of the substrate MGMM consumption in the reaction mixture containing 1.9 mM substrate and 0.026 mg of E. coli methionine aminopeptidase using C8 column. X-axis is the retention time in min and Y-axis is absorbance at 220 nm.

Table 1. The amount of enzyme, activity and specific activity of methionine aminotransferase demonstrated by using MGMM as substrate.

<table>
<thead>
<tr>
<th>Total concentration of substrate used in the assay</th>
<th>1.9 mM</th>
<th>1.9 μmol/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of substrate used in 66 μL reaction</td>
<td>(1.9 × 66) ÷ 1000</td>
<td>0.1254 μmol</td>
</tr>
<tr>
<td>Amount of substrate consumed in 5 min (half)</td>
<td>0.1254 μmol ÷ 2</td>
<td>0.0627 μmol</td>
</tr>
<tr>
<td>Amount of substrate consumed in 1 min</td>
<td>0.0627 ÷ 5</td>
<td>0.013 μmol</td>
</tr>
<tr>
<td>Amount of enzyme used in each reaction</td>
<td>(0.026 × 66) ÷ 200</td>
<td>0.0086 mg</td>
</tr>
<tr>
<td>Specific activity of the enzyme with MGMM</td>
<td>0.013 ÷ 0.0086</td>
<td>1.51 μmol/min/mg</td>
</tr>
</tbody>
</table>
et al., 1999; Xiao et al., 2010). Recombinant enzyme successfully processed recombinant human interferon as monitored by the HPLC-based assay system (Figure 4). A 3D model of INF was built by I-TASSER and validated by the protein structure validation server RAMPAGE (Lovell et al., 2002), which indicates the phi and psi deviations via Ramachandran. The difference in amino terminal methionine on the N-terminus resulted in detectable changes in the secondary and tertiary structures of INF with and without N-terminal as indicated by comparative analysis (Figure 5). The structural difference indicated by the comparative analysis makes the most of the recombinant fraction (with N-terminal methionine) different from the native (removed amino terminal methionine). We suggest a possible immune response by the human body against this structural difference between native and recombinant interferon. The idea is also supported by the literature (Mukovozov et al., 2008). The built structure was similar to those reported in previous studies (Ya et al., 2003). Our docking results showed that IFNAR2 is the natural protein partner of IFNa-2b (without methionine) as their docking complex exhibited ΔG = -784.53 kJ/mol. The IFNAR2/met-INFα2b complex exhibited a ΔG = -717.63 kJ/mol, indicating a comparatively low binding affinity of met-INFα-2b to the corresponding receptor IFNAR2 (Figure 6). The results are in correlation with the studies showing better response of native interferon as compared to the recombinant (Karlberg et al., 2010). The receptor for human interferon alpha consists of two subunits, i.e. IFNAR1 and IFNAR2. The subunit IFNAR2 has (nanomolar dissociation constant) greater affinity to IFNs without the presence of IFNAR1 (Gull et al., 2013) and is considered the major ligand-
binding component; rationally it gains more attraction in IFN docking studies. The NMR-based docking study of IFNAR2/INFα-2b also revealed participation of the N-terminus and A-helix of INFα2 and the C-domain of IFNAR2. Free energy change of MAP/met-INFα-2b complex (ES-complex) formation was $\Delta G = -683.07$ kJ/mol as determined by molecular docking studies. Active site pocket interacting with cobalt ions is indicated clearly as pink spheres (Figure 7). The role of active site amino acids and cobalt ions has been described by previous reports (Lowther et al., 1999; De Weerd et al., 2003). Here we provide a structural elaboration of Asp97, Asp108, His171,
Glu204, and Glu235 side chains interacting with cobalt ions. In summary, N-terminal methionine processing of human interferon α-2b has importance to obtain a single polypeptide species in the final product. Molecular docking studies revealed dissimilarities in the 3-D structure, putative receptor binding position, and binding free energy change of recombinant INF2b with and without N-terminal methionine. Based on these results, we advocate a functional superiority of recombinant INFα-2b without N-terminal methionine.

Acknowledgment
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References


Table ST1. Timewise change in the peak area and peak height of Met-interferon α-2b and interferon α-2b due to reaction with *E.coli* methionine aminopeptidase.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Protein</th>
<th>Peak area</th>
<th>Peak height</th>
<th>% of Met-Ifn converted to Ifn</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Ifn</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Met-Ifn</td>
<td>24242</td>
<td>202</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Ifn</td>
<td>3675</td>
<td>58</td>
<td>15%</td>
</tr>
<tr>
<td></td>
<td>Met-Ifn</td>
<td>20568</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Ifn</td>
<td>2944</td>
<td>57</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Met-Ifn</td>
<td>8828</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Ifn</td>
<td>4622</td>
<td>61</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>Met-Ifn</td>
<td>8403</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Ifn</td>
<td>6848</td>
<td>90</td>
<td>36%</td>
</tr>
<tr>
<td></td>
<td>Met-Ifn</td>
<td>12015</td>
<td>116</td>
<td></td>
</tr>
</tbody>
</table>

Table ST2. Quality evaluations of 3D protein model built by I-TASSER using various parameters and software.

<table>
<thead>
<tr>
<th>Protein</th>
<th>C-score*</th>
<th>TM-score&lt;sup&gt;+&lt;/sup&gt;</th>
<th>RMSD&lt;sup&gt;−&lt;/sup&gt;</th>
<th>IDEN&lt;sup&gt;−&lt;/sup&gt;</th>
<th>Cov&lt;sup&gt;−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-2b</td>
<td>1.44</td>
<td>0.895</td>
<td>1.88 Å</td>
<td>0.970</td>
<td>0.970</td>
</tr>
</tbody>
</table>

<sup>*Confidence (C) score for estimating the quality of predicted models (range –5 to 2). </sup>

<sup>Template modeling score (TM-score) measures the structural similarity between two structures (TM-score > 0.5 indicates a model of correct topology and a TM-score < 0.17 means a random similarity range 0 to 1). </sup>

<sup>Heavy atoms root-mean-square deviation (RMSD) with respect to the experimental structure. </sup>

<sup>IDEN is the percentage sequence identity in the structurally aligned region (range 0 to 1). </sup>

<sup>Cov represents the coverage of global structural alignment and is equal to the number of structurally aligned residues divided by length of the query protein cluster (range 0 to 1). </sup>

<sup>As compared to template ‘1itf’</sup>
Number of residues in favored region (~98.0% expected): 154 (93.9%)
Number of residues in allowed region (~2.0% expected): 8 (4.9%)
Number of residues in outlier region: 2 (1.2%)

Figure SF 1. The Ramachandran plot generated by the RAMPAGE server depicting the quality of the built protein INF alpha 2b with methionine cap.