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Characterization and bioassay of post-translationally modified interferon α-2b expressed in *Escherichia coli*

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ABSTRACT

Examples of N-terminal acetylation are rare in prokaryotic systems, but in this study, we report one such example in which N-terminal Cys residue of recombinant human interferon α-2b produced in *Escherichia coli* is a favourite site for N^{α}-acetylation. The recombinant protein following Q-sepharose chromatography gave a single band on PAGE analysis. However, on reverse phase HPLC the material separated into three peaks. These were characterized by mass spectrometric techniques as: (a) the direct translation product of the gene retaining the N-terminal methionine, (b) a species from which the methionyl residue had been removed by *E. coli* methionyl aminopeptidase to give the native interferon α-2b and (c) in which the N-terminal Cys residue of the latter contained an acetyl group. Tryptic digestion of interferon α-2b gave fragments linking Cys^{1} to Cys^{98} and Cys^{29} to Cys^{138}, while that of N^{α}-acetyl-interferon α-2b gave the Cys^{1}–Cys^{98} fragment with an additional mass of 42 attributed to an acetylated N-terminal. Bioassay of the derivatives showed that N^{α}-acetyl-interferon α-2b had 10% of the activity of interferon α-2b. The results suggest that the lower activity derivative seen here in *E. coli* may also be produced when the protein is produced in yeast.

1. Introduction

Interferon α-2b is a member of a large group of proteins which has a range of biological activities, notable amongst which is the antiviral action originally described in the pioneering work of Isaacs and Lindenmann (1957), (Isaacs et al., 1957; Lindenmann et al., 1957; Burke and Isaacs, 1958; Isaacs et al., 1958; Isaacs and Burke, 1958). Historically, along with proinsulin, interferon was one of the first proteins to be produced using the methods of recombinant technology by the groups of Pestka and Weissmann (Maeda et al., 1980; Goeddel et al., 1980; Weissmann et al., 1982; for a review see, Pestka, 1986). Subsequently, various members of the interferon family have been cloned and expressed in *Escherichia coli*, yeast or mammalian cells, and various forms of interferon are now available for clinical use (Pestka, 1981).

The expression of a protein in *E. coli*, by necessity requires the presence in the gene of the protein-initiation codon for methionine (Met). The primary translation product, of the recombinant protein thus contains N-terminal methionyl residue. *In vivo* processing of which by the host methionine aminopeptidase depends on the nature of the residue following the N-terminal Met (Hirel et al., 1989). The mature form of interferon α-2b has as its N-terminal a cysteine (Cys) residue linked to Cys^{98} by a disulphide bond (Fig. S1) (Pestka, 1986), requiring the removal of the starter metionine from the primary translation product. In this paper, we describe the isolation of the species expressed by the human interferon α-2b gene in *E. coli* and the elucidation of their key structural features using mass spectrometry.

2. Materials and methods

2.1. Isolation and cloning of DNA encoding human interferon α-2b

The isolation of DNA, expression of the encoded protein and its purification is described by Mahmood (2009). Briefly, human genomic DNA was isolated from the blood of a healthy student using the method of (Blin and Stafford, 1976) and served as template for further manipulations carried out using the protocols described by (Maniatis et al., 1978). In the first PCR reaction, 1F

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and 1R (Table S1) were used as forward and reverse primers to give a 705 bp product. The latter then served as a template for the second PCR using 2F and 2R as forward and reverse primers. The resulting 625 bp DNA contained the coding sequence for interferon α-2b as well as codons for N-terminal methionine, stop codon and downstream sequence of 119 nucleotides. The DNA was transferred *via* pTZ57R/T (Fermentas®) into the expression plasmid pET-21a(+) (Novagen®) to give pET-21a-*Ifn.* The latter plasmid was used to transform *E. coli* BL21-CodonPlus (DE3)-RIL cells.

2.2. Cell biomass production and solubilization of inclusion bodies

Batches of bacterial cultures, from the transformed colonies above, in 2–10 l were grown in orbital shake flask incubators at 37 °C and 100 rpm. LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) was used as the growth medium. The primary inoculum was grown overnight (for 12–16 h) in 10 ml of LB broth and transferred to 100 ml of LB broth for further growth for 3 h. This secondary culture was then used as inoculum to grow bacteria on large scale, with inoculum:medium ratio of 1:99. When bacterial culture optical density at 600 nm (OD_{600}) reached 0.4–0.6, lactose (0.8 mM) was added, as expression inducer, and incubation was continued for further 5 h so that maximum expression of the desired protein could be achieved.

The cells were harvested by centrifugation at 5000 × *g* for 5 min and the pellet suspended in 15 ml of sonication buffer (50 mM Tris–Cl pH 8.0, 1.5% Triton X-100, 200 mM NaCl, 10 mM EDTA, 0.1% sodium azide and 2 mM DTT) per 3.0 g of cell pellet. The suspension was then subjected to sonication for a total of 45 min in 9 cycles. One cycle consisted of 5 bursts of sonication, each of 60 s, followed by cooling in ice for 120–180 s. After every cycle the cell lysate was subjected to centrifugation at 5000 × *g*, 4 °C for 20 min. The supernatant was discarded while to the pellet again sonication buffer was added and the above operation repeated for further 8 cycles. The entire time including centrifugation took from 8 to 10 h, when an aliquot of final pellet, following solubilization in SDS (sodium dodecyl sulphate), as described below, showed a 260 nm:280 nm ratio of around 0.64:1.

For refolding, inclusion bodies were suspended in a small volume of solubilization buffer (8 M urea, 50 mM Tris–Cl (pH 8.0), 50 mM glycine and 4 mM DTT; glycine was included in the solubilization buffer to help avoid carbamylation of protein) and sonicated on ice for 5 cycles of 30 s pulse and a gap of 2 min. Final volume was adjusted with the solubilization buffer to give protein concentration of 5-7 mg/ml and the mixture incubated at 37 °C for 30 min to allow complete reduction of disulfide bridges. A clear solution, obtained after centrifugation of solubilized protein at 5000 × *g*, 4 °C for 20 min, was further processed for refolding.

2.3. Quantification of proteins

For the quantification of protein in final suspension, the inclusion bodies in 100 μl of representative sample was centrifuged and the pellet solubilized in 5% SDS solution. An appropriate dilution of the protein solution in 5% SDS was used for recording the spectrum from which absorbance at 260 nm and 280 nm was measured. Protein quantification, from absorbance at 280 nm, sometimes leads to erroneous over estimation of protein concentration due to the presence of nucleic acids. In the present work most of the contaminating nucleic acids are removed by repeated sonication and centrifugation cycles to give a 260 nm:280 nm ratio of around 0.64:1. If the E_{260}:E_{280} ratio is ≥0.64 then the estimate is corrected for actual protein quantity using the following formula, given in equation below, which is an approximation derived from the original data of (Warburg and Christian, 1941) and further elaborated by (Layne, 1957).

$$\text{Corrected protein concentration} = 1.55 \times (280\,\text{nm absorbance}) - 0.76 \times (260\,\text{nm absorbance})$$

2.4. Refolding and protein purification by Q-sepharose column chromatography

Following is illustrative of the refolding procedure used in the present study. To the refolding buffer (1250 ml; 100 mM Tris–Cl pH 8, 2 mM EDTA, 0.5 mM cystine, 5 mM cysteine, 0.1 mM PMSF) was added the protein (610 OD_{280} units, 100 ml; 6.1 OD_{280} units/ml) in 10 pulses containing 60 OD_{280} units of protein in each pulse, every 4 h. The reaction was gently stirred and maintained at 4 °C. The final concentration of the protein in the refolding sink was about 0.5 OD_{280} units/ml. The reaction mixture was dialysed against 20 mM Tris–Cl pH 8, with 4–5 changes of the buffer, or subjected to diafiltration to reduce the volume of the folding mixture. In both cases the reaction mixture was clarified by centrifugation at 5000 × *g* for 20 min, at 4 °C.

A suspension of Q-sepharose (50 ml) was poured into a column 2.5 cm × 20 cm (diameter × length) which was washed with autoclaved distilled water (200 ml) to remove any preservative. The equilibration of column was carried out with 500 ml of 50 mM Tris–Cl (pH 8.0). After equilibration, the refolded protein in refolding buffer was loaded on to the column manually. The flow rate of column was adjusted to 1 ml/min so that proper binding of protein to the resin could take place. The column was washed again with the same buffer (50 mM Tris–Cl pH 8.0) until the OD at 280 nm of the flow-through was zero. The protein was then eluted with different concentrations of NaCl (0.1 M to 1.0 M) in 50 mM Tris–Cl pH 8.0. The volume of each concentration of NaCl applied to the column was 15 ml. The protein profile in the collected fractions was monitored by taking OD at 280 nm and the quality of purification was judged by 15% SDS-PAGE.

The protein fractions, judged pure by SDS-PAGE analysis, were mixed together and dialyzed against 0.1% TFA till concentration of salt became approximately 0.001 M. The weight of the lyophilized powder was determined and the amount of protein per 1 mg of the powder measured by taking OD at 280 nm. For this estimation the extinction coefficient of human interferon α-2b as determined from online ExPASy Proteomics Server, ProtParam tool (ExPASy, Switzerland) was used to give 1 mg of the protein in 1.037 OD_{280} units/ml.

2.5. Protein purification by reverse phase liquid chromatography

For reverse phase high pressure liquid chromatography (RP-HPLC) separation, preparative columns from Thermo Scientific® (Thermo Scientific; BioBasic C-18; particle size: 5 μm; length 250 mm × internal diameter 10 mm) were used and gradient was generated with 0.1% TFA (trifluoroacetic acid) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). About 0.5–1.0 mg of the above solid was solubilized in solvent A and loaded on column with 0% solvent B for 5 min and then eluted with 40%–80% gradient of solvent B at 0.5% ramp/min. Different fractions of protein were eluted between 59% and 61% solvent B. Individual fractions were collected in separate containers and freeze dried for further mass spectroscopic analysis of intact proteins as well as their tryptic fragments.

2.6. Anti-proliferative bioassay of interferon α-2b by neutral red dye uptake method

The antiproliferative bioassay was carried out in 24-well culture plate with lid. The recombinant human interferon α-2b and its derivatives as well as reference protein interferon α-2a, in Dulbecco's Modified Eagle Medium (DMEM) were serially diluted from 20 ng to 1.25 ng in different wells while one well was kept as a control devoid of interferon. To each well with different amounts of the protein, known number of cells in suspension (40,000 cells/ml of DMEM) was added. The plates were placed in 5% CO_2 incubator at 37 °C. The cells were grown for 48 h in wells. After 48 h, 0.5 ml of 0.04% neutral red dye solution (in phosphate buffer saline, pH 7.4 and DMEM in a ratio of 1:9) was added to each well and cells incubated for 30 min. The cells were then washed with phosphate buffer saline thrice to remove any residual dye adhering to the outside of cells. The neutral red dye which was taken up by viable cells was extracted by 500 μl of acidified ethanol (water:ethanol:acetic acid = 49:50:1). The cells were kept in acidified ethanol for almost 3 min and then the liquid was pipetted out while keeping monolayer of cells intact. The OD at 540 nm was determined in order to monitor the cell growth in response to various concentrations of the protein under test.

2.7. Tryptic digestion of interferon and its derivatives

Sequencing grade porcine trypsin from Promega® was used for proteomic analysis of interferon and its derivatives. HPLC purified fractions (100 μg/100 μl each of peak 1 and 2; Fig. 1, in 50 mM ammonium bicarbonate pH 8.3) were directly subjected to tryptic digestion in solution (without reduction and alkylation) using 1:20 ratio of trypsin to protein. The reaction mixtures were incubated at 37 °C for 16–18 h and quenched with trifluroacetic acid (final concentration 1%; pH 2.0). 5 μl of the reaction mixture was then loaded on LC–MS column, analyzed on Agilent LC–MS and the raw data was processed (as described in Section 2.8).

2.8. Mass spectrometric analysis of interferon derivatives and their tryptic peptides

MALDI mass spectrometric analysis of interferon and its derivatives were performed on Autoflex™ TOF TOF Smartbeam 200 (Bruker, Germany GmbH), using dried droplet method. For this purpose, protein or peptide solution (1–2 μg/μl in 10 mM Tris–Cl pH 8.0) was mixed with sinapinic acid solution (9 μl, prepared from 10 to 12 mg of a matrix in 1:2 ratio of 100% acetonitrile to 0.1% trifluroacetic acid) and processed as described in Gardner et al. (2013).

On-line LC–MS analysis of purified recombinant proteins and tryptic digest, was performed on 6224 TOF LC/MS (Agilent Technologies, USA), equipped with a dual electrospray ionization source. The data were acquired using MassHunter Workstation software for quantitative analysis (version B.01.02). Positive ions were generated using a dual ESI voltage of 3.5 kV with the gas temperature at 325 °C, fragmentor voltage 175 V, skimmer 65 V, drying gas flow 5 l/min, and a nebulizer pressure of 30 psig. Data were collected at a scan rate of 1.03 spectra per second.

For online separation the reverse phase RP-HPLC column was from Agilent (Poroshell 300SB-C-18: particle size 3 μm, length 75 mm, internal diameter 2.1 mm). Blank (0.1% formic acid) was usually run before the sample analysis. The LC–MS analysis was performed by injecting 5 μl of a clear protein/peptide solution (0.5–1 μg), at a flow rate of 0.2 ml/min, using a gradient of 2–60% of 100% acetonitrile in 0.1% formic acid (solvent B) with 0.1% formic acid water (solvent A) in 36 min, washing of the column with 60–98% of solvent B for 5 min, and equilibration of the column with solvent A for 8 min. The TOF spectra range was adjusted from 100 to 3000 *m*/*z*. TIC (total ion current) chromatogram was generated and calibrated with a tuning mix (as provided with the instrument).

For intact proteins analysis, multiply charged spectrum was extracted from TIC in Agilent MassHunter Qualitative Analysis BioConfirm software (version B.02.00) and processed for deconvolution using optimized maximum entropy algorithms. In case of tryptic digest mixtures, TIC was processed in BioConfirmProteindigest layout, and optimized for molecular feature ions, for the complete identification of peptides and their derivatives.

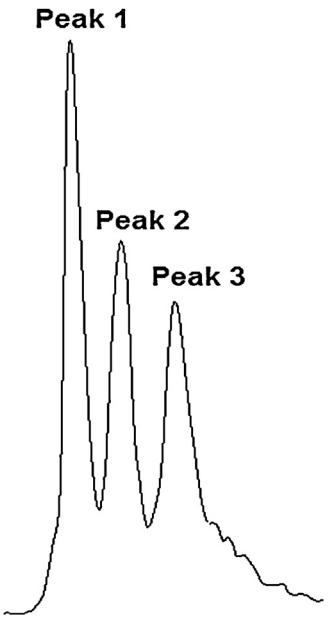

Fig. 1. HPLC analysis of Q-sepharose purified interferon α-2b. The material from Section 2.4 was subjected to fractionation as described in Section 2.5. The three peaks eluted between 59 and 61% acetonitrile containing 0.1% trifluoroacetic acid.

3. Results

The gene for human interferon α-2b was amplified from total genomic DNA, obtained from the blood of a student, using the forward and reverse primers, 1F and 1R (Table S1). The amplified DNA, following sequencing, acted as a template to give, using primers 2F and 2F (Table S1), a PCR product which was ligated into pET-21a to give pET-21a-*Ifn* and the latter used for the expression of the protein in *E. coli* BL21-CodonPlus (DE3)-RIL cells. The interferon gene was also modified in the 5′-region to introduce *E. coli* preferred codons for the first 10 residues of the translation product to give pET-21a-*IfnMod*. It was found that the expression using the latter construct was similar to that containing the original human DNA codons (lanes 1 and 2, Fig. S2) hence the latter was used for subsequent studies.

The interferon α-2b polypeptide, produced as inclusion bodies (Fig. S3), was solubilized, refolded and subjected to Q-sepharose chromatography. The flow through fractions contained oligomers of the protein, since PAGE analysis of this fraction, under non-reducing conditions gave a low mobility material, which under reductive conditions migrated at the expected position for the interferon α-2b polypeptide (Fig. S4). The protein, eluting in 0.3–0.5 M NaCl, had similar mobility under non-reductive and reductive conditions, corresponding to a monomer of interferon α-2b (Figs. S5 and S6). The fractions containing the monomeric material were dialysed and freeze-dried (for yields at various steps see Table S2).

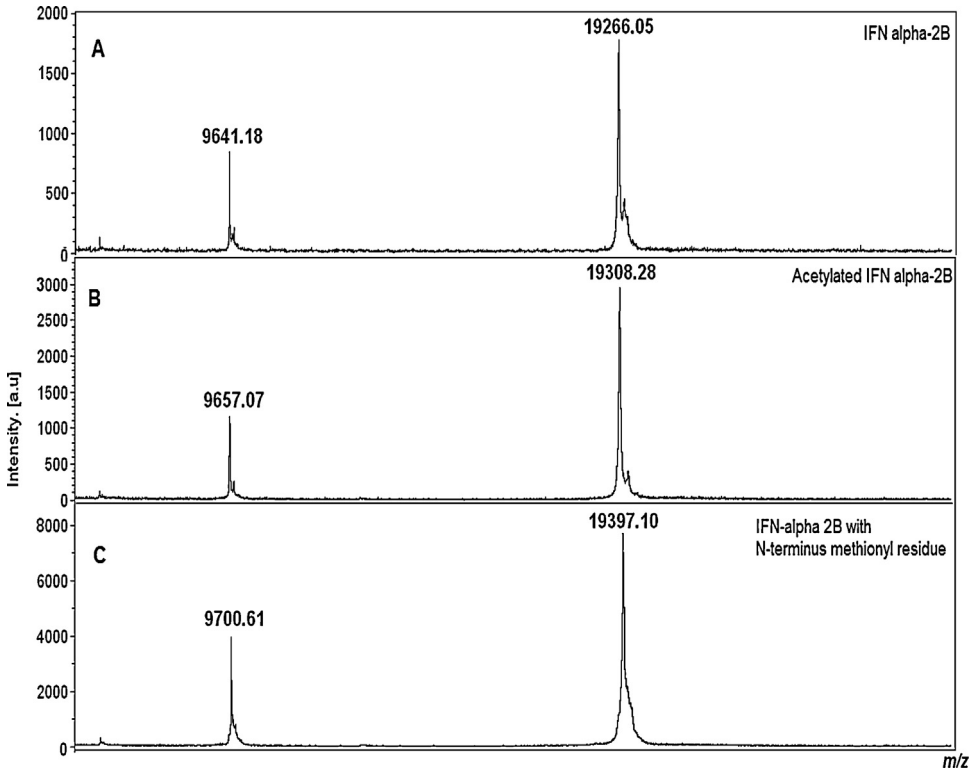

Fig. 2. MALDI spectrum showing $[M+1H]^+$ ions of proteins in the three peaks shown in Fig. 1. (A), peak 1; (B), peak 2 and (C) peak 3. Theoretical masses for the proteins in the three peaks are 19,265, 19,307, and 19,396 Da, respectively.

When the latter material was subjected to purification by HPLC the protein separated into three peaks, denoted 1, 2 and 3, in the order of their elution during HPLC separation (Fig. 1), which were analyzed by MALDI as well as ESI mass spectrometry. The material in peak 1 gave a $[M+1H]^+$ ion at 19,266.05 (Fig. 2A) corresponding to that of native interferon α-2b (**1**, Fig. 3), in which the N-terminal methionine residue of the primary translation product had been removed. The protein in peak 2 had the $[M+1H]^+$ ion at 19,308.28 which was 42 ± 1 units higher than that of interferon α-2b (Fig. 2B). A species with a similar mass was found as a minor contaminant in the recombinant interferon α-2b, produced in *E. coli*, by the Merck group (Liu et al., 2011), and characterized as an interferon α-2b derivative containing an acetyl group (**2**, Fig. 3). Peak 3 had the $[M+1H]^+$ ion at 19,397.10 (Fig. 2C), for the primary translation

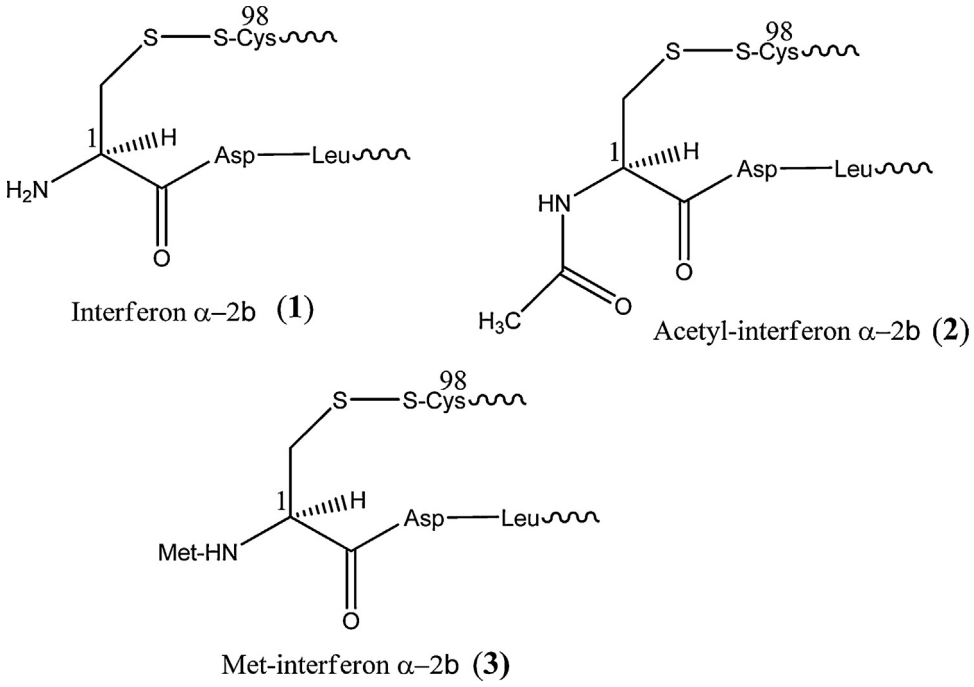

Fig. 3. Partial structures of interferon α-2b derivatives. Only one of the two —S—S— bonds (C^1–C^{98}) of the protein is shown here, for the full sequence see Fig. S1.

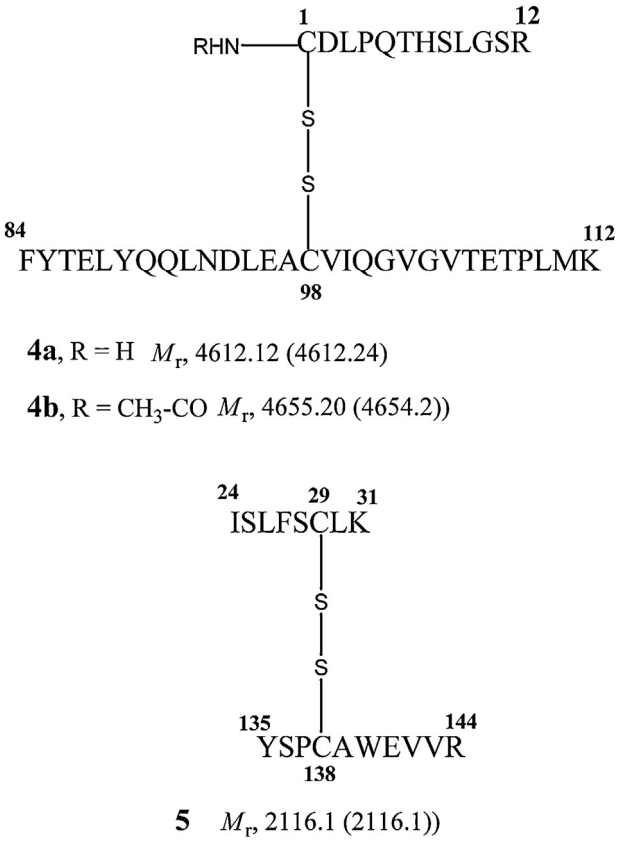

Fig. 4. Structures of the disulphide-bond containing fragments.

product of the gene used in this study, corresponding to Met-interferon α-2b (**3**, Fig. 3).

For further characterization, the material in peak 1 was directly (without prior reduction and alkylation) treated with trypsin and the digest analyzed by LC–MS. From the masses of the peptides in Table S3, 96% of the theoretical tryptic fragments could be identified in this analysis. The peptides 14 and 15 (Table S3A) had masses corresponding to the two disulphide bonds, C^{1}–$C^{98,}$ and C^{29}–C^{138} present in interferon α-2b (**4a** and **5**, Fig. 4). The material in peak 2 was similarly processed and the analysis of the tryptic digest showed the C^{1}–C^{98} disulphide bond containing fragment 14 in Table S3B, had a mass 42 ± 1 Da higher, than the equivalent fragment obtained from peak 1, corresponding to a modification by an acetyl group (**4b**, Fig. 4). The mass of the fragment C^{29}–C^{138} containing the second disulphide bond between C^{29} and C^{138} (**5**, Fig. 4) was the same as found in the digest of peak 1, fragment 15, Table S3A and B.

The biological activity, involving the anti-proliferative action of interferon was determined, in-house, using MDBK (Madin-Darby bovine kidney) cells, and by National Institute of Biological Standards and Control (NIBSC, UK) on Daudi cells (CCL-213, ATCC). Fig. 5 shows the course of cell growth inhibition using the protein prepared in this work, and purified up to the Q-sepharose chromatography step, along with a reference sample of commercially available interferon α-2a (Roferon). The two samples showed similar dose-response profiles with MDBK cells. NIBSC analysis, for the anti-proliferative effect on Daudi cells (Silva et al., 2005), of similar samples, shipped from Pakistan to UK at room temperature, gave an activity of 1×10^{8} IU/mg relative to 1.4×10^{8}/mg for their carefully stored standard.

Anti-proliferative bioassay was also performed on the material contained in the three purified peaks, along with a biological standard from PBL (USA). Interferon α-2b, from peak 1 (Fig. 1) and

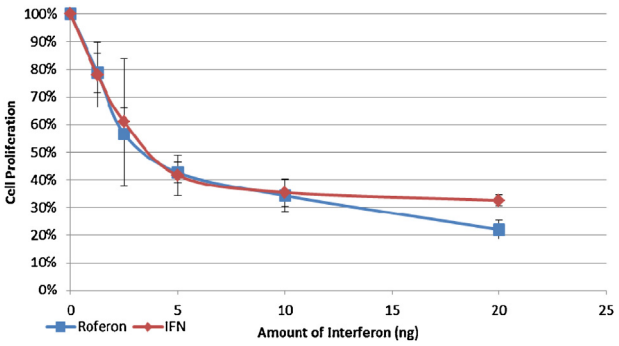

Fig. 5. Antiproliferative bioassay of interferon α-2b. The assay used MDBK cell line where the cell growth inhibition was measured by the neutral red dye uptake method. Sample prepared in this work (IFN) and reference sample from commercially available interferon α-2a (Roferon). Each point on the curve denotes mean value from three replica experiments. Error bars represent standard deviation.

Met-interferon α-2b, peak 3, had the same specific activity as the PBL standard. However, N-acetyl-interferon α-2b was one-tenth as active as interferon α-2b. N^{α}-acetylation abolishes the positive charge on the N-terminal α-amino group, thus a significant loss of activity accompanying the modification shows the importance of a positive charge at the N-terminal of the protein (Table 1).

4. Discussion

Generally the recombinant proteins expressed in *E. coli*, are expected to produce faithfully the translation product containing the amino acid sequence encoded by the gene. From time to time, however, post-translation modifications are observed. Notable amongst these is the removal of N-terminal Met by methionine aminopeptidase (Ben-Bassat et al., 1987). Enzymes with such an activity are found ubiquitously and show preference for the removal of N-terminal Met provided that the next residue in the sequence has a small side chain (glycine, alanine, serine, cysteine, threonine, proline and valine) (Hirel et al., 1989). In the present work this rule was broadly followed resulting in the predominant generation of the N-terminal Cys found in mature interferon α-2b. However, a substantial amount of the latter species was further converted into acetyl-interferon α-2b, presumably by an acetyltransferase (Bradshaw et al., 1998).

Protein N-terminal acetylation, originally described in 1958, is a major modification of eukaryotic proteins (Bradshaw et al., 1998; Narita, 1958; Arnesen, 2011). It was estimated that 80% of the soluble proteins from Ehrlich escites cells were acetylated (Brown and Roberts, 1967). Studies on the N-terminal acetyltransferases of yeast have shown the presence of three closely related enzymes, NatA, NatB and NatC, each acting on a set of protein, showing specificity for different classes of amino acids (Polevoda and Sherman, 2000). However, the nature of the N-terminal amino acid itself is not sufficient for specificity, residues down-stream of the acetylation site also play an important role. For example, while the N-terminal

Table 1
Inhibition of cell growth by different types of interferon proteins. The inhibition of cell growth by different types of interferon for a range 1.0 ng–0.001 ng was determined by neutral red dye uptake assay. The assay was performed as in Fig. 5. The reference protein used in this assay was from the ELISA kit of PBL Biomedical Laboratories for the assay of interferon α-2b.

Protein type	Amount at which 50% inhibition observed (ng) (±SD)
Interferon α-2b	0.062 ± 0.01
M-interferon α-2b	0.062 ± 0.01
N-acetyl-interferon α-2b	0.5 ± 0.05
Reference (PBL)	0.062 ± 0.01

Met in the sequence Met-Asn-Asp-Leu-Ala- was optimally acetylated by NatB, a similar sequence, Met-Asn-Asp-Leu-Lys-, in which Ala at fifth position, was replaced by Lys, did not acetylate. In their extensive screening of the specificity of N-terminal acetyltransferases of yeast (Polevoda and Sherman, 2000), an example where Cys is acetylated was not found. So far in *E. coli*, the examples of N^{α}-acetylation are rare and only 5 proteins of *E. coli*, mostly ribosomal subunits, are known to be N-acetylated (Smith et al., 1996; Tanaka et al., 1989; Yoshikawa et al., 1987; Arai et al., 1980). In the present study, using *E. coli*, N-acetylation of N-terminal Cys, in interferon α-2b, was inferred to be the major post-translational modification, for the following reason. In the portion of interferon α-2b, giving rise to the disulphide bond containing fragment **4** (Fig. 4), there are two amino groups which can be N-acetylated; its N-terminal, Cys^{1}, and the ε-amino group of Lys^{112}. In the light of the known specificity of trypsin, if the latter was N-acetylated the bond between residue 112 and 113 would not be cleaved by trypsin, therefore, it must be the N-terminal residue in the protein of peak 2 (Fig. 1) that was N-acetylated to produce the disulphide bond containing tryptic fragment **4b** (Fig. 4), with a mass of 43 Da higher than that of the equivalent fragment obtained from interferon α-2b in peak 1. Hence, the parent protein in peak 2 (Fig. 1) may be formulated as N^{α}-acetyl-interferon α-2b. In a recent PEGylation of interferon α-2b at one of the disulphide bonds, the MALDI spectrum in the paper, of the precursor interferon α-2b, was shown to have a mass of 19,306 Da (Balan et al., 2007) which corresponds to the material in peak 2, identified as N^{α}-acetyl-interferon α-2b (**2**, Fig. 3) and not interferon α-2b itself that would have a mass of 19,265 Da. The interferon used in the latter work was reported to be obtained from a yeast expression system. It would thus appear that one of the yeast N-acetyltransferases, for which Cys modification was not observed with the proteins selected for screening (Polevoda and Sherman, 2000), recognizes a signal away from the N-terminal in interferon α-2b which is acetylated in yeast and also by a yet uncharacterized *E. coli* enzyme, as shown here. The facile formation of N-acetyl-interferon α-2b, in *E. coli* as well as yeast, the two microorganisms used for the production of interferon, together with the observation in this work that the species has reduced biological activity, should require a careful monitoring, for the presence of this contaminant, in commercial samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.05.001.

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