Pak. J. Biochem. Mol. Biol., 2017 50(1): 22- 27

Recombinant Production of Therapeutically Important Cytokine, Interleukin-24

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ABSTRACT: This study describes the recombinant production of a therapeutically important cytokine, i.e., interleukin-24 (IL-24) in *E. coli*-based expression system. IL-24 has a unique ability to selectively and efficiently kill the cancer cells leaving the healthy cells unharmed and therefore has enormous potential in cancer treatment. Human peripheral blood mononuclear cells, isolated from blood of healthy individual, were stimulated with concanavalin-A for production of mRNA of different cytokines and total RNA was isolated from these stimulated cells. The coding sequence for IL-24 mature peptide was amplified from mRNA by RT-PCR using gene specific primers and subsequently cloned in T7*lac* promoter-based pET-28b(+) vector to construct pIL-24. *E. coli* strain BL21 (DE3) codon plus was used to transform with pIL-24 for high level expression of recombinant IL-24. Expression of IL-24 was achieved by induction with IPTG in LB medium and analyzed by 15% SDS PAGE. A prominent band of ~18kDa protein representing >35 % of the total *E. coli* cellular proteins was observed. Sub-cellular fractions of the induced cells were analyzed by disrupting the cells by sonication and then centrifugation to get the soluble fraction and inclusion bodies. IL-24 was found in the inclusion bodies. IL-24 inclusion bodies were washed with triton X-100. *In silico* analysis was done using different bioinformatics tools to determine the secondary and 3-D structure of IL-24 protein. IL-24 forms a compact 3-D structure with four major alpha helices. IL-24 can further be purified and its bioactivity can be determined for its potential use in cancer therapy.

Keywords: Interleukin, cytokine, autoinduction, inclusion bodies, refolding
Received: January 10, 2017 **Accepted:** February 13, 2017
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INTRODUCTION

Since its discovery interleukin-24 (IL-24, also known as *mda-7*), has been a primary focus of the advanced research worldwide. Besides its bystander antitumor and antiangiogenic effects in diverse maladies of cancer, it has a unique property to selectively kill a broad spectrum of cancer cells without any toxic effects on the healthy cells [1-6]. This astonishing attribute of cancer cells selectivity of IL-24 has provided a foundation for the development of novel course of therapy by Introgen Corporation with the label of INGN-241, a replication-defective adenovirus that have ability to express IL-24 [7, 8]. The mechanism how IL-24 selectively kills the cancer cells still remains to be explored, however apoptotic activity of IL-24 allow this novel gene to be accepted in health and medical sciences as effective therapy against cancer and allied maladies.

The expression of IL-24 is limited to tissues that are related to the immunity like thymus, spleen, and peripheral blood leukocytes as the levels of endogenous IL-24 in a cell are regulated at mRNA level and post translational events [9-11]. So far, IL-24 has been expressed *in vivo* in the tumor cells through replication defective adenoviruses produced to achieve high level expression of IL-24. Technique of homologous recombination between two vectors has been employed to produce *in vivo* IL-24 expressing replication defective adenoviruses that have ability to grow only inside the specific cancer cells [1]. Cloning of full length cDNA of IL-24 amplified by PCR between specific restriction sites to construct expression vectors that use promoters of cytomegalovirus (CMV) or rous sarcoma virus (RSV) has been utilized [12]. However, temporary expression of the gene transferred through replication defective adenoviruses and risks associated with are the limitations of such *in vivo* expression systems.

IL-24 undergoes several post translational modifications, however, the fact that non glycosylated IL-24 has the ability to kill cancer cells selectively, permits the development of new strategies for recombinant production of IL-24 in *E. coli* for the cost effective and economic manufacturing of this important cytokine. Bacterial expression vectors have been employed to achieve expression of fusion products of IL-24 with Glutathione-S-transferase (GST) as GST-IL-24 and with thioredoxin (Trx) as Trx-IL-24. Expression of these fusion recombinant proteins was obtained in *E. coli* host strain BL21 (DE3) as insoluble inclusion bodies [3, 13].

The present research attempts to over-express this pharmaceutically important cytokine using bacterial expression system for subsequent use in therapeutics. We also employed *in silico* studies approach to sequence characterize the variant forms of human IL-24. Further secondary structure characteristics and 3-D conformation of human IL-24 were also predicted using online 3-D modelling servers.

MATERIALS AND METHODS

Reagents and Chemicals

Plasmid minipreparation and gene purification kits, restriction enzymes, T4 DNA ligase, DNA/ Protein markers were purchased from Thermo Scientific (Ontario, Canada). QIAgen RNeasy Mini Kit (QIAGEN Incorporation) was used for RNA isolation. All other chemicals and reagents used in this study were purchased from Sigma Aldrich (Tuofkirchen, Germany).

Plasmids, Bacterial Strains and Culture Media

pTZ57R/T used for T/A cloning of the IL-24 coding sequence was purchased from Fermentas Life Sciences (Maryland, USA). T7 promoter based expression plasmid pET28b(+) (Novagen Madison, USA) was used for the expression of IL-24. Recombinant vector was maintained in *E. coli* strain DH5α and over expression of recombinant IL-24 was obtained in *E. coli* strain BL21 (DE3) Codon Plus. Both strains were purchased from Novagen (Madison, USA). Details of strains and plasmids used are given in Table 1.

Table 1: List of *E. coli* strains and plasmids used in this study.

Strain/ plasmid	Characteristics	Reference
***E. coli* Strains**		
DH5α	F–*Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)* U*169* recA*1* endA*1* hsdR*17 (rK–, mK+)* phoAsupE*44 λ–* thi-*1* gyrA*96* relA*1*	Novagen Madison, USA
BL21 Codon plus (DE3) RIPL	F^-*ompThsdS*B (rB^-mB^-) (DE3), plasmid encoding *argU*, *ileY*, and *leuW*	Novagen Madison, USA
Plasmids		
pTZ57R/T	[a]AmpR, 3'-ddT overhangs for TA cloning of PCR products, *lacZ* for blue/white screening	Fermentas Life Sciences Maryland, USA
pTZ-IL-24	[a]AmpR, cloning and sequence analysis of IL-24 cDNA	This work
pET28b(+)	[b]KanR, protein overexpression vector,T7 promoter	Novagen Madison, USA
pIL-24	[b]KanR, IL-24 over expression vector	This work

[a]AmpR, ampicillin-resistance; [b]KanR, Kanamycin-resistance.

In routine, *E. coli* strains DH5α and BL21(DE3) Codon Plus were grown in Luria Bertani (LB) medium [tryptone 1%, yeast extract 0.5%, sodium chloride 1%, pH adjusted to 7.0] supplemented with kanamycin (100 µg mL^{-1}). Solid medium for plating was prepared by adding 1.5 % agar to the LB broth prior to sterilization. To sterilize the media it was autoclaved for 15 minutes at 121°C under steam at 15 psi pressure.

Primer Designing

For PCR amplification of IL-24 mRNA (NCBI Accession No: NM_006850.3), the gene specific primers were designed using NEBcutter[14] and Primer3plus programs [15]. The properties of primers like melting temperature (Tm), composition of bases, formation of dimers and hairpin structure and stability were further analyzed using OligoCalc properties calculator [16]. Restriction sites of the enzymes *Nde*I and *Bam*HI were added in the primers to facilitate directional cloning of IL-24 coding sequence in pET28b(+) (Table 2).

Table 2: List of primers used for the amplification of sequence coding for IL-24 mature peptide.

Primer	Sequence (5' to 3')	Tm (°C)	GC (%)	Restriction Enzyme*
IL-24 F	GCAG**<u>CATATG</u>**CAAGAATTCCACTTTGGGCC	63	50	*Nde*I
IL-24 R	GCGT**<u>GGATCC</u>**CTATCAGAGCTTGTAGAATTTCTGC	65	49	*Bam*HI

*Sites of restriction enzymes are shown as bold and underlined.

Isolation of Total RNA from PBMC

Human peripheral blood mononuclear cells (PBMC) separated from the blood of healthy individual, were stimulated with concanavalin-A as described by Poindexter and its total RNA isolated[17]. Briefly, PBMCs isolated from citrated blood by density gradient centrifugation over Histopaque (Sigma Aldrich, Germany) were cultivated in RPMI-l640 medium supplemented with L-glutamine, HEPES, penicillin/streptomycin, and heat-inactivated fetal calf serum followed by stimulation with concanavalin-A for 10-12 hours until maximum levels of cytokine mRNA expression was achieved. Following stimulation the PBMC were harvested by centrifugation and the total RNA was isolated using the QIAgen RNeasy Mini Kit according to the protocol described by manufacturer (QIAGEN Incorporation), to be used as template for subsequent RT-PCR based amplification (Fig 1).

RT-PCR Amplification of IL-24 Coding Sequence

The total RNA extracted from the PBMCs (5µL) was used as template to synthesize cDNA coding for IL-24 by reverse transcription-PCR. In a sterile 0.20 mL PCR tube, placed on ice, 50µL reaction mixture was prepared by adding cDNA made by reverse transcription of total RNA, forward and reverse primers (200 nM of each), dNTPs mix (200 µM), 1x reaction buffer containing Mg^{+2}, nuclease free water (for volume make up) and 1.25 U *Taq* DNA polymerase. A reaction with same composition without template DNA was also prepared as negative control. An iCycler Thermal Cycler (Bio-Rad, USA) was programmed to perform one cycle of initial denaturation at 95°C for 5 minutes, 25 cycles of denaturation (95ºC), annealing (65ºC) and extension (72ºC) each with a hold time of 45 sec and a final extension at 72°C for 10 minutes and the PCR tubes along with reaction mixture were placed in thermal cycler for amplification of template DNA.

Cloning of IL-24 Gene in Expression Vector

The amplification of the IL-24 cDNA was followed by agarose gel electrophoreses and subsequent purification using DNA gel extraction kit. The purified PCR product was initially cloned in pTZ57R/T vector by T/A cloning. DNA ligase was used to ligate the purified cDNA and linearized pTZ57R/T vector to construct pTZ-IL-24 recombinant plasmid. Secondly purified PCR product and expression plasmid pET-28b(+) both were restricted with *Nde*I and *Bam*HI restriction enzymes to generate sticky ends followed by purification and ligation using T4 DNA ligase to generate the pIL-24 recombinant plasmid (Fig 2a). The recombinant plasmids pTZ-IL-24 and pIL-24 were maintained in *E. coli* (DH5α). *E. coli* (DH5α). Cells were chemically made competent and transformed according to the standard protocol[18]. The transformants were identified by their resistance to antibiotic ampicillin/kanamycin and further confirmed by colony PCR and restriction analysis of recombinant plasmids isolated from transformed *E. coli* cells.

Expression Analysis of IL-24

Chemically competent *E. coli* (BL-21 CodonPlus) cells were transformed with pIL-24 and the positive transformants were identified by their ability to acquire antibiotic resistance against kanamycin antibiotic. A well-isolated, healthy colony carrying pIL-24 was transferred to 10 mL LB broth supplemented with kanamycin (100 µg mL^{-1}) and was incubated for 16 hours at 37° C. This overnight grown culture was thereafter used to inoculate fresh LB broth (100 mL) at 1:33 dilutions and placed in an orbital shaker at 37° C with 150 RPM. Cells were induced with 0.5 mM IPTG when OD_{600} reached 0.4-0.6 and expression was analyzed by SDS-PAGE after 3 hours of induction[19].

Localization of IL-24 in *E. coli* was analyzed by SDS-PAGE analysis of the total cell lyaste of induced *E. coli.* Briefly the induced cells were suspended in lysis buffer [Tris-Cl 50 mM (pH 9.5), EDTA 0.5 mM (pH 8.0), NaCl, 100 mM, PMSF 1 mM] in 1:10 (w/v) followed by sonication. Total cell lysate was centrifuged for 30 minutes at 7000 rpm and 4ºC. The insoluble fraction was resuspended in washing solution [Tris-Cl 50 mM (pH 9.5), Triton X-100 1%, NaCl, 100 mM, PMSF 1 mM] and centrifuged for 30 minutes at 7000 rpm and 4ºC. The washed inclusion bodies were analyzed by SDS-PAGE analysis.

In silico analysis of IL-24

The nucleotide sequences for IL-24 variant-1, -3 and -4 (NCBI Accession No: NM_006850.3, NM_001185156.1 and NM_001185157.1 respectively) were aligned using online accessible Clustal Omega[20] to study the similarities and differences among these sequences. Secondary structure of IL-24 was predicted by Protein Structure Prediction Server (PSIPRED)[21] to get insight into the residues involved in the basic structural conformation and its bioactivity. Three dimensional structure of IL-24 protein was predicted by Protein Homology/analogY Recognition Engine (Phyre)[22] and the resultant file was analyzed by using Visual Molecular Dynamics (VMD) software. VMD is user friendly software to visualize the molecular dimensions and its dynamic structure in different forms to easily understand the structural basis of protein[23].

RESULTS

RT-PCR Amplification of IL-24 Coding Sequence

PBMC secrete various cytokines including IL-24 upon chemical stimulation and hence possess sufficient mRNA population to be isolated for subsequent use in cDNA synthesis.

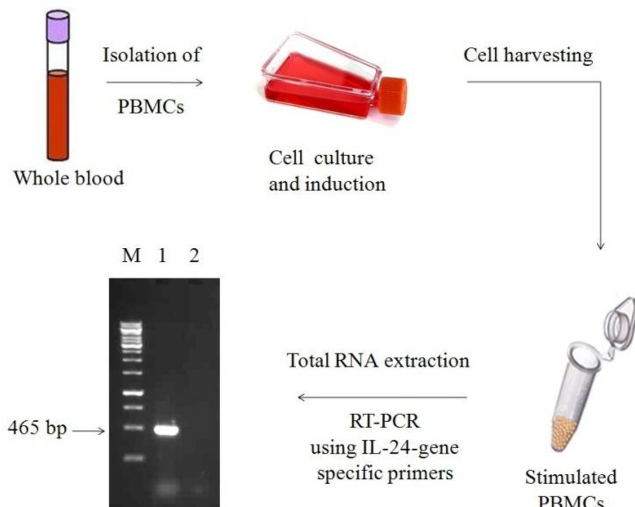

Figure 1: Induction of IL-24 in cultured PBMCs and RT-PCR amplification of IL-24 cDNA with gene specific primers. Lane M, DNA size marker; Lane 1 represents 0.465 kb, PCR product, Lane 2 represents negative control.

Total RNA isolated from these cells was thereafter used as template for the RT-PCR amplification of IL-24 cDNA. cDNA generated from total RNA and subsequently amplified by PCR at 65ºC appeared as a single, intact and prominent band of ~465 bp, when analyzed by agarose gel electrophoresis (Fig 1).

Cloning of IL-24 Gene in Expression Vector

~465 bp long cDNA for IL-24 was initially cloned in pTZ57R/T vector by T/A cloning and then subcloned in pET-28b(+) followed by transformation of *E. coli* DH5α. The efficiency of transformation was very high as a fairly good number of colonies appeared on LB agar plate supplemented with ampicillin/kanamycin. Out of several well-isolated and healthy colonies, five colonies were screened for the presence of IL-24 by colony PCR and all were found positive for presence of insert (Fig 2b). Plasmids isolated from three colonies, found positive, were further analysed for the presence of IL-24 by digestion with *Nde*I amd *Bam*HI restriction enzymes. A prominent band of ~500 bp was observed when components of restriction reaction mixture were resolved by agarose gel electrophoresis.

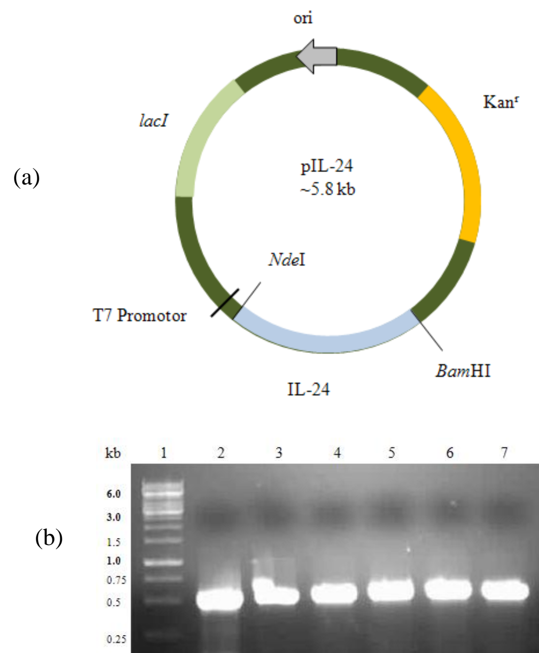

Figure 2 (a) Construction of recombinant plasmid pIL-24 by cloning an ~0.465 kb long amplicon in pET-28b(+) expression vector between *Nde*I and *Bam*HI restriction sites. **(b)** Analysis of pIL-24 transformants by colony PCR. Lane 1, DNA size marker; Lane 2, positive control; Lane 3-7 amlifications from colony no 1-5.

Expression analysis of IL-24

E. coli transformed with pIL-24 were induced with chemical inducer IPTG and expression was analyzed by SDS-PAGE. Maximum expression was observed when *E. coli* were induced with 0.5 mM IPTG in the logarithmic phase (at an OD_{600} of 0.6) for three hours at 37°C where a clear intense band at ~18kDa was observed showing expressed IL-24 protein at level of about more than 35 % of total cell proteins. Life time and cell density of primary cell culture had a great influence on the expression of IL-24 in *E. coli* where the maximum expression was obtained with a primary culture of 10 hours and OD_{600} of 3. Starting OD_{600} also affected the growth of *E. coli* transformed with pIL-24. To obtain maximum expression the starting OD_{600} was set to 0.015 that reached in 2 hours to an OD_{600} of 0.6. Soluble fraction

and insoluble fractions of total cell lysate when further analyzed on SDS PAGE, all the IL-24 protein was found to be associated with insoluble fraction of total cell lysate in the form of inclusion bodies shown in (Fig 3).

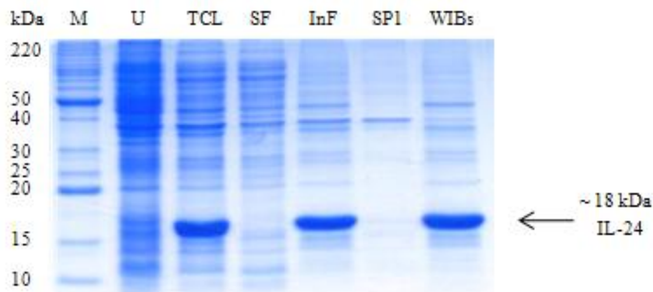

Figure 3: 15 % SDS-PAGE analysis of *E. coli* harbouring IL-24. Lanes; M, Protein size marker; U, uninduced; TCL, total cell lysate; SF, soluble fraction; InF, insoluble fraction; SP1, supernatant of first wash; WIBs, washed inclusion bodies.

In silico analysis of IL-24

Human IL-24 has three variant forms that range in size from 12 kDa to 18 kDa. The nucleotide sequences of IL-24 variant 1 and variant 3 were found exactly similar and no point of difference was found whereas 159 nucleotides (150-309) were not present in IL-24 variant 4.

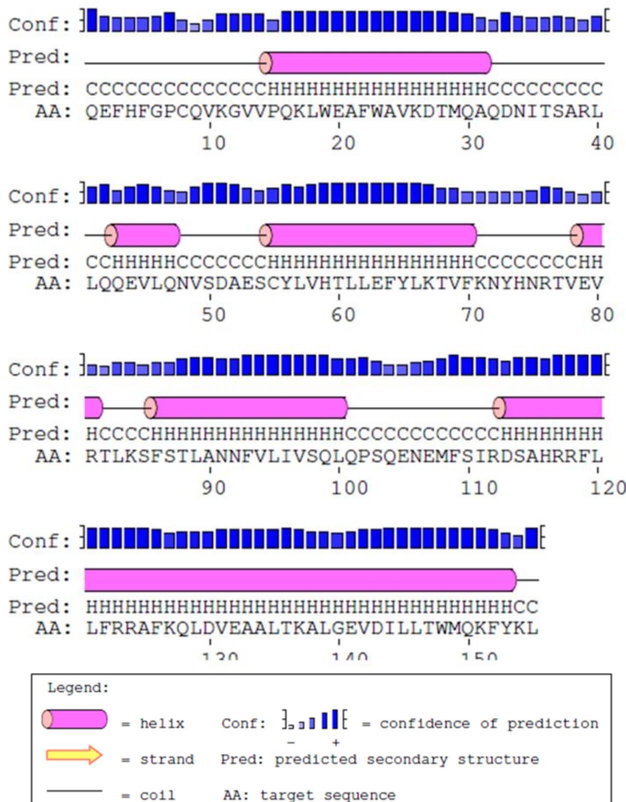

Figure 4: Secondary structure of IL-24 predicted by Protein Structure Prediction Server.

Secondary structure analysis of IL-24 by Protein Structure Prediction Server (PSIPRED) showed that this protein have five major alpha helices of different lengths connected through random coils. There were no beta sheets in this structure (Fig 4).

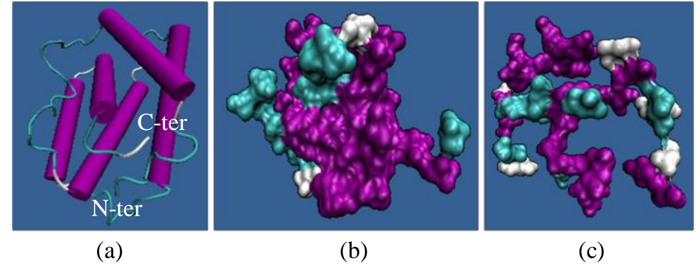

Figure 5: 3-D structure of IL-24 protein created by Protein Homology/analogY Recognition Engine (Phyre) and visualized in Visual Molecular Dynamics (VMD) **(a)** Loop and helix structure **(b)** Hydrophobic residues exposed on the surface of molecule **(c)** Charged residues exposed on the surface of molecule.

Phyre was used to construct the three dimensional structure of IL-24 protein by homology modeling. Three dimensional structure of IL-24 was visualized by VMD showing the five alpha helices arranged side by side with their ends connected by coil structures (Fig 5a). The surface structure of IL-24 showed the presence of greater number of hydrophobic amino acids as compared to number of charged amino acids (Fig 5b and c). The distribution of amino acids on the surface of a protein affects its solubility. IL-24 is hydrophobic as predicted by Peptide Property Calculator tool (Fig 6a). The isoelectric point of IL-24 predicted by ProtParam[24] is 8.5. IL-24 protein is negatively charged above pH 8.5 (Fig 6b).

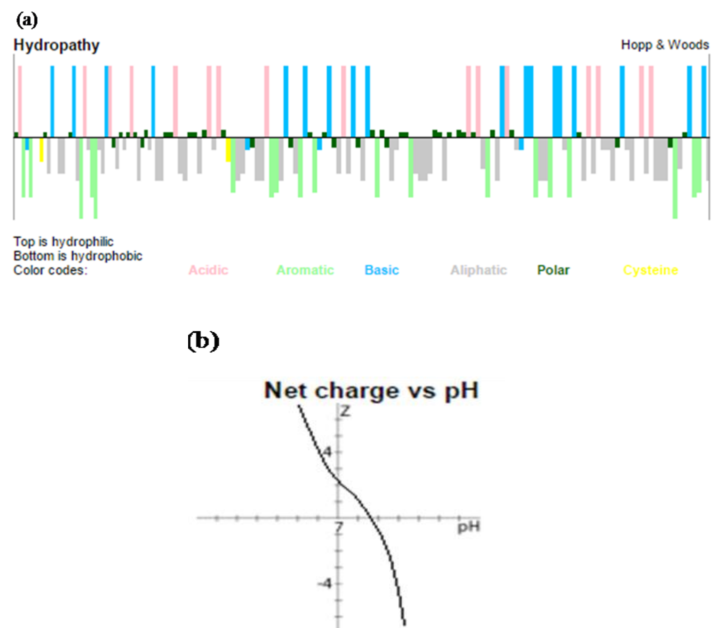

Figure 6: Solvent accessibility and effect of pH on net charge of IL-24 (a) Hydropathy profile of IL-24 shows that presence of aromatic and aliphatic residues make it a hydrophobic protein **(b)** Isoelectric point of IL-24 is 8.6 above this pH the protein is negatively charged and below this pH the protein is positively charged.

DISCUSSION

The development of suitable methods for the recombinant production of pharmaceutically important proteins is one of the major objectives of the biotechnology industry. Among the hosts for the recombinant production of important therapeutic proteins using recombinant DNA technology *Escherichia coli* is recognized as an economic and efficient host [25, 26]. In the present study IL-24, a pharmaceutically important cytokine was cloned and expressed in *E. coli*.

Total RNA extracted from cells at a particular time includes mRNA population coding for a particular protein expressed at that particular time and can be used to synthesize cDNA for subsequent amplification. IL-24 is synthesized as 206 amino acid precursor protein having a molecular mass of ~23 kDa and before secretion, a signal peptide is cleaved from precursor IL-24 protein producing mature IL-24 protein having molecular mass of ~18 kDa. In some earlier reports different lengths of signal peptide, 48 amino acids and 49 amino acids have been reported [27, 28]. In the present study SignalP 4.1 [29] was used to predict the signal peptide, which was found to be 51 amino acid long producing a mature IL-24 protein of 155 amino acids.

cDNA was synthesized followed by amplification using PCR for subsequent use as IL-24 coding sequence to produce recombinant IL-24. pTZ57R/T cloning vector offers advantages of T/A cloning and blue white screening using *lac*Z gene of *lac* operon. IL-24 mature peptide coding sequence was initially cloned in pTZ57R/T and then IL-24 coding sequence was subcloned into pET28b(+) downstream T7 promoter which is a strong promoter widely used for the over expression of recombinant proteins. To ensure the in frame directional insertion of IL-24 coding sequence in pET28b(+) expression vector, the *Nde*I and *Bam*HI restriction sites were used in the forward and backward primers, respectively.

The expression of IL-24 was achieved in *E. coli* strain BL21 (DE3) Codon Plus RIPL. IPTG is widely used chemical inducer added to the *E coli* cultures in logarithmic phase in order to achieve over expression of recombinant proteins within less time. 0.5 mM IPTG was found sufficient for over expression IL-24 protein within 3 hours at 37°C when *E coli* cultures were induced at an OD_{600} of 0.6. IL-24 was successfully over expressed with >35% expression level of total cell proteins. IL-24 was found to be associated with the insoluble fraction of the total cell laysate and was expressed as inclusion bodies in *E. coli*. Over expression of several proteins in *E. coli* is associated with formation of biologically inactive inclusion bodies which can be solubilized using mild detergents and renatured after purification[30, 31]. The IL-24 inclusion bodies were further washed with Triton X-100 and analyzed by SDS-PAGE.

Results from *in silico* analysis of human IL-24 mature peptide sequence revealed that human IL-24 is a protein folded into specific secondary and three dimensional structures similar to IL-10 family cytokines with high contents of alpha helices. Secondly, distribution of greater number of hydrophobic amino acids on the surface of folded protein, high isoelectric point and characteristic charge distribution are the factors accounting for the expression of human IL-24 as insoluble inclusion bodies in *E. coli* using conventional molecular cloning and expression techniques (Fig 5 and 6).

CONCLUSION

The gene encoding IL-24 mature peptide was amplified, cloned in T7*lac* promoter-based pET-28b(+) vector and successfully expressed in *E. coli* BL21 (DE3) Codon Plus. *In silico* studies showed that IL-24 has helical secondary structures arranged in a typical 3-D conformation. At the protein level, this protein shares 70% and 74% sequence identity with *Mus musculus* and *Mus caroli* IL-24 respectively. IL-24 is an important therapeutic cytokine, further studies on its purification and bioactivity will help to develop efficient biopharmaceutics.

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