Optimized conditions for high-level expression and purification of recombinant human interleukin-2 in E. coli

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Interleukin-2 (IL-2), a potent cytokine has been used in anti-cancer therapy for over a decade now. IL-2, originally identified as a growth factor for T lymphocytes is a 15 kDa hydrophobic glycoprotein that induces the activation, clonal proliferation and differentiation of T and B-lymphocytes and enhances the cytotoxicity of monocytes and natural killer (NK) cells. Here, we report a simple method for the cloning, high-level expression and purification of IL-2 protein, which can be easily extended to other bioactive therapeutic proteins. The IL-2 gene was amplified from human spleen cDNA and cloned in a prokaryotic (E. coli) expression system. An optimal expression of the IL-2 protein was determined by varying the expression conditions like temperature, inducer concentration and duration of induction. The protein was expressed as inclusion bodies and a panel of reagents including detergents, urea and guanidine hydrochloride were used to solubilize it. After solubilization, the protein was renatured and subjected to a single step gel-filtration chromatography to yield immuno-bioactive IL-2 protein with >99% purity.

Keywords: Interleukin-2, Inclusion bodies, Cytokines, Refolding

Modulation of immune response using recombinant cytokines is one of the strategies that have revolutionized the treatment of many important diseases and a number of cytokine immunotherapies are available in the market today. These include erythropoietin for the treatment of anemia, granulocyte-macrophage colony stimulating factor (GMCSF) as white blood cell growth factor, interleukin-2 (IL-2) for the treatment of cancer, interferon-α (IFN-α), for anti-viral therapy and IFN-β for the treatment of multiple sclerosis.

The therapeutic use of the IL-2 to stimulate T-cell proliferation has been exploited for the treatment of metastatic renal cell carcinoma and melanoma. Human IL-2 described originally as T-cell growth factor is a lymphokine produced and released by antigen-activated T-cells. It mediates its activity through binding to its receptors. T-cell receptors (TCRs) upon exposure to foreign antigens signal the expression of IL-2 receptors. NK cells also produce IL-2 receptors, albeit with a much lower affinity than those expressed by T-cells. NK cells once stimulated by IL-2 produce cytokines like IFN-γ, tumor necrosis factor α (TNF-α) and GMCSF, which stimulate macrophages. NK cells-mediated macrophage activity is an important frontline approach in preventing the spread of infection before antigen-specific T-cell responses emerge, so the potential role of IL-2 in promoting their activity is critical.

Natural human IL-2 is composed of 133 amino acid residues that are organized into four main anti-parallel, amphipathic α helices. It is extremely stable in extremes of pH and heat, with a strong hydrophobic core and a hydrophilic exterior. It is variably glycosylated, but the carbohydrates are not involved in mediating its biological activity.
A recombinant form of IL-2 is manufactured by Novartis (brand name: proleukin) and is the only FDA-approved therapy for the treatment of metastatic renal cell carcinoma (kidney cancer) and melanoma (skin cancer). The in vivo administration of proleukin in animals and humans produces multiple immunological effects in a dose-dependent manner. These effects include activation of cellular immunity with profound lymphocytosis, eosinophilia, thrombocytopenia and the production of cytokines including TNF, IL-1 and IFN-γ.

The effectiveness of IL-2 in treating IL-2 in HIV therapy are in progress and results are encouraging. Clinical studies using IL-2 in HIV therapy are in progress and results are encouraging. The effectiveness of IL-2 in treating above-mentioned conditions lies in its ability to enhance B- and T-cell responses, as well as to mobilize NK cells. As IL-2 plays a pivotal role in the clonal expansion of both activated T and B-lymphocytes, it is also useful as a vaccine adjuvant. On other hand, many immunosuppressive drugs used in the treatment of autoimmune diseases and organ transplant work by inhibiting IL-2 production by antigen-activated T-cells.

IL-2 is one of the most important cytokine for immunomodulatory functions and is produced by recombinant DNA technology. In the present study, we provide an efficient, rapid and cost-effective strategy can be further extended to other therapeutic proteins.

**Material and methods**

**Reagents**

DNA restriction and modification enzymes (NEB, USA), DNA markers (Promega, USA), PCR master mix (Qiagen, USA), concanavalin (Con A) A, protein markers (Bangalore Genei, India), Bradford’s reagent (BioRad, USA) were used. All other reagents were purchased from Sigma Chemical Co., USA.

**hIL-2 Plasmid construction**

The IL-2 gene encoding the mature polypeptide chain was amplified from human spleen cDNA (Clontech, USA) and cloned into pET22b (+) (Novagen) expression vector as a Nde1/Xho1 fragment. The sequences of the synthetic primers (Sigma-Genosys) used for cloning were: forward primer: 5'-aatatatccggactctcaagtcttacaaa-3', and reverse primer: 5'-aatctatatggtaggatgtgagatag-3'. The positive clones were identified by restriction enzyme digestion (using Nde1 and Xho1) and sequenced using standard sequencing protocols.

**Expression of hIL-2**

The positive clone was selected and transformed in the *E. coli* cells (BL21DE3). Standard procedure for induction of the target protein using isopropyl thiogalactoside (IPTG) was followed. Briefly, a single colony was inoculated in a 5 ml luria broth (LB) media containing 100 µg/ml ampicillin and grown overnight at 37ºC/ 200 rpm. The overnight culture was diluted 100 times in super broth (SB) media containing 100 µg/ml ampicillin and grown at 37ºC/200 rpm. When absorbance at 600 nm (A600) reached 0.6-0.8, culture was induced with 1 mM IPTG and harvested 3 h post-induction. An aliquot from the harvested cells was taken and analyzed by SDS-PAGE. Optimal expression of protein was standardized by varying temperature, duration of induction and inducer concentration. Immunoblot of crude extract with standard monoclonal antibodies against IL-2 further confirmed the expression of protein.

**Isolation and purification of inclusion bodies (IBs)**

The induced *E. coli* cells were centrifuged at 8000 × g for 15 min and the cell pellet was resuspended in 100 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 1 mM PMSF. Cells were lysed by sonication and centrifuged at 12000 × g for 20 min at 4ºC to isolate IL-2 protein inclusion bodies (IBs). The IB pellet was then washed with 100 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 2% deoxycholate and centrifuged at 12000 × g for 20 min at 4ºC. The pellet was washed with distilled water to remove contaminating salt and detergent and centrifuged at 12000 × g for 20 min at 4ºC to yield IL-2 enriched IBs.

**Solubilization of IL-2 protein from IBs**

IL-2 IBs were solubilized in a variety of detergents at their critical micellar concentration (CMC) viz., cetyltrimethyl ammoniumbromide (CTAB, 6 mM), 3-[3-cholamidopropyl] dimethylammonio]-1-propane-sulfonate (CHAPS, 1 mM), sodium dodecyl sulfate (SDS, 8.5 mM), N-lauryl sarcosine (NLS, 14.6 mM), nonidet-P40 (NP-40, 0.059 mM), Tween-20 (0.06 mM) and Triton-X-100 (TX-100, 0.5 mM) and varying concentrations of urea (0 to 8 M) and guanidine hydrochloride (0 to 6 M). Equal amount of IL-2 IBs pellet was resuspended in 1 ml of the above-mentioned solubilizing buffers. The suspensions were incubated at room temperature with gentle vortexing for 30 min. The sample was then centrifuged at 12000...
× g for 5 min. The pellets and the supernatants were analyzed on SDS-PAGE to check for the solubility of IL-2 protein.

Refolding and purification of hIL-2 protein

The IBs were solubilized in 6 M guanidine hydrochloride (GuHCl) solution (prepared in 0.1 M Tris buffer, pH 8.0) and incubated for 30 min at room temperature with gentle vortexing, followed by centrifugation at 12000 × g for 20 min at 4°C. The supernatant was diluted with refolding buffer (0.1 M Tris buffer, pH 8.0 containing 10 mM reduced and 1 mM oxidized glutathione in a ratio of 10:1), so as to obtain a protein concentration and GuHCl of 0.1 mg/ml and 2 M respectively. Subsequently, the solution was kept for 16 h at room temperature for slow refolding of IL-2. The insoluble protein was removed by centrifugation at 12000 × g for 20 min at 4°C. The supernatant was concentrated using Millipore, concentrator and loaded on a gel filtration column Superdex 75 HR (31 cm × 1 cm), equilibrated with 0.1 M Tris buffer containing 2 M GuHCl and connected to an FPLC system (GE Biosciences). The 1 ml fractions were collected at the rate of 0.5 ml/min and the fractions, which showed single band (corresponding to IL-2 protein) on SDS-PAGE were pooled together, dialyzed against Milli-Q water and lyophilized. Lyophilized protein was further used for immunological and bioactivity assays.

Immunological and biological assays

The immunoactivity of purified protein was determined by direct ELISA using mouse anti-human IL-2 monoclonal antibodies (R & D Systems, USA). Standard IL-2 (R & D Systems, USA) was used as a control.

Murine spleen cell proliferation assay was used to measure the bioactivity of purified IL-2 protein12,13. Briefly, splenocytes of female C57BL/6J mice (6-8 weeks old) were isolated and seeded at a density of 0.3 million (0.3 × 10⁶) cells per well in a 96-well plate. The cells were then stimulated with concanavalin A (Con A) for 48 h and subsequently treated with varying concentrations of purified IL-2 (6 pg/ml-4 ng/ml) for 24 h at 37°C and proliferation was measured by a standard MTT assay. Appropriate controls were included in the assay. The bioactivity of purified IL-2 protein was determined based on the extent of proliferation of splenocytes.

Results and Discussion

Production of biologically active proteins in E. coli requires standardization of conditions for expression as well as the optimization of the solubilizing and refolding conditions after expression. Recombinant IL-2 is traditionally expressed in E. coli as IBs14,15 and requires several steps of purification to obtain a bioactive and immunoactive protein. Our aim was to develop a simple method for solubilization, refolding and purification of IL-2 protein.

Cloning and expression of hIL-2 in E. coli system

The IL-2 gene was amplified from human spleen cDNA (Clontech), cloned as a NdeI/Xho1 fragment in a prokaryotic expression vector pET-22b(+) and further expressed in E. coli cells. Following induction at 37°C, a faint band of 15 kD, corresponding to the size of IL-2 protein was observed in bacterial cell lysate (data not shown). The expression of IL-2 protein was studied by varying the temperature, inducer concentration and duration of induction. Optimal temperature for maximal induction was determined by induction of the IL-2 clone at different temperatures, viz. 30, 37, 42 and 45°C. The expression of IL-2 protein at different temperatures was analyzed on a SDS-PAGE. No or little expression was observed at 30 and 37°C, while at 42 and 45°C, there was a substantial increase in the expression, but was found to be leaky in nature, where a low level of expression was also observed in uninduced control cells (data not shown).

The next step was to optimize the concentration of IPTG, an inducer of lac repressor-regulated promoters. Since there was a leaky expression of IL-2 protein as expected, an increase in the IPTG concentration from 0.1 to 1.5 mM did not show any significant improvement in the expression. But, as minimal amount of IPTG reduced the expression of contaminating proteins, 0.1 mM IPTG was used for induction of IL-2 (data not shown). To determine the time-course of induction, kinetic studies were carried out at 42°C and samples were taken every hour from the culture and analyzed on SDS-PAGE. Maximum expression of the IL-2 protein was attained at 3 h post-induction, and thereafter, there was a decrease in the expression (data not shown).

Thus, the optimal expression of IL-2 protein was observed at induction temperature, 42°C, inducer concentration, 0.1 mM and duration of induction, 3 h. Fig. 1a and b shows the SDS-PAGE and immunoblot
respectively of the IL-2 protein expressed under these conditions.

**Purification of IL-2 inclusion bodies (IBs)**

Usually proteins overexpressed in prokaryotic expression system are unable to fold to proper conformation and hence tend to aggregate forming IBs. Indeed, IL-2 protein also formed IBs, resulting from such aggregations, which could be both non-covalent and covalent (due to three cysteine residues) in nature. Fig. 2a outlines the strategy followed for the purification of IL-2 IBs. The IBs were extracted from *E. coli* cells and purified by sequential washing with 2% deoxycholate and Milli-Q water. These washing steps removed the contaminants, especially non-specific proteins and proteases that might have been adsorbed on to the hydrophobic IBs during processing. The purified IBs thus obtained contained ~45% of monomeric 15 kD IL-2, as determined by densitometric scanning of SDS-PAGE gel (Fig. 2b).

**Solubilization of IL-2 IBs**

Solubilization of the IBs is a critical step towards obtaining maximal amount of the desired protein in solution without inducing any chemical or deleterious modifications to it. Thus, solubility of IL-2 IBs was studied in a wide range of reagents consisting of detergents and denaturants like urea and GuHCl. The amount of solubilized IL-2 was measured by SDS-PAGE analysis.

The solubility of IL-2 IBs was checked in seven different detergents, each used at their critical micellar concentrations (CMCs) as the utility of detergents at and above their CMC is significantly hampered due to difficulty in their removal by simple methods. Among detergents, solubilization of recombinant proteins in SDS, CTAB, and sarcosyl has been widely reported. Proteins solubilized in detergents have a more ordered structure than with urea or GuHCl, which may yield active protein, thus eliminating the refolding step. In an earlier study, detergents like n-cetyl pyridinium chloride (CPC), etc were used for the solubilization of porcine growth hormone and no additional dialysis or dilution steps of the solubilization agent were required to obtain a refolded protein.

Based on the electrophoretic profile, IL-2 protein present in the IBs was found to be highly soluble (>95%) in NLS, partially soluble in CTAB (~40%), little soluble (15%) in NP-40, SDS, Tween-20 and Triton-X-100 and completely insoluble in CHAPS (Fig. 3).

The solubility of IL-2 protein was also checked in strong chaotropic agents (denaturants) like urea and
GuHCl. Interestingly, an increase in the urea concentration from 0 to 8 M did not show any significant improvement in the solubility of IL-2 protein present in the IBs, with only around 28% solubilization in 8 M urea (Fig. 4a). Whereas in GuHCl, there was a gradual increase in protein solubility from 0% in 1.5-2 M to 40% in 4 M to almost 90% in 6 M GuHCl concentration (Fig. 4b).

Thus, optimal solubilization of IL-2 IBs was achieved with NLS and GuHCl. Amongst these, GuHCl was preferred reagent for solubilization, as removal of detergents from protein is usually a cumbersome process and leads to partial denaturation of proteins25.

Refolding and purification of hIL-2 protein from IBs

Refolding of a protein is a delicate process, where a controlled removal of the excess of denaturants and reducing agents create an appropriate environment for protein to spontaneously fold into a correct conformation. It is interesting to note that no defined protocol for refolding has been found suitable for all proteins. Therefore, methods like dilution, dialysis, diafiltration, gel filtration, and immobilization on to a solid support are commonly used for the renaturation of solubilized protein26.

In case of GuHCl, solubilized IL-2 protein refolding was initiated by diluting the solution to a final protein concentration of 0.1 mg/ml and a GuHCl concentration of 2 M. Since IL-2 protein contains three cysteine residues, for proper disulfide bond formation, a redox reagent consisting of reduced and oxidized glutathione was added to the refolding buffer. Best conditions for refolding of disulfide-bonded proteins are commonly established when the reduced form is present in excess and the pH is slightly alkaline. These conditions allow rapid disulfide exchange reactions until the protein reaches the most stable disulfide-bonded configuration.
the elution profile of protein and SDS-PAGE analysis revealed that peak II contained purified IL-2 protein (Fig. 6a). This was further confirmed by immunoblot (Fig. 6b). The yield of protein was ~3 mg/L of culture (determined by Bradford’s method) and its purity was >99% as determined by densitometry (Table 1).

**Table 1—Step-wise protein recovery and IL-2 enrichment using GuHCl as a solubilization reagent**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Purity (%)</th>
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<tbody>
<tr>
<td>Inclusion bodies (IBs)</td>
<td>56.8</td>
<td>45</td>
</tr>
<tr>
<td>Solubilized protein</td>
<td>36.81</td>
<td>61.4</td>
</tr>
<tr>
<td>Refolded protein</td>
<td>22.07</td>
<td>64.7</td>
</tr>
<tr>
<td>Peak II obtained by gel filtration chromatography (Superdex-75 HR)</td>
<td>2.87</td>
<td>99.2</td>
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a Determined using Bradford’s protein estimation method; b determined by densitometry of Coomassie brilliant blue-stained SDS-gels; c derived from 1 L shake flask culture; total yield amounted to ~5 g wet cells

**Fig. 6—** (a): SDS-PAGE analysis of purified IL-2 protein [Different peaks obtained after purification of IL-2 IBs on Superdex-75 HR were analyzed by SDS-PAGE. Lanes 1, 2 and 3 represent peak III, II and I, respectively. Lane M represents the standard molecular weight markers. The IL-2 protein (15 kDa) is marked with an arrow]; and (b): Immunoblot analysis of purified IL-2 protein [Purified IL-2 protein, (peak II) (lane 1) and standard IL-2 (lane 2) were probed with anti- IL-2 antibody. The IL-2 protein (15 kDa) is marked with an arrow]

**Fig. 7—** IL-2 dependent proliferation of murine splenocytes [Murine spleen cells pre-stimulated with Con A were treated with varying concentrations of purified IL-2 and standard IL-2 and proliferation of splenocytes was measured by standard MTT assay] to be ~0.5 and ~0.25 ng/ml for purified and standard IL-2 proteins, respectively (Fig. 7).

In conclusion, we report an efficient, rapid and cost-effective method of obtaining recombinant IL-2 protein expressed as IBs in *E. coli*. This can be extended to other therapeutically important proteins both for research and clinical trials.

**References**