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Production and purification of refolded recombinant human IL-7 from inclusion bodies

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Abstract

A recombinant form of human rhIL-7 was overexpressed in *Escherichia coli* HMS174 (DE3) pLysS under the control of a T7 promoter. The resulting insoluble inclusion bodies were separated from cellular debris by cross-flow filtration and solubilized by homogenization with 6 M guanidine HCl. Attempts at refolding rhIL-7 from solubilized inclusion bodies without prior purification of monomeric, denatured rhIL-7 were not successful. Denatured, monomeric rhIL-7 was therefore initially purified by size-exclusion chromatography using Prep-Grade Pharmacia Superdex 200. Correctly folded rhIL-7 monomer was generated by statically refolding the denatured protein at a final protein concentration of 80–100 μg/mL in 100 mM Tris, 2 mM EDTA, 500 mM L-arginine, pH 9.0, buffer with 0.55 g/L oxidized glutathione at 2–8 °C for at least 48 h. The refolded rhIL-7 was subsequently purified by low-pressure liquid chromatography, using a combination of hydrophobic interaction, cation-exchange, and size-exclusion chromatography. The purified final product was >95% pure by SDS–PAGE stained with Coomassie brilliant blue, high-pressure size-exclusion chromatography (SEC-HPLC), and reverse-phase HPLC. The endotoxin level was <0.05 EU/mg. The final purified product was biologically active in a validated IL-7 dependent pre-B-cell bioassay. In anticipation of human clinical trials, this material is currently being evaluated for safety and efficacy in non-human primate toxicology studies.

Keywords: Interleukin-7; Purification; Refolding; Chromatography

Interleukins are a class of cytokines that stimulate and regulate the differentiation and proliferation of lymphoid and myeloid cells. IL-7, originally discovered as a pre-B-cell growth factor [1,2], has many diverse effects on the immune and hematopoietic systems. These effects include mobilization, maturation, and maintenance of IL-7 responsive immune cells [3,4].

Recombinant humanized IL-7 is a single-chain polypeptide, 152 amino acids long, with a molecular weight of 17.4 kDa. A proposed structural model for the rhIL-7[1] molecule, based on computer modeling and comparison to structural elements of interleukin 4, has been published. This model predicts an up–up down–down secondary structure seen in other cytokines and tertiary structures with a hydrophobic surface exposed to bulk solvent on helix D [5]. rhIL-7 has a high isoelectric point (9.3 by CZE, 8.45 by AAA), three potential N-linked

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[1] *Abbreviations used*: rhIL-7, recombinant human interleukin-7; CZE, capillary zonal electrophoresis; AAA, amino acid analysis; NK, natural killer; AIDS, autoimmune deficiency syndrome; BMT, bone marrow transplantation; CGMP, current good manufacturing practice; PCR, polymerase chain reaction; rpm, revolution per minute; OD, optical density; TSAG, tryptic soy agar plus glucose; scfm, standard cubic feet per minute; DO, dissolved oxygen; IPTG, isopropyl-β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PETG, polyethylene terephthalate copolyester; BPG, bioprocess glass; DTE, 1,4-dithioerythritol; UV, ultraviolet; HIC, hydrophobic interaction chromatography; LAL, limulus amebocyte lysate; TCA, trichloroacetic acid; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; dH_2O, distilled water; SEC, size-exclusion chromatography; TEA, tetraethylammonium; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; MCB, master cell bank; ELISA, enzyme linked immunosorbent assay; EU, endotoxin unit.

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14. ABSTRACT

A recombinant clone of human rhIL-72 thata codes for 152 amino acids was cloned into Escherichia coli HMS 174(DE3) pLys S under the control of a T7 promoter. The cloned rhIL-7 has a molecular weight of 17.4 kDa with three disulfide bonds and three sites for glycosylation. The molecule has a hydrophobic nature and heparin affinity. The isoelectric point of this recombinant protein is 9.3 by CZE and 8.45 by AAA. The insoluble inclusion bodies produced during fermentation were solubilized by homogenization with 6M guanidine HCl. The rhIL-7 inclusion bodies were enriched for monomer by size exclusion chromatography using a Superdex 200 Prep Grade solumn under denaturing conditions. The enriched, denatured rhIL-7 monomer was reduced and stratically refolded at a final protein concentration of 80 - 100 µg/ml and a final guanidine HCl concentration of 0.06 M at 2-8°C for 48 - 72 hours. The refolded monmeric rhIL-7 was subsequently purified by low pressure liquid chromatography using hydrophobic interaction cation exchange and size exclusion chromatography. The purified final product was >95% pure by Coomassie brilliant blue -stained SDS-PAGE, high pressure size exclusion chromatography and reverse phase HPLC. The endotoxin level was < 0.05 EU/mg. The final purified product was biologically active in a validated IL-7 dependent pre-B cell bioassay. In anticipation of human clinical trials this material is currently being evaluated for safety and efficacy in non-human primate toxicology studies.

15. SUBJECT TERMS
interleukin, cytokines, production, purification

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glycosylation sites, and a signal peptide of 25 amino acids. The molecule contains six cysteine residues that form three disulfide bonds indispensable for biological activity [6].

Studies on the IL-7 receptor have shown it to be a member of the hematopoietin receptor superfamily [7–9]. The IL-7 receptor shares common structural components with other interleukin receptors (IL-2, IL-4, IL-9, and IL-15), suggesting roles in T-cell regulation [10–12]. Studies have revealed that IL-7 was a potent stimulator of T-cell development [13,14]. Besides being a potent proliferative cytokine, IL-7 induces cytotoxic activity in macrophages, NK cells, and cytotoxic T-lymphocytes [15–18].

The effects of IL-7 on T- and B-cell development and its possible role in various clinical pathologies have been shown in numerous studies. In humans, IL-7 is required for development of mature T-cells, but apparently, it is not essential for B-cell development [19]. The ability of IL-7 to induce or augment antigen-specific T-cell responses and the broader cytotoxic activities of both NK cells and T cells [15–17] have possible implications for antitumor and antiviral therapy. Recent research has shown that IL-7 can be used in vitro to stimulate tumor-specific cytotoxicity [20–22]. The proliferative properties of IL-7, with its possible use as a vaccine adjuvant due to its enhancement of T-cell responses to low-affinity antigens [3,25], have also been investigated in vaccine-based therapeutic strategies [13,14,22–24]. The ability of IL-7 to mobilize hemopoietic stem cells has been demonstrated to be a major contributor to the reconstitution of T-cell homeostasis after radiation, chemotherapy, and bone marrow/stem cell transplantation [3,26]. The results from these research experiments suggest that IL-7 has promising therapeutic potential for T-cell depleted patients (cancer, bone marrow transplant, and AIDS). This paper describes the development of a robust and reproducible fermentation and purification process for large-scale CGMP manufacturing of IL-7 for non-human primate toxicology studies and Phase I clinical trials.

Materials and methods

Generation of the IL-7 clone

Dr. John Murphy, Children's Hospital, Boston, provided the rhIL-7 construct. The design and the synthesis of the gene encoding rhIL-7 used *Escherichia coli* codon-usage bias. The 456 bp gene was originally inserted into pET-11d (Novagen) by a PCR-based cloning strategy. The IL-7 coding sequence was subsequently inserted into a pET-24a plasmid (Novagen). The resulting expression vector pLC124 is kanamycin-resistant. The expression system HMS174 (DE3) pLysS (pLC124) has a lac operon in conjunction with the T7-promoter to insure stronger control over target gene expression.

Large-scale fermentation (1000 L)

The seed buildup was conducted in two stages. In the first stage, three 500-mL shake flasks were batched with 100 mL of sterile seed medium (12 g/L tryptone, 24 g/L yeast extract, 6.3 g/L glycerol, 12.5 g/L K_2PO_4, 3.8 g/L KH_2PO_4, 5 g/L glucose, 0.195 g/L $MgSO_4$, 50 μg/mL kanamycin, and 10 μg/mL chloroamphenicol). Each first-stage seed flask was aseptically inoculated with 1 mL (1%, v/v) of pooled material from four thawed vials of a working cell bank. Seed flasks were placed on a rotary shaker at 250 rpm and incubated at 37 °C. One of the flasks was selected to obtain time-course samples at 1 h intervals. The time-course samples were processed for OD_{600}, pH, and TSAG plate (observed by wet mount). Based on the growth curve (OD_{600} value vs. time), the first-stage seed culture was considered ready to scale to the second-stage seed when mid-late log phase or early stationary phase was observed ($OD_{600} = 7.5 \pm 2.0$). In the second-stage seed, three 2000-mL shaker flasks were batched with 1200 mL of the sterile seed medium previously described. Each second-stage seed flask was aseptically inoculated with 18 mL (1.5%, v/v) of the first-stage seed culture and grown under identical conditions. The criteria to scale the second-stage seed to the production fermenter were the same as those used for the first-stage seed.

The fermentation consisted of two production stages. The first-stage production was conducted in a 113-L fermenter with a working volume of 80 L and was batched with the medium previously described. P2000 antifoam was added to 0.1%, v/v. The fermenter was maintained at a temperature of 37 °C with an agitation rate of 250 rpm increasing to 550 rpm, an aeration rate of 0.6 scfm increasing to 2.4 scfm, and a vessel pressure of 5 psig. The %DO within the fermenter was maintained at >20% by first adjusting the sparging and then the agitation rate incrementally. The fermenter was inoculated aseptically with 1.2 L (1.5%, v/v) of the second-stage seed. The criteria for scaling the first-stage production to the second-stage production fermenter were based on the resulting growth curve (mid-late log phase or early stationary phase) and a requirement to reach a specific OD_{600} (7.5 ± 2.0). The second-stage production was conducted in a 1000-L fermenter (a working volume of 720 L). The second-stage production fermenter was batched with the same medium as the 80-L fermenter. P2000 antifoam was added to 0.1% (v/v) and kanamycin was added to a final concentration of 50 μg/mL. The fermenter was maintained at a temperature of 37 °C with an agitation rate of 150 rpm increasing to 350 rpm, an aeration rate of 2.5–9 scfm, and a

vessel pressure of 5 psig. The %DO and its maintenance were identical to the first-stage fermenter. The fermenter was aseptically inoculated with 11 L (1.5%, v/v) of the first-stage fermenter. The culture was induced with filter-sterilized, dioxane-free IPTG at a 1-mM final concentration when the OD_{600} reached 9.5 (± 2.0). The culture was prepared for harvest 3 h after IPTG induction.

The cell paste was recovered, using two tubular bowl centrifuges (Sharples AS16) in parallel, with a speed of 16,500 rpm. The fermentation yielded 9.3 kg of cell paste that was divided into approximately 1 kg aliquots and stored at −70 to −90 °C until proceeding to the inclusion body recovery phase of the process.

Cell recovery and inclusion body solubilization

Cell paste (7.4 kg) was resuspended in 50 mM sodium phosphate, pH 7.4, 100 mM sodium chloride, and 20 mM EDTA at a ratio of 2.33 L of buffer per kg. The cells were disrupted by passing the suspension three times through a high-pressure homogenizer (Gaulin 15M8TA) at 55 MPa (8000 psig). The OD_{600} was tested after each pass to monitor the efficiency of the process. The lysate was concentrated to 1/2–1/3 of the starting volume using a 0.2-μm hollow fiber cartridge, and then diafiltered with six volumes of 50 mM sodium phosphate, 100 mM sodium chloride, 20 mM EDTA, 1% Triton X-100, pH 7.4, followed with 10 volumes of the same buffer without Triton X-100. Samples of the wash were analyzed by reducing SDS–PAGE.

Washed inclusion bodies (3500*g*) were centrifuged for 30 min at 12,000*g*) in a 2–8 °C refrigerated centrifuge (Beckman Avanti J25I) to remove excess liquid. The total weight of the pellets after centrifugation was 1409*g* (19% recovery).

The inclusion body solubilization consisted of resuspending 1409 g of inclusion body pellets by homogenizing them in a total volume of 8400 mL of 6 M guanidine HCl (approximately 6 mL of guanidine HCl per gram of pellet). The suspension was stored overnight at 2–8 °C. The volume of the final suspension was 8895 mL (10.9 mg/mL). The solubilized inclusion bodies were dispensed into 15 × 560.0 mL aliquots in sterile Nalgene 1-L PETG bottles. The material was stored at or below −70 °C.

Resin depyrogenation

Column resins were depyrogenated by equilibrating the resin overnight with 0.1 N NaOH.

Prerefolding column chromatography

Size-exclusion chromatography: column 1

A 14.0-cm (i.d.) BPG chromatography column [Amersham Pharmacia Biotech (APB), Uppsala, Sweden] was packed with 14 L of depyrogenated Superdex 200 resin (APB). The column was equilibrated with 3- to 5-column volumes of 6 M guanidine HCl at a flow rate of 30 cm/h. Four aliquots (2320 mL total, of solubilized inclusion bodies) were separately reduced with DTE (10 mg DTE per mL of solubilized IL-7) overnight at room temperature. The reduced, solubilized material was centrifuged for 30 min at 12,000*g* in a 2–8 °C refrigerated centrifuge (Beckman Avanti J25I). The supernatant was then filtered through a 0.45-μm Millipack cellulose acetate filter (Millipore) to remove particulates. Product was loaded at 4.1% (±0.2%) column volume at a linear flow rate of 30 cm/h. The product was eluted from the Superdex 200 column with equilibration buffer. The UV absorbing (OD_{280} nm) protein was collected into sterile pyrogen-free containers. The denatured IL-7 eluted from the Superdex 200 column in a volume of 2.8–2.9 L. Three Superdex 200 column runs were performed, yielding a total of 14.8 g of monomeric IL-7 (79% recovery) from 19.2 g of solubilized inclusion bodies. The monomeric IL-7 from the Superdex 200 runs were 94–95% pure, as determined by SDS–PAGE densitometry (data not shown). Each pool was concentrated by using an Amicon concentrator with a PM-10 membrane (Millipore) at 14 psi to a final protein concentration of 10–12 mg/mL. Aliquots of denatured monomeric IL-7 in 240.0-mL volumes were stored at −80 °C.

Static refolding

The refolding conditions consisted of a 1:100 dilution of the Superdex 200 purified denatured rhIL-7 to a final protein concentration of 80–100 μg/mL. A 240.0-mL aliquot (2.4 g) of denatured Superdex 200 purified rhIL-7 was reduced with 10 mg DTE per mL for 16–18 h at room temperature. Oxidized glutathione was added to 25 L of refolding buffer (0.1 M Tris, 0.5 M L-arginine, and 2 mM EDTA, pH 9) to a final concentration of 0.55 g/L and mixed thoroughly with an overhead impeller. The reduced rhIL-7 solution was added to the completed refold buffer with a Mannostat pump at 110 mL/min with continuous mixing. The solution was incubated without mixing at 2–8 °C for 48–72 h before proceeding to the HIC chromatography step.

Refolded IL-7 purification

HIC chromatography: column 2

A 20-cm (i.d.) BPG chromatography column (APB) was packed with 1.5 L of depyrogenated Butyl Sepharose 4 Fast Flow resin. The optimum column load for the HIC step was approximately 7.6 mg/cm^2 at 2–8 °C. The HIC column was equilibrated with 10-column volumes of load buffer (1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7) at 20 cm/h. For each 25-L batch, refolded IL-7 was diluted 1:2 with 3 M ammo-

nium sulfate and 50 mM sodium phosphate, pH 7. The diluted material was loaded onto the column at 30 cm/h. The column was washed with 5- to 7-column volumes of load buffer until the UV absorbance (OD_{280} nm) returned to a stable baseline. The rhIL-7 was eluted from the HIC column with a 10-column volume linear reverse gradient from 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7, to 50 mM sodium phosphate, pH 7, at a flow rate of 20 cm/h. Fractions were analyzed by non-reducing SDS–PAGE. Gradient fractions containing greater than 90% IL-7 monomer were pooled. The final pool volume (~2 L) was stored overnight at 2–8 °C.

Buffer exchange: column 3

A 20-cm (i.d.) BPG (APB) was packed with 7 L of depyrogenated Sephadex G-25 resin (APB). The column was equilibrated with 25 mM sodium phosphate and 10 mM sodium chloride, pH 6.8, at a linear flow rate of 20 cm/h. The Butyl HIC pool was loaded at 15% (±2%) column volume at a linear flow rate of 20 cm/h. The product was eluted from the column with 1-column volume of equilibration buffer. The UV-absorbing (OD_{280} nm) product was collected into a sterile pyrogen-free container. The pool was stored at 2–8 °C overnight.

Cation-exchange chromatography: column 4

Under these running conditions, the rhIL-7 binds to the cation-exchange resin while endotoxin and DNA flow through the column. A 2.6-cm (i.d.) XK chromatography column (APB) was packed with 110 mL of depyrogenated Source 15S resin (APB) and equilibrated with 25 mM sodium phosphate, 10 mM sodium chloride, pH 6.8, at 90 cm/h. The Sephadex G-25 pool was loaded onto the Source 15S column at a flow rate of 90 cm/h with a load capacity of 56 mg/cm^2. The column was washed with 10-column volumes of 25 mM sodium phosphate, 10 mM sodium chloride, pH 6.8. The rhIL-7 was eluted from the column with a 30-column volume linear gradient from 25 mM sodium phosphate, 10 mM sodium chloride, pH 6.8, to 25 mM sodium phosphate, 1 M sodium chloride, pH 6.8. Non-reducing SDS–PAGE gels were used to analyze gradient fractions corresponding to UV absorbance (OD_{280} nm) chromatogram peaks. Gradient fractions containing greater than 90% rhIL-7 monomer were pooled. The purified material was stored as intermediate bulks at −70 °C.

Preformulation chromatography ion exchange flow-through and concentration columns: columns 5 and 6

A Q-Sepharose column was attached to the Source 15S column in tandem as a guard column to ensure complete removal of endotoxin. A 10-cm (i.d.) BPG (APB) was packed with 1460 mL of depyrogenated Q-Sepharose Fast Flow resin (APB). The Q-Sepharose column was equilibrated with 10-column volumes of 25 mM sodium phosphate, 10 mM sodium chloride, pH 6.8, at a linear flow rate of 15 cm/h. A 5.0-cm (i.d.) XK chromatography column (APB) was packed with 214 mL of depyrogenated Source 15S resin (APB). The Source 15S column was equilibrated with 10-column volumes of 25 mM sodium phosphate and 10 mM sodium chloride, pH 6.8, at a linear flow rate of 61 cm/h. The pooled material from column 4 was diluted to a conductivity of 7.5 mS/cm with 25 mM sodium phosphate and 10 mM sodium chloride, pH 6.8. The diluted IL-7 was loaded at a flow rate of 61 cm/h. After the load was completed, the system was purged with a 1-column volume of 25 mM sodium phosphate and 10 mM sodium chloride, pH 6.8. The Q-Sepharose column was disconnected and the Source 15S column was then washed with 4.6-column volumes of 25 mM sodium phosphate and 10 mM sodium chloride, pH 6.8. The rhIL-7 monomer was eluted with a 4.5-column volume linear gradient from 25 mM sodium phosphate, 10 mM sodium chloride, pH 6.8, to 25 mM sodium phosphate, 1 M sodium chloride, pH 6.8. Gradient fractions corresponding to UV-absorbing (OD_{280} nm) chromatogram peaks were analyzed by SDS–PAGE gels. Fractions containing rhIL-7 monomer were pooled and assayed for endotoxin with kinetic LAL assay.

Product formulation: column 7

A 5.0-cm (i.d.) XK chromatography column was packed with 1776 mL of depyrogenated Superdex 75 Prep-Grade resin. The column was equilibrated with 5-column volumes of 25 mM sodium phosphate and 250 mM sodium chloride, pH 6.8, at 30 cm/h. The Superdex 75 Prep-Grade column was loaded at 4.2% column volume at a linear flow rate of 30 cm/h. The product was eluted with 1-column volume of equilibration buffer at 30 cm/h. The UV-absorbing (OD_{280} nm) formulated product was collected into sterile pyrogen-free PETG containers (Nalgene).

The final product was stored at 2–8 °C for no longer than 48 h before snap-freezing in a dry ice/methanol bath. The final frozen product was stored at or below −70 °C.

Testing for potency, safety, and purity

Total protein analysis

Total protein concentrations of samples were determined using the Protein Coomassie Blue Assay kit, Bradford method (Pierce Chemical, Rockford, IL), according to the manufacturer's instructions, using bovine serum albumin (Pierce) as the reference standard.

SDS–PAGE

Samples taken at various stages of purification were analyzed by SDS–PAGE using 4–12% Bis–Tris

polyacrylamide gels (Invitrogen, Carlsbad, CA). Samples were diluted 1:2 with NuPage sample buffer (non-reducing conditions) or with NuPage sample buffer and (10%, v/v) β-mercaptoethanol (reducing conditions) and electrophoresed at 200 V (constant voltage) for 30 min. Gels were stained with Coomassie brilliant blue or silver stain, according to manufacturer's specifications.

TCA precipitation

Samples taken from the Superdex 200 step were TCA-precipitated before SDS–PAGE to remove guanidine-HCl. Sixty μL of sample was precipitated with 6 μL of 4–8 °C 6 M trichloroacetic acid (Sigma). Samples were vortexed and incubated at 4–8 °C for 30 min. To each sample tube, 180 μL of 0.6 M TCA (4–8 °C) was added; the samples were vortexed and centrifuged in an Eppendorf microfuge (Model 5415C, Brinkmann Instruments; Westbury, NY) at 10,000 rpm for 10 min at ambient temperature. The supernatant was decanted; 1 mL of −20 °C acetone was added to each sample tube and vortexed vigorously. The acetone-washed pellets were then centrifuged at 10,000 rpm for 5 min at ambient temperature, using an Eppendorf microfuge (Model 5415C, Brinkmann Instruments; Westbury, NY). The pellets were dried at room temperature for 30 min, resuspended in 60 μL Tris–EDTA–sodium chloride buffer (Biowhittaker, Walkersville, MD), and vortexed.

Reverse-phase HPLC

Samples were analyzed by reverse-phase HPLC using an AGILENT 1100 HPLC system. Fifteen μg of sample was injected onto a 150×3.9-mm C18 column (Deltapack, Waters, MA) at 1.0 mL/min. Protein was eluted from the C18 column with a linear gradient from 90% buffer A (0.1% TFA in dH_2O) to 70% buffer B (0.1% TFA, 99.99% acetonitrile). The separation was performed at ambient temperature at 1 mL/min over 30 min monitoring at a wavelength of 210 nm. Chromatograms were integrated and the area under the rhIL-7 peak was reported as a percentage of the total area detected.

SEC-HPLC

SEC was performed in phosphate-buffered saline (Biowhittaker, Walkersville, MD) with 0.5 M sodium chloride, pH 7.4, using a Waters HPLC system controlled with Millenium software. Fifty μg of sample was injected onto a 7.8-mm × 30-cm $G3000SW_{XL}$ column (TosoHaas, Montgomeryville, PA) with a SWXL guard column, at a flow rate of 0.5 mL/min. Peaks were detected by monitoring at a wavelength of 280 nm. The purity of rhIL-7 was calculated as a percentage of the total peak area detected.

Capillary zonal electrophoresis

rhIL-7 was analyzed by capillary zone electrophoresis (CZE) at 22 °C, using a Beckman P/ACE 5510 instrument equipped with a 37-cm-long fused silica capillary column with a 50-μm i.d. The capillary column was coated with 10% polyacrylamide for elimination of electro-osmotic flow and absorption of protein on the wall. The running buffer was 50 mM phosphate buffer adjusted to pH 2.2 with TEA. The running voltage was 12 kV and the current generated was 25 μA. The detection wavelength was 200 nm. The sample was diluted fivefold with water to reduce the salt concentration.

2E8 cell proliferation assay

Potency testing for the final purified material was conducted using a 2E8, IL-7 dependent murine pre-B-cell line. Samples were diluted to an initial concentration of 200 ng/mL and serially diluted down to 3.05 pg/mL. Using 96-well plates, 0.1 mL of diluted IL-7 and 0.1 mL of 2E8 cells (2×10^5 cells) were added in triplicate, resulting in a test range from 100 ng/mL to 1.53 pg/mL. The cells were incubated at 37 °C for 48–58 h and then pulsed with 1 μCi of tritiated thymidine for 16–20 h. Harvesting the cells over glass fiber filters captured the incorporated [^{3}H]thymidine. The filters were then counted in a β counter.

Other methods

Residual endotoxin levels were analyzed using a kinetic LAL method (Biowhittaker, Walkersville, MD). The level of contaminating-bacterial DNA in the final purified product was determined using a randomly primed ^{32}P-labeled *E. coli* DNA probe. Spike recovery, positive and negative controls, were analyzed in parallel with the final product and measured with an AMBIS Radioanalytical Imaging System (Charles River Laboratories, Malvern, PA). MALDI-TOF mass spectrometry was performed using a Brucker Daltonics Reflex Time-of-Flight Mass Spectrometer operating in reflection mode. Quality control analysis was performed in the NCI-Frederick, Biopharmaceutical Quality Control Laboratory. *E. coli* host cell protein was analyzed using a Cygnus ELISA kit (Cygnus Technologies, Plainville, MA).

Results and discussion

Fermentation, harvesting, and recovery

Initial studies indicated that there was significant colony-to-colony variability in rhIL-7 productivity. A single high-productivity clone was identified by screen-

ing a large number of subclones. A highly productive clone was identified, banked, and used for all studies described below. Twenty-liter pilot-scale studies showed that 1.5% inoculum volume was found comparable to that of a 5% inoculum and was used for the experiments described below.

Two 1135-L CGMP-scale fermentations of rhIL-7, with a working volume of 720 L, were performed. The pH was monitored but not controlled during the fermentation. Fig. 1 shows the increase in rhIL-7 expression up to 3 h after IPTG induction and the total rhIL-7 percentage. Induction times up to 3 h resulted in increased rhIL-7 expression with no change in production yield for induction times greater than 3 h. There was no significant difference between the maximum DO levels (80%) and lower DO levels (50%). Each of the two CGMP production runs yielded 9.3 and 11.5 kg of cell paste.

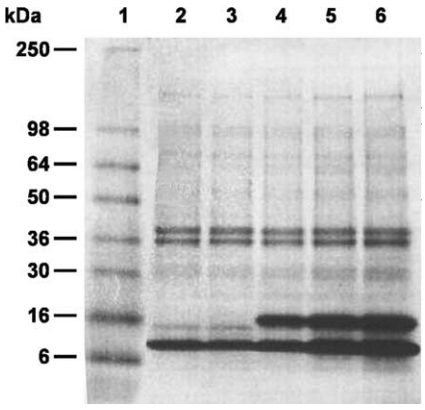

Fig. 1. Coomassie SDS–PAGE gel (reduced) Fermentation IPTG Induction Time Course samples. Lane 1: molecular weight standards; lane 2: cell lysate before induction (10 μL); lane 3: cell lysate 0 h after induction (10 μL); lane 4: cell lysate 1 h after induction (10 μL, 53.3% IL-7 by SDS–PAGE Densitometry); lane 5: cell lysate 2 h after induction (10 μL, 54.8% IL-7 by SDS–PAGE Densitometry); and lane 6: cell lysate 3 h after induction (10 μL, 59.8% IL-7 by SDS–PAGE Densitometry).

Although rhIL-7 was produced in an insoluble form, generally resistant to proteolysis, it was essential to develop an efficient, high-throughput process for the recovery of the inclusion bodies from the cell paste. Preliminary studies indicated that the IL-7 inclusion bodies were not well formed and were sensitive to high-pressure stress. Under the optimized conditions described in this study, there was an excellent recovery compared to other less-scalable methods. Further, the use of tangential-flow filtration permitted large-scale removal of debris and other adsorbed contaminants. A pronounced 10-kDa host cell contaminant was removed during the inclusion body recovery and washing steps. Fig. 2A shows the inclusion bodies at various phases of the recovery and washing process. Three recovery runs were performed, yielding 1.5 kg of washed inclusion bodies from 4 kg of cell paste.

Refolding

Preliminary development studies showed that the refolding efficiency of rhIL-7 was very poor in the presence of high molecular weight contaminants such as

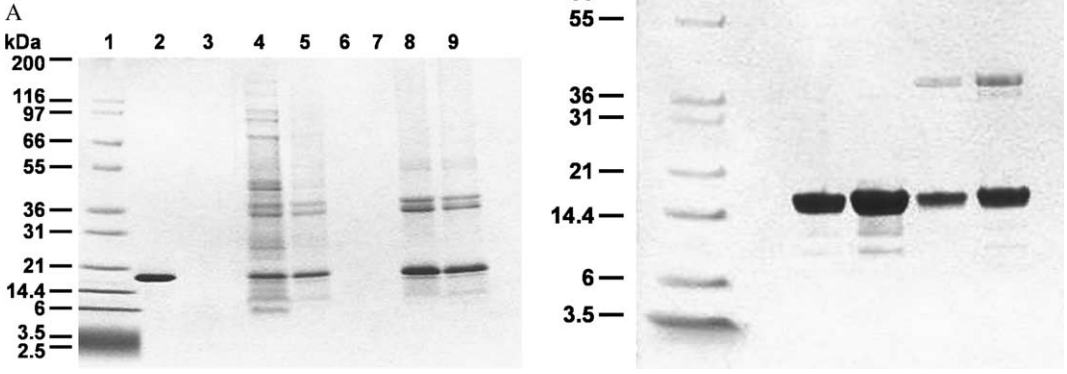

Fig. 2. Upstream removal of protein contaminants before refolding (A) Coomassie SDS–PAGE gel (reduced) Fermenter harvest and Inclusion Body Recovery samples. Lane 1: molecular weight standards; lane 2: IL-7 3 μg; lane 3: blank; lane 4: cell paste (10 μL, 20.8% IL-7 by SDS–PAGE Densitometry); lane 5: lysate (10 μL, 49.0% IL-7 by SDS–PAGE Densitometry); lanes 6 and 7: blank; lane 8: diafiltrate #2 after six volumes (10 μL, 72.7% IL-7 by SDS–PAGE Densitometry); and lane 9: washed inclusion bodies (10 μL, 62.5% IL-7 by SDS–PAGE Densitometry). (B) Coomassie SDS–PAGE (reduced) of rhIL-7 after S-200 column. Lane 1: molecular weight standards; lane 2: blank; lane 3: S-200 run #1 pool (5 μg, 95.4% IL-7 by SDS–PAGE Densitometry); lane 4: S-200 run #1 pool (10 μg, 95.1% IL-7 by SDS–PAGE Densitometry); lane 5: washed inclusion bodies (5 μg); and lane 6: washed inclusion bodies (10 μg).

DNA. The removal of these contaminants significantly improved renaturation efficiency and overall yield. Consequently, monomeric rhIL-7 was purified under denaturing conditions by size-exclusion chromatography. Fig. 2B shows the enrichment for monomeric rhIL-7 after Superdex 200 chromatography.

Various redox refolding conditions were evaluated during development. IL-7, refolded under neutral or basic pH conditions in a Tris-based buffer, maximally stimulated the proliferation of IL-7 dependent murine pre-B cells, indicating correct S–S pairing breach of the 3-disulfide bonds. The addition of 0.5 M L-arginine to the refolding buffer increased the yield of biologically active monomeric rhIL-7 by a factor of 10. The addition of 1 M guanidine HCl did not significantly improve refolding efficiency and led to a poorer recovery at the Butyl HIC chromatography. A final protein concentration of 80–100 μg/mL and 0.06 M guanidine HCl yielded the best recovery. Volume reduction by tangential flow filtration was attempted using a 30-kDa membrane. This was abandoned due to shear-induced aggregates. There was no observed increase in correctly refolded rhIL-7 beyond 48 h. Recoveries were optimal if the refolding temperature was maintained between 2 and 8 °C.

Purification

Since TFF was not a viable option for reducing the volume of the feed stream, the first step in the chromatography train was selected to concentrate and partially purify the refolded IL-7 from the redox-refolding buffer. The downstream purification process is summarized in Table 2. The high ionic-strength of the refolding solution precluded using ion-exchange chromatography. Hydrophobic-interaction chromatography was found to reversibly bind IL-7 while allowing the other components of the refolding buffer to pass through. The Butyl Sepharose Fast Flow resin from Pharmacia was determined to yield the best recovery and was able to separate monomeric IL-7 from its higher molecular weight degradation products. The Butyl Sepharose resin was evaluated with ammonium sulfate concentrations from 0.75 to 1.75 M with and without 0.5 M guanidine HCl for recovery and separation from contaminants. The latter experiments were performed as part of the evaluation of refolding conditions with 1 M guanidine HCl. Chromatography conditions of 1.5 M ammonium sulfate without guanidine HCl were selected based on optimal recovery and purity of monomeric rhIL-7. Two critical parameters for the HIC chromatography step were the quantity of protein loaded per column area (protein/cm^2) and temperature. Increasing the amount of protein loaded at or above 30 mg/cm^2 induced increased aggregation to the HIC resin and poor recovery, even when the load per mL was held constant. Temperature variations above the range of 2–8 °C resulted in a delayed elution profile for the HIC step. Fractions corresponding to UV absorbance (OD_{280} nm) chromatogram peaks were analyzed by non-reducing SDS–PAGE. Gradient fractions containing greater than 90% IL-7 monomer were pooled. For a 25 L batch (2.4-g total input protein), the recovery for the HIC step was 371 mg (15.5%).

The high salt concentrations in the HIC pool needed to be reduced before proceeding to the cation-exchange chromatography step in order for the rhIL-7 to bind. Based upon its low backpressure characteristics, high recovery, and acceptable resolution from salts at 20–25% column load volumes, size-exclusion chromatography with Sephadex G-25 resin was selected for the buffer exchange step. Recovery was typically 85% (285 mg of protein). The rhIL-7 monomer was then purified over a cation-exchange (Source 15S, APB) resin. Under these running conditions, rhIL-7 binds while negatively charged contaminants, such as endotoxin and DNA, do not bind to the resin. Further, trace amounts of residual dimeric IL-7 were easily separated from the main monomer pool when the product was eluted with a shallow 20-column volume gradient. The pool from the Source 15S column had no detectable contaminant bands by reducing and non-reducing-SDS–PAGE (data not shown). The endotoxin burden at this point in the process was 15 EU/mL. The intermediate bulk material has been stored at −70 °C for over 12 months before final polishing and formulation (12% yield).

A preclinical lot composed of a total of four 25-L batches was manufactured to produce about 1 g of purified rhIL-7 monomer. Three refold/purification campaigns starting with 2.4 g of denatured S-200 purified rhIL-7 monomer (25-L scale) and one starting with 1.0 g of denatured S-200 purified rhIL-7 monomer (10-L scale) were performed. The average yield of monomeric rhIL-7 for a 25-L campaign was about 300 mg. A total of 955 mg of purified monomeric rhIL-7 was produced from 8.16 g of solubilized inclusion bodies processed. The recoveries and yields for the overall process are listed in Table 1. The molecular weight and purity of samples from the four intermediate bulks were determined to be comparable by non-reducing SDS–PAGE (data not shown). The four intermediate bulks were pooled and further processed as described below.

Due to variability in the endotoxin burden in inclusion body preparations between different fermentation production runs, an anion-exchange column was added to the purification method to ensure removal of this contaminant. Small-scale development experiments were conducted with different anion-exchange resins and evaluated for endotoxin removal and rhIL-7 recovery. Based on the results from these experiments, Q-Sepharose Fast Flow (APB) resin was selected. The Q-Sepharose Fast Flow resin binds 14–18 EU/mL resin with a recovery of rhIL-7 greater than or equal to 90%. The Q-Sepharose Fast Flow resin was added as a flow-

Table 1
Process summary and yields

Sample	Concentration (mg/mL)	Volume (mL)	Total protein (g)	% Recovery	% Yield
Cell paste			803		
Washed/pelleted inclusion bodies			153	19	19
Solubilized inclusion body	10.9	972	10.6	6.9	1.3
S-200 purified IL-7 monomer	10–12	680–816	8.16	77	1.0
Refold/purification	0.94	1016	0.955	12	0.1
Formulation	1.18	768	0.906	95	0.1

through column upstream of the Source 15S concentration column with a capacity set at 10 EU/mL resin. The product from this phase of the purification process has been found to be extremely stable with no observed loss of potency or physical integrity for over 24 months.

Before formulating the purified rhIL-7 into the final formulation buffer by SEC, the product had to be concentrated to meet the target protein concentration of 1mg/mL. Since TFF was not a viable option, step elution from Source 15 Sepharose was selected. The preformulation Source 15S concentration column was scaled down 1.5-fold from the purification column, with an IL-7 binding capacity of 4.5 mg/mL. The rhIL-7 following elution was concentrated sevenfold to 6.6 mg/mL. There was no evidence of particulates and the protein solution was clear.

Dimerization of IL-7 was most pronounced at acidic pH. During storage of the intermediate bulks at −70 °C, we have observed the formation of dimer due to pH excursions during freezing and thawing in a sodium phosphate-based buffered solution. Thus, SEC was added as a final polishing and formulation step. Superdex 75, Superdex 200, and Sephadex G-25 resins were evaluated for the formulation of purified IL-7. Formulation of purified IL-7 into low pH buffer conditions had acceptable stability but had lower recovery (76%) due to non-specific adsorption to the Superdex resins. Sephadex G-25 resin had acceptable recovery (91%) but the resin's fractionation range of 1–5 kDa lacks the ability to separate rHIL-7 monomer from multimeric forms. The Superdex 75 PG resin has fractionation capability within the range of molecular weight sizes for monomeric and multimeric forms of rHIL-7 with recoveries of 92% or better using 25 mM sodium phosphate, 250 mM sodium chloride, pH 6.8, buffer. Based on these criteria, the Superdex 75PG resin was selected for the formulation step. A summary of the overall process for the refolding and intermediate bulk purification for one of the 25 L toxicology purification campaigns with individual step yields is presented in the upper half of Table 2. The final purification and formulation chromatography for the combined intermediate bulks are shown in the bottom portion of Table 2.

Purity, safety, and potency analysis of the final product

The final purified product was evaluated for safety, purity, and biological activity to ensure the quality of material for toxicology. SDS–PAGE Coomassie brilliant blue-stained gels run under reducing and non-reducing conditions showed a single band at 17.4 kDa (data not shown). Non-reduced silver-stained SDS–PAGE gels (Fig. 3C) showed a single band at the same molecular weight. Under reducing conditions, some

Table 2
Purification summary Lot A

Sample	Concentration (mg/mL)	Volume (mL)	Total protein (mg)	% Starting	% Previous	EU/mL
S-200 purified inclusion bodies	10	239.6	2396	5.3	77	
Refold						
	Pool #1–0.143					Pool #1–7.34
Butyl HIC		2750	371	15.5	15.5	
	Pool #2–0.127					Pool #2–39.56
Desalting (Sephadex G-25)	0.092	3100	285	11.9	76.7	0.81
Source 15S (cation exchange)	0.98	312	305	12.7	107	4.6
Combined four intermediate bulks together						
Intermediate bulks	0.94	1021	958.4			13
Diluted intermediate bulks	0.178	5500	979	101	101	1.54
Q-Sepharose fast flow (anion exchange)[a]						
Source 15S (cation exchange)	6.6	150	990	101	101	<0.1
Formulation (Superdex 75PG)	1.18	768	906	92.5	91.5	<0.05

[a] Q-Sepharose column attached in tandem to Source 15S column.

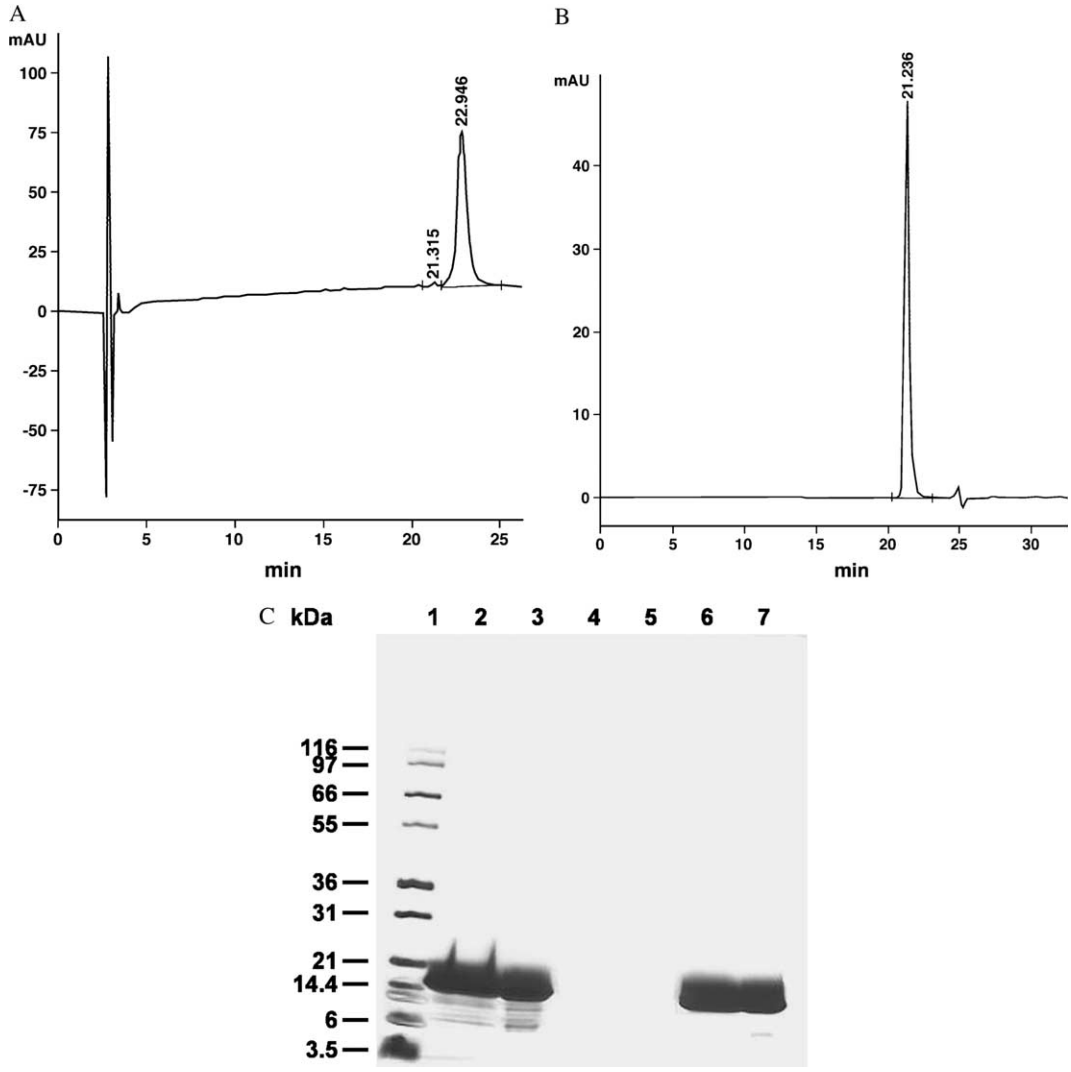

Fig. 3. Purity of the final purified bulk IL-7 following polishing on Superdex 75. (A) Reverse-phase HPLC. Eighteen μg of purified IL-7 was analyzed on a C18 column; detection was at OD_{210} nm, peak purity was >98%. (B) SEC-HPLC. Ten μg of purified IL-7 was analyzed on a G3000SwxL column; detection was at OD_{280}, peak purity was >99%. (C) silver stain. Lane 1: molecular weight standards; lane 2: 4 μg of purified IL-7 under reducing conditions; lane 3: 4 μg of purified IL-7 reference standard under reducing conditions; lanes 4 and 5: blank; lane 6: 4 μg of purified IL-7 under non-reducing conditions; and lane 7: 4 μg of purified IL-7 reference standard under non-reducing conditions.

minor contaminants were detected in the 6–14.4 kDa range. MALDI-TOF provides supporting data for the molecular weight of the final product with peaks corresponding to a mass of 17,556.9 and 8771.7 Da (doubly ionized) and two minor peaks at 35,104 and 5859.3 Da (data not shown). The 35,104 Da peak corresponds to dimer, an artifact of ionization, since there was no observed dimer by SDS–PAGE or SEC. The 5859.3 Da peak could be an indication of triply charged rHIL-7 or could be some of the lower molecular weight contaminants present in the reduced silver-stained SDS–PAGE. These observed molecular weights are consistent with the predicted molecular weight. The MALDI mass spectrometry data for the toxicology material and development reference standard are comparable. No detectable contaminant peaks were present in the CZE analysis of purified rhIL-7 (data not shown). The final purified product was greater than or equal to 98% purity by C18 reverse-phase HPLC and SEC (Figs. 3A and B). The endotoxin burden was less than 0.05 EU/mL (0.042 EU/mg). The activity in the bioassay was comparable to the reference standard (Fig. 4). Analysis for *E. coli* host cell protein contaminants by ELISA was 4.1 ng/mL (3.5 ng/mg).

Stability

Purified rhIL-7 was evaluated for physical stability at 4 and 37 °C in 50 mM sodium acetate, pH 4.7; 50 mM citrate, pH 6.0; and 25 mM sodium phosphate, pH 6.8. rhIL-7 was tested for up to 96 h and evaluated by SDS–PAGE silver-stained gels. There was no evidence of

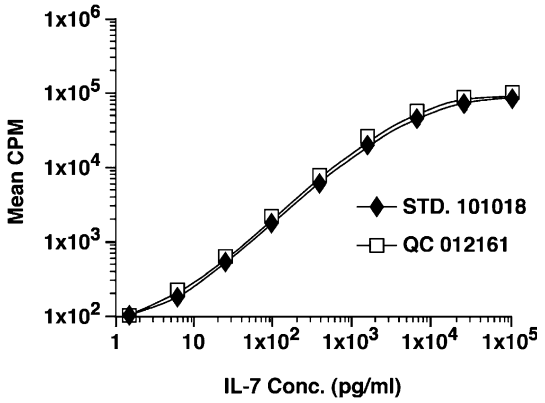

Fig. 4. Bioactivity of the final purified bulk IL-7 following polishing on Superdex-75. Log–log plots of independent 2E8 bioassay for the IL-7 reference standard (STD 101018) and the purified IL-7 (QC012161).

significant degradation at these temperatures and buffer conditions relative to the zero time-point control. Silver-stained gels were used to assess the physical stability of rhIL-7 under −70 °C storage conditions. Samples of the purified rhIL-7 intermediate bulks were stable for more than 24 months under these storage conditions. The bioactivity of the toxicology lot of rhIL-7 was comparable to the reference standard. No significant decrease in the bioactivity of the reference standard was observed during the past 2 years (data not shown).

Summary

IL-7 is a potent proliferative cytokine for the stimulation of the adaptive immune response. Early studies in mice identified IL-7 as a non-redundant cytokine necessary for B-cell and T-cell development [27]. In humans, IL-7 is necessary for development of T cells but not B cells. IL-7 has potential therapeutic applications for the lymphoid reconstitution of immuno-depressed patients (chemotherapy, radiation, AIDS, and bone marrow transplant).

This paper described a robust and reproducible CGMP-compliant process for the manufacture of material for non-human primate toxicology and human Phase I clinical studies. The resulting final product was biologically active with high purity (>95%) and low endotoxin (<0.05 EU/mL). The process yielded 10 mg of purified IL-7 per liter of fermenter product.

Disclaimer

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