

Scale-up Purification of a Mutant of Recombinant Human Interleukin 2 by Reverse-phase High Performance Liquid Chromatography

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ABSTRACT

Reverse-phase high performance liquid chromatography (RP-HPLC) is the final step of a large-scale purification procedure for recombinant human [Ala-¹²⁵]interleukin 2 (rhIL-2A¹²⁵). This step was 25-fold scaled up to a 5 x 25 cm C₄-bonded reverse phase column and the rhIL-2A¹²⁵ recovery was about 90%. rhIL-2A¹²⁵ was over 97% pure in collected fractions as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry scanning of the gels. Contaminating proteins from *Escherichia coli* were less than 2 µg/mg of rhIL-2A¹²⁵ according to dot-blot immunoassay. Analytical RP-HPLC of purified rhIL-2A¹²⁵ evidenced three peaks: P_A, representing less than 7% of total protein; P_B, the major peak, and P_{B'}, representing less than 1% of total protein, where P_A + P_B + P_{B'} = 95%. The specific bioactivity of the protein purified by preparative RP-HPLC ranged from 1.1 to 2.0 x 10⁷ IU/mg. Analysis by isoelectric focusing (IEF) distinguished four rhIL-2A¹²⁵ species in the purified protein fraction: two major bands corresponding to the N-terminal alanine and N-terminal methionine rhIL-2A¹²⁵, and two minor species that apparently correspond to C-terminal modified and Met-104-sulfoxide forms. The results described in the present paper demonstrate the feasibility of using preparative RP-HPLC for obtaining large amounts of purified rhIL-2A¹²⁵.

Keywords: interleukin 2, RP-HPLC, scale-up purification

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RESUMEN

Escalado de purificación de un mutante de la interleuquina 2 humana recombinante mediante cromatografía líquida de alta resolución en fase inversa. Un mutante de la interleuquina 2 humana recombinante (rhIL-2A¹²⁵) se purificó a escala preparativa. La cromatografía líquida de alta resolución en fase inversa (RP-HPLC) se usó como paso final y se escaló 25 veces hasta una columna de 5 x 25 cm. El recobrado de rhIL-2A¹²⁵ en este paso fue superior a 90%. La pureza del material obtenido fue superior a 97%, según se estimó mediante el análisis densitométrico en geles desnaturalizantes de poliacrilamida. La determinación de proteínas contaminantes de *Escherichia coli* por inmunoblot demostró niveles inferiores a 2 µg/mg de rhIL-2A¹²⁵. En el estudio analítico por RP-HPLC se detectaron tres picos de rhIL-2A¹²⁵: P_A, que representa menos de 7% de la proteína total; P_B, pico fundamental; y P_{B'} ≤ 1% de la proteína total, de manera que P_A + P_B + P_{B'} ≥ 95%. La actividad específica del material purificado en la escala preparativa estuvo en el rango de 1,1 a 2,0 x 10⁷ UI/mg. El análisis por isoelectroenfoque permitió identificar cuatro especies de rhIL-2A¹²⁵ en la fracción proteica purificada: dos bandas principales que corresponden a moléculas con alanina N-terminal y metionina N-terminal, y otras dos especies minoritarias que parecen corresponder a formas modificadas en el extremo carboxilo y a moléculas con Met-104 sulfoxido. Los resultados presentados demuestran la factibilidad del empleo del RP-HPLC preparativo para obtener la rhIL-2A¹²⁵ en cantidades de gramos.

Palabras claves: escalado de la purificación, interleuquina 2, RP-HPLC

Introduction

Interleukin 2 (IL-2) was initially described as a T cell growth factor [1, 2] and since then several functions have been ascribed to this lymphokine which plays a central role in immunity. The isolation of the gene encoding IL-2 [3] permitted the production of enough recombinant protein for clinical trials, which has made IL-2 to gain a considerable attention since the last decade as a therapeutic agent for some types of cancer [4, 5]. In recent years, its safe use for immunotherapy has been recognized, and clinical trials denote that IL-2-based therapy is promising for chronic infectious diseases, especially for HIV infection and hepatitis C [6–8]. Therefore, therapeutic use strongly demands recombinant sources and the scaling up of purification processes.

Different procedures have been utilized for the purification of human recombinant IL-2 (rhIL-2) from insoluble protein aggregates [9–12]. The purification of a mutant rhIL-2 where the Cys-125 was substituted for serine has also been reported [13]. Reverse-phase high performance liquid chromatography (RP-HPLC) is frequently used as the final step in these procedures, which usually includes other purification steps like ionic exchange or affinity chromatography. In general, all these procedures use RP-HPLC at micro- or semi-preparative scale.

Herein we present a procedure for scaling up the RP-HPLC purification step for a recombinant analogue of hIL-2 produced by replacing Cys-125 with alanine (rhIL-2A¹²⁵). This article also provides a method for purification of this analogue of human

1. Morgan DA, Ruscetti FW, Gall R. Selective *in vitro* growth of T lymphocytes from normal human bone marrows. *Science* 1976;193:1007–8.

2. Gillis S, Smith KA. Long-term culture of tumor-specific cytotoxic T cells. *Nature* 1977;268:154–6.

3. Taniguchi T, Matsui H, Fujita T, Takaoka, Kashima N, Yoshimoto, R, Hamuro J. Structure and expression of a cloned cDNA for human interleukin-2. *Nature* 1983;302:305–10.

4. Rosenberg SA. Interleukin-2 and the development of immunotherapy for the treatment of patients with cancer. *Cancer J Sci Am* 2000;6:S2–7.

IL-2 at the gram scale to render a molecule suitable for preclinical testing.

Materials and Methods

Equipment

The HPLC system included two 2248 pumps (Amersham Pharmacia Biotech, Sweden) and a 7010 loop (Rheodyne, USA) for the analytical scale. Effluents were monitored by measuring absorbance at 280 nm with a 2238 Uvicord S II (LKB-Bromma, Sweden).

Recombinant hIL-2A¹²⁵ production and up-stream purification

The mutant IL-2 protein was expressed at high levels in *Escherichia coli* JM101 harboring the pIL-2mA12 plasmid. This plasmid was obtained by cloning the gene encoding the mature mutant protein in the pFP-15 plasmid [14]. Recombinant hIL-2A¹²⁵ was expressed as insoluble aggregates in inclusion bodies and was mainly isolated from contaminant proteins by a cell pellet washing procedure. Insoluble proteins were then solubilized with 6 M guanidine hydrochloride (Fluka, USA) and renaturation was carried out using gel filtration chromatography (GFC) on a Sephadex G-25 column equilibrated with 10 mM Tris-HCl, 25 mM NaCl, pH 8.0 at 4 °C.

Reverse-phase high performance liquid chromatography

All RP-HPLC steps were carried out at room temperature. RP-HPLC was performed using a solvent system including: solvent A, 0.1% TFA (Fluka, USA) in H₂O and solvent B, ACN (Riedel-deHaën, Germany) containing 0.05% TFA. RP-HPLC columns were used in accordance with the purification scale: semi-preparative, 1 x 25 cm (Vydac, USA) and preparative, 5 x 25 cm (Vydac, USA). Bonded phase was C₄ in all cases. Flow rates were selected for each column following the manufacturer's recommendation (semipreparative column, 2 mL/min and preparative column, 45 mL/min). For applying onto Vydac columns, the pH of the effluent from GFC was adjusted to 3 with acetic acid (Riedel-deHaën, Germany) at room temperature, centrifuged at 12,000 *xg* for 15 min at 4 °C and filtered through a 0.2 µm membrane (Sartorius, Germany). Protein mixtures were loaded onto the RP columns at concentrations around 0.1 mg/mL. Sample loads were up to 30 mg for the semipreparative, and up to 1500 mg for the preparative scale. rhIL-2A¹²⁵ was eluted with segmented lineal gradients from 15% to 80% of solvent B (Table 1).

Analytical procedures for characterizing the purified protein

Purified protein peaks were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electro-

phoresis (SDS-PAGE), dot-blot immunoassay for quantitative analysis of *E. coli* contaminant proteins, and analytical RP-HPLC. The biological activity was also measured and isoelectric focusing was performed to several purification batches.

Protein determination. Protein concentration was determined by a modification of the method of Lowry *et al.* [15], using trichloroacetic acid for protein precipitation [16, 17].

SDS-PAGE. Thirty micrograms of protein were vacuum-dried, dissolved in sample buffer (50 mM Tris HCl pH 6.9, 7 mM 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heated at 100 °C during 5 min before electrophoresis. SDS-PAGE was performed on 15% polyacrylamide gels as described by Laemmli [18]. The protein weight marker (CIGB, Cuba) contained BSA (67 kD), IgG (50 and 25 kD), recombinant alpha interferon (19.4 kD) and lysozyme (14 kD).

Dot-blot for *E. coli* proteins. The presence of *E. coli* contaminant proteins was determined as follows: 30 µg of protein were vacuum-dried, dissolved in 50 µL of PBS and transferred to nitrocellulose filters using a Minifold apparatus (BioRad, USA). Two-fold dilution curves of *E. coli* contaminant proteins from 1 mg/well were included. Filters were blocked by incubating with 5% skim milk in PBS during 90 min at 42 °C. A polyclonal antiserum against *E. coli* JM101 proteins, raised in rabbits by a cascade procedure, was used for detecting contaminant proteins. Binding of specific antibody was visualized by protein A-colloidal gold complex formation.

Analytical RP-HPLC. One hundred micrograms of purified rhIL-2A¹²⁵ were three-fold diluted with solvent A and loaded onto the analytical column (0.46 x 25 cm; JT Baker Research Products, USA) at a flow rate of 0.8 mL/min. The elution was carried with the lineal gradient for analytical scale described in Table 1.

Isoelectric focusing (IEF). Several randomly selected batches from the preparative scale procedure were analyzed by IEF. IEF was performed on Ampholine polyacrylamide gel plates (pH 3.5–9.5) using a flat bed apparatus 2117 Multiphor (LKB Bromma, Sweden). Samples were vacuum-dried and dissolved in distilled water to a final concentration of 1 mg/mL. Between 10–15 µg of protein were applied onto the gels. Electrophoresis was carried out at 7 °C for 100 min at a constant power of 25 W. IEF standards were purchased from Amersham Pharmacia Biotech (Sweden). Gels were stained with Coomassie Blue R-250 following fixation in 12% trichloroacetic acid.

Biological assay for IL-2. To determine the biological activity of the purified protein, RP-HPLC peaks were first dialyzed, formulated at 0.5 mg of protein/mL in 0.01% SDS and freeze-dried at 2 mL/vial. The biological activity of rhIL-2A¹²⁵ was estimated with an IL-2-dependent cell proliferation assay using the murine cytotoxic T-cell line CTLL-2 (ATCC PIB 214) [19]. Briefly, 40 000 cells/well were seeded in 96-well microtiter plates and cultured for 48 h in the presence of serial log2 dilutions of a standard IL-2 preparation or the experimental samples. Cell viability was then assessed by the modified method of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [20]. One unit of IL-2 biological activity (U/mL) is defined as the reciprocal of the dilution required to sustain half-maximal responses.

5. Lindsey KR, Rosenberg SA, Sherry RM. Impact of the number of treatment courses on the clinical response of patients who receive high-dose bolus interleukin-2. *J Clin Oncol* 2000;18:1954–9.

6. Davey RT, Murphy RL, Graziano FM, Boswell SL, Pavia AT, Cancio M, *et al.* Immunologic and virologic effects of subcutaneous interleukin 2 in combination with antiretroviral therapy: a randomized controlled trial. *JAMA* 2000;284(2):183–9.

7. Chun TW, Engel D, Mizell SB, Hallahan CW, Fischette M, Park S, *et al.* Effect of IL-2 on the pool of latently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. *Nature Medicine* 1999;5(6):651–5.

8. Pardo M, Castillo I, Oliva H, Fernández-Flores A, Barcena R, de-Peuter MA, *et al.* A pilot study of recombinant interleukin-2 for treatment of chronic hepatitis C. *Hepatology* 1997;26(5):1318–21.

9. Bailon P, Weber DV, Keeney RF, Fredericks JE, Smith C, Famillethi PC, *et al.* Receptor-affinity chromatography: a one-step purification for recombinant interleukin-2. *Biotechnology* 1987;5:1195–8.

10. Sano C, Ishikawa K, Nagashima N, Tsuji T, Kawakita T, Fukuhara K, *et al.* Crystallization and preliminary X-ray studies of human recombinant interleukin-2. *J Biol Chem* 1987;262:4766–9.

11. Weir MP, Sparks A, Chaplin AM. Micro-preparative purification of recombinant human interleukin-2. *J Chromatography* 1987;396:209–15.

12. Hora MS, Rana RK, Wilcox CL, Katre NV, Hirtzer P, Wolfe SN, Thomson JW. Development of a lyophilized formulation of interleukin-2. *Dev Biol Stand* 1992;74:295–306.

13. Wolfe SN, Dorin GJ, Davis JT, Smith F, Lim A, Weissburg R, inventors; Cetus Corporation, assignee. Process for recovering purified, oxidized, renatured recombinant interleukin-2 from microorganisms. US patent 5,162,507. 1992 Nov 10.

14. Novoa LI, Machado JA, Fernández JR, Benitez JV, Narciandi RE, Rodríguez JL, *et al.*, inventors; CIGB, assignee. Method for the expression of heterologous proteins produced in fused form in *E. coli*, use thereof, expression vectors and recombinant strains. European Patent 0416673. 1995 Nov.

15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265–75.

16. Bensadoun A, Weinstein D. Assay of proteins in the presence of interfering materials. *Analytical Biochemistry* 1976;70:241–50.

17. Peterson GL. A simplification of the protein. *Analytical Biochemistry* 1977;83:346–56.

18. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.

19. Gillis S, Ferm MM, On W, Smith KA. T cell growth factor: parameters of production and a quantitative microassay for activity. *J Immunol* 1978;121:2168–73.

20. Tada H, Shiho O, Kuroshima K, Koyama K, Tsukamoto K. An improved colorimetric assay for interleukin-2. *J Immunol Methods* 1986;93:157–65.

Table 1. Gradient conditions for eluting proteins loaded onto C₄ reverse-phase columns of different dimensions.

	Columns		
	0.46 x 25 cm	1 x 25 cm	5 x 25 cm
Gradient conditions	35–73% B	15–45% B	15–45% B
	40 min	10 min	20 min
	0.8 mL/min	6 mL/min	45 mL/min
		45–80% B	45–75% B
		47 min	52.6 min
		2 mL/min	45 mL/min

Results and Discussion

Scale up of RP-HPLC rhIL-2A¹²⁵ purification

Human IL-2A¹²⁵ was obtained by replacing the cysteine at position 125 with alanine using site-directed mutagenesis, so that the intramolecular disulfide bond could only be formed between Cys-58 and Cys-105, as necessary for obtaining fully active molecules. This procedure avoided contamination of incorrectly folded species, which in intact IL-2 preparations mainly results from isomerization of disulfide bonds due to the presence of the free, reactive sulfhydryl group in Cys-125. In addition, through this substitution IL-2 oxidative dimerization is largely abrogated. These non-desired molecular species that are commonly found in recombinant IL-2 preparations, reduce the refolding yield and sometimes the specific activity of the final products.

Although the use of RP-HPLC has been broadly reported for the purification of recombinant IL-2 at the analytical and semipreparative scale, gram-scale RP-HPLC as the main purification step of rhIL-2 is scarcely reported. Herein we evaluated the feasibility of using preparative RP-HPLC for the purification of rhIL-2A¹²⁵ to be tested for therapeutic use.

Effluents from GFC contained soluble rhIL-2A¹²⁵ about 65% pure. This purity is essentially reached through the cell pellet washing procedure as GFC has a relatively low purification rate. Renaturation was achieved by dilution and gel filtration in an aqueous environment at pH 8. However, as Vydac columns used for RP chromatography have a working pH range between 2 and 6.5 (manufacturer's instructions), rhIL-2A¹²⁵ solubility over this range was evaluated to choose the optimum pH for loading GFC pools onto RP columns.

Samples from GFC eluate were adjusted to a different pH by adding concentrated acetic acid and centrifuged to separate pellet and supernatant in order to determine protein concentration and for analysis by SDS-PAGE. rhIL-2A¹²⁵ solubility was referred to as the percentage of soluble protein relative to the total amount of protein in each sample (Figure 1). Solubility diminished as pH decreased from 8.0 (70%) to 5.0 (19%), but by further decreasing pH the percentage of soluble protein increased up to 85% at pH 3.0. This is in agreement with previous reports which described that IL-2 only dissolves at extreme pH values in the absence of detergents [21]. These results defined pH 3.0 as the working pH for loading rhIL-2A¹²⁵ onto RP-HPLC columns.

The purification parameters were first adjusted at the semipreparative scale where a two-step lineal gradient was established (Table 1). The first step was designed for quick elution of the less retained proteins, corresponding to non-IL-2 species. The second step, where the rhIL-2A¹²⁵ fraction is eluted, was performed by increasing 0.74% of solvent B/min (flow rate: 2.5 cm/min) and 4.8 column volumes were passed on. Recombinant hIL-2A¹²⁵ was eluted at 38.8 min corresponding to 66.5% of solvent B. The protein peak was collected in different fractions in order to reduce the presence of minor contaminating IL-2 forms that elute just at the front of the main peak (Figure 2, peak A). This species correspond to Met-104 sulfoxide rhIL-2A¹²⁵ (rhIL-2A¹²⁵M¹⁰⁴) and C-terminal modified forms [22].

Scaling up to preparative columns was set up at a flow rate of 2.3 cm/min extending the running time to

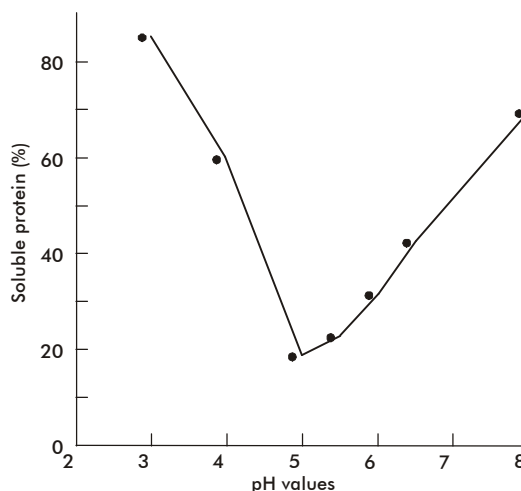


Figure 1. Solubility behavior at different pH of rhIL-2A¹²⁵. The pH of GFC effluent was adjusted to different values in the range from 8.0 to 3.0 by adding acetic acid. Samples were centrifuged and protein quantity was estimated in pellets and supernatants. Results are presented as percentage of soluble protein.

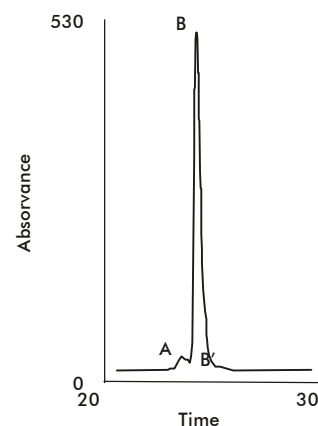


Figure 2. Analytical RP-HPLC of a purified rhIL-2A¹²⁵ fraction obtained by preparative RP-HPLC. 100 µg of total protein were loaded onto a C₄ analytical column (0.46 x 25 cm). Elution was carried out with a lineal gradient from 35% to 75% ACN containing 0.05% TFA.

52.6 min to maintain total gradient volume. The protein peak corresponding to rhIL-2A¹²⁵ was eluted at 53 min (63.8% solvent B) at gradient increments of 0.57% of solvent B/min. The rhIL-2A¹²⁵ recovery for this chromatographic step was over 90%.

At the preparative scale, resolution of rhIL-2A¹²⁵ from other proteins was high enough to permit at least a 2.4-fold increase in protein load per millimeter relative to semipreparative scale. This was accomplished just by accommodating gradient steepness to bed geometry as proposed by other authors for IL-2 analogues [21]. The substitution of alanine at position 125 stabilizes protein structure during RP retention, as suggested by the previously described slight decrement of the protein stationary phase interaction for this mutant [23], which produced an increase in RP retention. Other factors that contribute to rhIL-2A¹²⁵ resolution in RP-HPLC purification include the purity over 65% of GFC pools as well as the hydrophilic retention of major *E. coli* contaminants in RP columns. A good resolution at semi-preparative RP-HPLC has been described for native IL-2 and the Ser-125 analogue, although it has already been described in processes including up-stream chromatographic steps that increase purity over 85% prior to RP-HPLC [11, 13].

In the present study, preparative RP chromatography shows a resolution power high enough to be used as a single chromatographic purification step for obtaining this IL-2A¹²⁵ analogue. This single step procedure increases protein purity by 1.5-fold and copes with IL-2 amounts over 900 mg.

Characterization of purified rhIL-2A¹²⁵

Effluent fractions from preparative RP-HPLC corresponding to rhIL-2A¹²⁵ peaks contain more than 0.8 µg of protein per millimeter. Recombinant hIL-2A¹²⁵ monomers were pure over 97% as demonstrated by densitometric measurements of SDS-PAGE, and oligomers were less than 1% of total protein. A slow migrating band (Figure 3, lane E), representing less than 2% of total protein, was also present. This protein band was identi-

21. Kunitani M, Hirtzer P, Johnson D, Halenbeck R, Boosman A, Kohs K. Reverse-phase chromatography of interleukin-2 mutants. *J Chromatography* 1986;359: 391-402.

22. Moya FG. Caracterización de especies modificadas de la interleuquina-2 humana recombinante (IL-2hrA¹²⁵) [dissertation]. Havana: Havana Univ.; 2001.

23. Kunitani M, Johnson D, Snyder LR. Model of protein conformation in the reverse-phase separation of interleukin-2 mutants. *J Chromatography* 1986;371: 313-33.

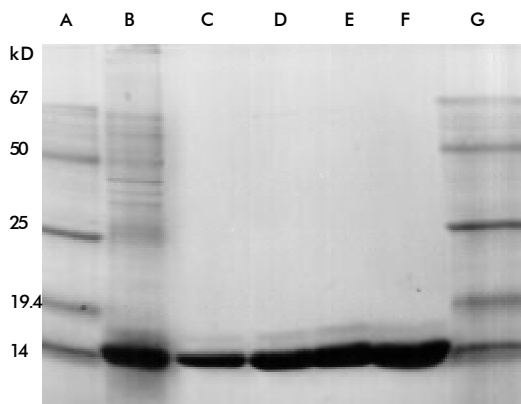


Figure 3. SDS-PAGE of rhIL-2A¹²⁵ purified by preparative scale RP-HPLC. Fifteen percent polyacrylamide gels were used in conjunction with the Tris/glycine discontinuous buffer system. Gels were stained with Coomassie Brilliant Blue R-250. Lanes A and G: molecular weight markers (67, 50, 25, 19.4 and 14 kD); lane B: 25 µg GFC pool; lanes C-F: IL-2 peak (C: 20 µg, D: 30 µg, E: 40 µg, F: 40 µg [non-reducing conditions]).

fied by western-blot as a high-molecular-weight form of rhIL-2A¹²⁵ and further characterized by mass spectrometry and sequencing as molecules containing C-terminal extensions of 11 aminoacids [22]. Similar C-terminal modifications have been described for human IL-2 and murine IL-6 expressed at high levels in *E. coli* [24, 25].

Contaminants under 2 µg/mg of rhIL-2A¹²⁵ were evidenced by the dot blot immunoassay for quantifying *E. coli* proteins, which represents a purity higher than 99.8%.

Analytical RP-HPLC showed three IL-2 peaks (Figure 2): P_A = 7% (Mean ± SD; 4.1 ± 2.7); P_B = 1% (0.2 ± 0.3); and the major P_B (93.5 ± 4.3), representing the cystine IL-2A¹²⁵ monomer as characterized [22]. This analysis consistently demonstrated that [P_A + P_B + P_B'] = 95% (97.9 ± 1.8). These three peaks have also been reported for other rhIL-2 preparations but with different percentages for P_A (= 2%) and P_B (= 6%) [13]. Resolution of IL-2 forms and cystine IL-2A¹²⁵ was not attained in this preparative RP-HPLC procedure due to the large protein loads, although selective fractioning of the IL-2 peak could remarkably reduce the presence of the such forms (unpublished results).

Isoelectric focusing showed four different bands (Figure 4). Two major species of pI 7.94 and 7.68 correspond to N-terminal alanine (rhIL-2A¹²⁵) and N-terminal methionine (rhIL-2M¹A¹²⁵) proteins, respectively. This heterogeneity at the N-terminus occurs as a result of incomplete post-transcriptional processing [26, 27]. Recombinant hIL-2M¹A¹²⁵ represents 70.3% of the expressed protein, as estimated by amino terminal sequence analysis. The other two minor bands of pI 7.46 and 7.36, although requiring further characterization, may correspond to the other IL-2 species aforementioned: the C-terminal modified and Met-104-sulfoxide forms.

A range of pI from 6 to 8.5 has been reported for human IL-2 [28] and the differences depend on the preparation method and protein source material. In general, similar species of the recombinant native molecule have a slightly higher pI (IL-2M¹, 7.7; IL-2A¹, 8.0; Met-104-sulfoxide IL-2, 7.4; C-terminal extended forms approximately one unit less than that of IL-2) [11, 24, 26]. Although an inverse effect on pI could be expected by

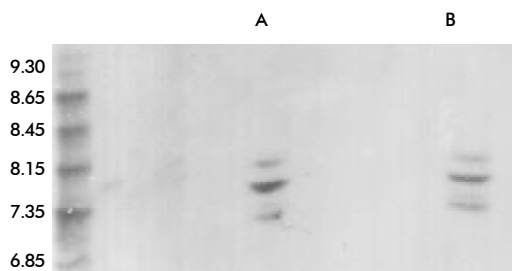


Figure 4. Gel isoelectric focusing of rhIL-2A¹²⁵ purified by preparative scale RP-HPLC. Lane A: 10 µg; lane B: 15 µg. pI values are from marker proteins. The gel was stained with Coomassie Brilliant Blue R-250.

replacing cysteine with alanine, several reports demonstrated that the sulfhydryl group of Cys-125 is buried in the molecule [29] and therefore its direct contribution to the surface net charge is limited. The conformational change provoked by the amino acid replacement should account for the differences observed in pI relative to the native molecule.

The ability of IL-2 to support the proliferation of CTLL-2 cells [19] was used as the basis of the biological assay. The specific bioactivity of the purified rhIL-2A¹²⁵ preparation was about 1.5 × 10⁷ IU/mg (ranged 1.1–2.0 IU/mg). This is in agreement with previous reports concerning the activity of other recombinant IL-2 preparations [26], including Cys-125 substituted analogues [21, 30]. The presence of different IL-2 species in variable proportions, as described above, does not affect the specific activity of the total preparation, since a bioactivity similar to that of N-terminal alanine rhIL-2 has been reported for the other present M-104-sulfoxide or the N- and C-terminal modified rhIL-2 forms [24, 31].

Recent results demonstrate that by fractioning the IL-2 peak it is possible to obtain an IL-2 preparation free of N- and C-terminal-modified rhIL-2 forms. This procedure contributes to increase the homogeneity of the final IL-2 preparation.

Conclusion

The data herein presented demonstrate the suitability of high-scale RP-HPLC as the main purification step for obtaining homogeneous preparations of the recombinant IL-2 analogue IL-2A¹²⁵. The hydrophobic nature described for IL-2 and the fact that this particular analogue substituted at position 125 conserves the protein tertiary structure or folding to a great extent while it is on the surface of the stationary phase, make this chromatographic procedure an excellent choice for preparative purification. The IL-2 preparations obtained through this procedure have adequate chemical and biological properties. Minimal improvements of this procedure will allow testing of this preparation in preclinical and clinical studies. In the case of hIL-2A¹²⁵, this is the first report of a practical approach to obtain large amounts of the biologically active analogue of IL-2.

Acknowledgments

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24. Ahmad Z, Ciolek D, Pan YC, Michel H, Khan FR. Purification and characterization of a high-molecular-weight form of recombinant human interleukin-2. *J Protein Chem* 1994;13:591–8.

25. Tu GF, Reid GE, Zhang JG, Moritz RL, Simpson RJ. C-terminal extension of truncated recombinant proteins in *Escherichia coli* with a 10Sa RNA decapeptide. *J Biol Chem* 1995;270:9322–6.

26. Liang SM, Allet B, Rose K, Hirschi M, Liang CM, Thatcher DR. Characterization of human interleukin 2 derived from *Escherichia coli*. *Biochem J* 1985;229:429–39.

27. Watanabe T, Kuriyama M, Honda S, Sawada H, Kitano K. Initiation methionine of recombinant interleukin-2 is completely processed *in vivo* by replacement of second proline residue. *J Fermentation and Bioengineering* 1995;80:213–7.

28. Mark DF, Doyle MV, Kothe K. Human interleukin-2. In: Steven G, editor. *Recombinant lymphokines and their receptors*. New York: Marcel Dekker, Inc. Basel; 1987. p. 1–28.

29. Bazan JF. Unraveling the structure of IL-2. *Science* 1992;257:410–2.

30. Arakawa T, Boone T, Davis JM, Kenney WC. Structure of unfolded and refolded recombinant derived [Ala125] interleukin 2. *Biochemistry* 1986;25: 8274–7.

31. Yamada T, Kato K, Kawahara K, Nishimura O. Separation of recombinant human interleukin-2 and methionyl interleukin-2 produced in *Escherichia coli*. *Biochem Biophys Res Commun* 1986; 135:837.