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 C18-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: Pα, representing less than 7% of total protein; Pδ, the major peak, and Pβ, representing less than 1% of total protein, where Pα + Pδ + Pβ ≤ 95%. The specific bioactivity of the protein purified by preparative RP-HPLC ranged from 1.1 to 2.0 x 10^7 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

ABSTRACT

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

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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 reversa. Un mutante de la interleuquina 2 humana recombinante (rhIL-2A125) se purificó a escala preparativa. La cromatografía líquida de alta resolución en fase reversa (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 desnaturizantes 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: Pα, que representa menos de 7% de la proteína total; Pδ, el pico fundamental; y Pβ ≤ 1% de la proteína total, de manera que Pα + Pδ + Pβ ≥ 95%. La actividad específica del material purificado en la escala preparativa estuvo en el rango de 1.1 a 2.0 x 10^7 UI/mg. El análisis por isoelectrofofoque 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 sulfóxido. Los resultados presentados demuestran la factibilidad del empleo del RP-HPLC preparativo para obtener 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 interlukin-2.

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-2A**

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 pF15 plasmid [14]. Recombinant hIL-2A 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-deHahn, 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 C₁₈ in all cases. Flow rates were selected for each column following the manufacturer’s recommendation (semi-preparative 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 4 by using a 1 M KOH solution for 5 min before elution. SDS-PAGE was performed on 12.5% 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 PBS and transferred to nitrocellulose filters using a Minifold apparatus (Biotrack, 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-collodial gold complex formation.

**RP-HPLC**

One hundred micrograms of purified rhIL-2A 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 linear 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 Coomasie 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-2A 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.

**Table 1.** Gradient conditions for eluting proteins loaded onto C₁₈, reverse-phase columns of different dimensions.

<table>
<thead>
<tr>
<th>Gradient conditions</th>
<th>Columns</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.46 x 25 cm</td>
<td>1 x 25 cm</td>
</tr>
<tr>
<td>35–73% B</td>
<td>15–45% B</td>
</tr>
<tr>
<td>40 min</td>
<td>10 min</td>
</tr>
<tr>
<td>0.8 mL/min</td>
<td>6 mL/min</td>
</tr>
<tr>
<td>45–80% B</td>
<td>45–75% B</td>
</tr>
<tr>
<td>45–80% B</td>
<td>45–75% B</td>
</tr>
<tr>
<td>47 min</td>
<td>52 min</td>
</tr>
<tr>
<td>2 mL/min</td>
<td>45 mL/min</td>
</tr>
</tbody>
</table>


Results and Discussion

Scale up of RP-HPLC rhIL-2A<sup>125</sup> purification

Human IL-2A<sup>125</sup> 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/2Ala<sup>125</sup> to be tested for therapeutic use.

Effluents from GFC contained soluble rhIL-2A<sup>125</sup> 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-2A<sup>125</sup> 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-2A<sup>125</sup> 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-2A<sup>125</sup> onto RP-HPLC columns.

The purification parameters were first adjusted at the semipreparative scale where a two-step linear 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-2A<sup>125</sup> 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 rhIL-2A<sup>125</sup> 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-2A<sup>125</sup> (rhIL-2A<sup>125</sup>M<sub>104</sub>) 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 52.6 min to maintain total gradient volume. The protein peak corresponding to rhIL-2A<sup>125</sup> was eluted at 53 min (63.8% solvent B) at gradient increments of 0.57% of solvent B/min. The rhIL-2A<sup>125</sup> recovery for this chromatographic step was over 90%.

At the preparative scale, resolution of rhIL-2A<sup>125</sup> from other proteins was high enough to permit at least a 2.4-fold increase in protein load per millimeter relative to semi-preparative 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-2A<sup>125</sup> 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-2A<sup>125</sup> 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-2A<sup>125</sup>

Effluent fractions from preparative RP-HPLC corresponding to rhIL-2A<sup>125</sup> peaks contain more than 0.8 µg of protein per millimeter. Recombinant hIL-2A<sup>125</sup> 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-
Although an inverse effect on pI could be expected by 104-sulphoxide IL-2, 7.4; C-terminal extended forms correspond to the other IL-2 species aforementioned: hIL-2A and N-terminal alanine (rhIL-2A) responding to N-terminal alanine (rhIL-2A) 0.2±0.3; and the major P = 1% (97.9±1.8). These three peaks have also been reported for other rhIL-2 preparations but with different percentages for P = 7% (Mean ± SD; 4.1±2.7); P = 1% (97.9±1.8). These three peaks have also been reported for the rhIL-2 preparation was about 1.5 x 10^1 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 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 hIL-2A [25] as the main purification step for obtaining homogeneous preparations of the recombinant IL-2 analogue hIL-2A [25]. 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-2A [25], this is the first report of a practical approach to obtain large amounts of the biologically active analogue of IL-2.

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