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MICROPREPARATIVE PURIFICATION OF RECOMBINANT HUMAN INTERLEUKIN-2

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SUMMARY

Recombinant Interleukin-2 (IL-2) is expressed in *E. coli* as insoluble aggregates; a protocol has been developed for solubilization, renaturation and purification of IL-2 from such aggregates at the 5–10-mg level. IL-2 aggregates were isolated from soluble proteins by centrifugation, subjected to a 1 *M* guanidine hydrochloride wash and a butan-1-ol wash (the latter to remove lipid), dissolved in 8 *M* guanidine hydrochloride–10 m*M* dithiothreitol and partly purified by gel permeation chromatography. Refolding/oxidation was then performed by dilution into Tris–HCl, pH 8.5 containing 1.5 μM copper sulphate to accelerate autoxidation. Final purification was by successive cation-exchange and reversed-phase high-performance liquid chromatographic steps, yielding over 99.5% pure IL-2 with an overall recovery of 20%.

INTRODUCTION

Interleukin-2 (IL-2) is a hydrophobic protein (molecular weight 15 500) of central importance to immune regulation; its various activities include promotion of T-cell proliferation and of natural killer cell activity[1]. It has one disulphide bridge (Cys58/Cys105) and one free cysteine, residue 125 (ref. 2). When cloned in *E. coli* IL-2 is expressed as insoluble aggregates of inactive protein[3]; the characterization of recombinant IL-2 derived from such aggregates has been described[4,5] and in a previous paper we have described denaturant [6 *M* guanidine hydrochloride (GuHCl)] extraction, refolding/oxidation and purification of IL-2 from *E. coli* on an analytical scale[6]. These investigations showed that fully active IL-2 could be recovered in 20–30% yield from the reduced molecule in GuHCl provided refolding was carried out by dilution into pH 8.5 buffer at 1–5 μg/ml IL-2 concomitant with copper(II)-catalysed air oxidation of the cysteines. This paper comprises a method for purification of recombinant IL-2 at the 5-mg level based around these earlier analytical results, representing a hundred-fold scale-up.

EXPERIMENTAL

Chemicals and solvents

Tetramethylenediamine, sodium dodecylsulphate (SDS), ammonium persul-

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phate and butan-1-ol were AnalaR grade, (BDH); acrylamide and bisacrylamide were from BioRad; all other chemicals were from Sigma. GuHCl was grade 1 purity. Acetonitrile (HPLC Grade S) was obtained from Rathburn Chemicals (Peebleshire, U.K.) trifluoroacetic acid and (TFA) (Sequanal grade) from Pierce. Water for high-performance liquid chromatography (HPLC) was glass distilled and Milli-Q purified (Millipore).

Growth of E. coli and preparation of cell pellet

E. coli RB791 (50 ml) containing a plasmid bearing a synthetic gene coding for the mature 133-residue protein[3] were grown overnight and used to inoculate 5 l of medium in shake flasks. Following induction and harvesting, cells (12–15 g wet weight) were disrupted by sonication in 50 ml 10 m*M* phosphate buffer, pH 7.4, containing 0.15 *M* sodium chloride–10 m*M* dithiothreitol (DTT) and the IL-2 aggregates pelleted by centrifugation at 10000 *g* for 15 min in a Beckman J2-21 centrifuge with a JA-17 rotor. The pellet was resonicated in an equal volume of buffer and recentrifuged at 7000 *g* for 10 mins. Some contaminating protein was removed by washing the pellet with 50 ml 1 *M* GuHCl–10 m*M* DTT. Then the pellet was extracted for 30 min in 50 ml butan-1-ol–10 m*M* DTT to remove lipid, centrifuged at 10000 *g* for 10 min and dissolved for 1 h at 37°C in 15 ml 8 *M* GuHCl–10 m*M* DTT–50 m*M* Tris–HCl, pH 8.5, prior to gel permeation chromatography (GPC).

Gel permeation chromatography

GuHCl-solubilized pellet was chromatographed on a 90 × 5 cm Sepharose CL-6B column equilibrated in 6 *M* GuHCl–1 m*M* DTT–20 m*M* acetate buffer, pH 5. The flow-rate was 70 ml/h and 9.5-ml fractions were collected. IL-2, as detected by SDS polyacrylamide gel electrohoresis (SDS-PAGE), had approximately the same retention time as reduced, unfolded lysozyme (mol. wt. 14400) indicating that IL-2 was effectively unfolded under these conditions.

Renaturation

The pool (180 ml) of semi-purified IL-2 from GPC was refolded/oxidized by 25-fold dilution into 50 m*M* Tris–HCl, pH 8.5–1.5 μM copper sulphate to give final concentrations of 0.04 m*M* DTT, 0.24 *M* GuHCl and about 1.4 μg/ml IL-2. Oxidation was complete within 2 h at 20°C.

Ion-exchange chromatography (IEC)

Renatured IL-2 (4.51) was adjusted to pH 4 with acetic acid and loaded at 70 ml/min onto a 10 × 5 cm column (200 ml) of S-Sepharose Fast Flow cation-exchange resin (Pharmacia) equilibrated with 10 m*M* phosphate–citrate buffer, pH 4; following a 500-ml wash proteins were eluted with a 0 to 0.9 *M* sodium chloride gradient in the same buffer at 10 ml/min, and the column regenerated after each run with 0.1 *M* sodium hydroxide.

Reversed-phase (RP)-HPLC

The IL-2 pool from IEC (220 ml) was finally purified by RP-HPLC on a 25 × 1 cm Synchropak RP-P C_{18} column[7] 300 Å pore size, obtained from Synchrom, Linden. A Varian 5020 liquid chromatograph fitted with a UV-100 detector was

employed. The pool was loaded onto the RP-P column at 6 ml/min; all of the IL-2 was adsorbed under these conditions. Following a 100-ml wash with 0.1% TFA, proteins were eluted with a gradient of acetonitrile–0.1% TFA (see legend Fig. 3 for details).

Gel electrophoresis

SDS-PAGE was performed with 15% gels as described by Laemmli[8]. Sample buffer was 190 m*M* Tris–HCl, pH 6.8–3% SDS–30% glycerol–0.75% bromophenol blue–5% 2-mercaptoethanol; samples were loaded on a protein basis, as determined by the Bradford method (Pierce Protein Assay Reagent), or from 280 nm absorbance in the case of pure IL-2[5]. The SDS-PAGE protein standard (Pharmacia) contained phosphorylase b (mol. wt. 94000), bovine serum albumin (mol. wt. 67000), ovalbumin (mol. wt. 43000), carbonic anhydrase (mol. wt. 30000), soyabean trypsin inhibitor (mol. wt. 20100) and lactalbumin (mol. wt. 14400). Gels were silver stained by the method of Wray *et al.*[9].

Isoelectric focusing (IEF) was performed with an LKB Multiphor flat bed apparatus with precast pH 3.5–9.5 "Pagplate" gels (LKB); conditions for electrophoresis of a 11 × 12 cm gel were 1.5 kV, 15 mA, 10 W and 5°C cooling temperature with a 45-min prefocus time and a 105-min run time. p*I* standards were obtained from Pharmacia. Gels were stained with Coomassie Blue R-250 following fixing in 20% trichloroacetic acid.

RESULTS AND DISCUSSION

Analytical scale purification of recombinant IL-2 was successfully performed by GPC in GuHCl of crude unwashed broken cell pellet followed by renaturation and then RP-HPLC as described previously[6]. Reversed-phase column lifetimes were short when loads were increased, due to adsorption of lipid-like material. This situation was improved in the preparative method described here by removal of lipid by butanol washing of the pellet prior to GuHCl solubilization, a process which incurred negligible losses of IL-2. An additional 1 *M* GuHCl wash was also introduced to remove contaminating protein.

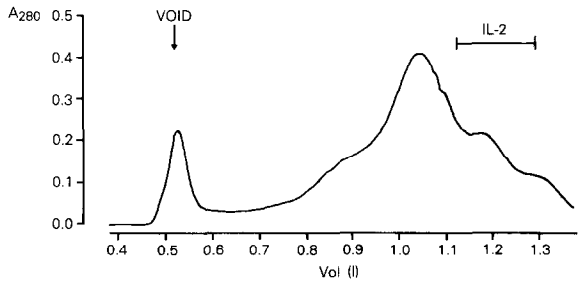
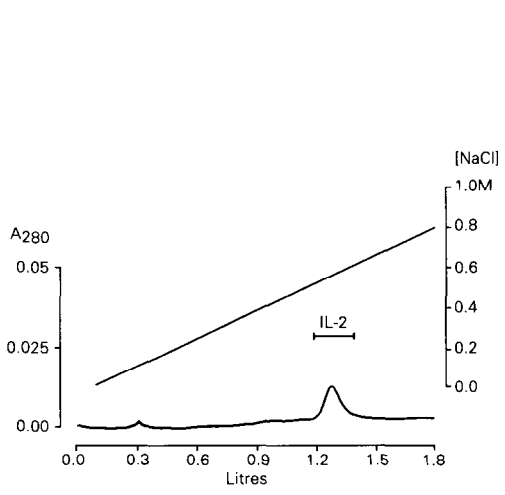


Fig. 1. Gel permeation chromatography of GuHCl-solubilized cell pellet from 12 g *E. coli* cell paste on a 90 × 5 cm Sepharose CL-6B column equilibrated with 6 *M* GuHCl–1 m*M* DTT, pH 5. The flow-rate was 70 ml/h. IL-2 containing fractions were pooled and stored at −70°C prior to renaturation. The purification fold was 2.5.

GPC was retained as the first step in the scaled up procedure following pellet solubilization; although a limiting factor in the process, it was found to be essential for efficient renaturation in spite of its relatively low purification factor (Fig. 1). Refolding by dilution of crude pellet extract gave a flocculant precipitate, and recoveries of IL-2 were 5–10-fold decreased; furthermore IL-2 eluted as a very broad peak by IEC, probably due to adherence to other material. The interfering contaminants may have been membrane fragments, proteins or possibly trapped nucleic acid[10].

Renaturation by dilution of the GPC pool gave a slightly cloudy solution. Dissolved oxygen was sufficient to give rapid oxidation in the presence of copper(II), with less than 10% of the wrong disulphide isomers (Cys58/Cys125 and Cys105/Cys125) being formed as judged by RP-HPLC, which resolved the three disulphide species[11]. Recovery was drastically reduced if oxidation occurred in GuHCl due to formation predominantly of the Cys105/Cys125 species by virtue of the proximity of these two thiols[11]; in an aqueous environment at pH 8.5 the conformational energy of the protein presumably forced oxidation to the correct disulphide configuration. However, overall renaturation yields were only 20–30% and decreased if the IL-2 concentration was raised[6], indicating that the losses were due to aggregation[12].

IL-2 could be rapidly recovered from the dilute renaturation medium by adsorption onto S-Sepharose fast flow ion exchange resin at pH 4. At this pH IL-2 is soluble and ex-column recovery was 70% following sodium chloride gradient elution (Fig. 2). Furthermore, most *E. coli* proteins did not stick to the resin and a very good purification was achieved, the eluate being about 85% pure IL-2. The eluate was stored overnight at 4°C before loading onto RP-HPLC (Fig. 3). Resolution of IL-2



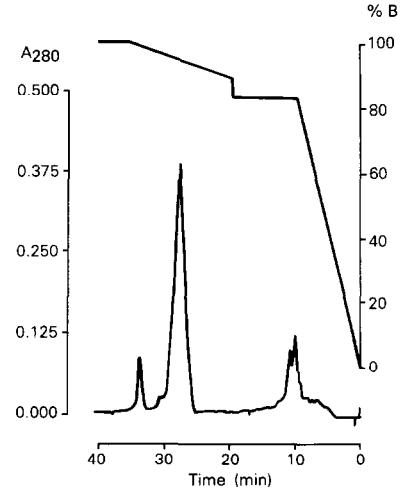


Fig. 2. Ion-exchange chromatography of renatured material from GPC (see Fig. 1) on a 10 × 5 cm column of S-Sepharose fast flow at pH 4. The flow-rate was 10 ml/min. IL-2 eluted at approximately 0.55 *M* sodium chloride, and was 85% pure at this stage.

Fig. 3. Semi-preparative RP-HPLC of IEC-purified IL-2 on a 25 × 1 cm Synchropak RP-P C_{18} column. A = 0.1% TFA, B = 65% acetonitrile–TFA, flow-rate 1.5 ml/min. The main IL-2 peak eluted at 60% acetonitrile (28 min); a small amount of residual reduced IL-2 was seen at 34 min. The peak at 10 min contained no IL-2 species.

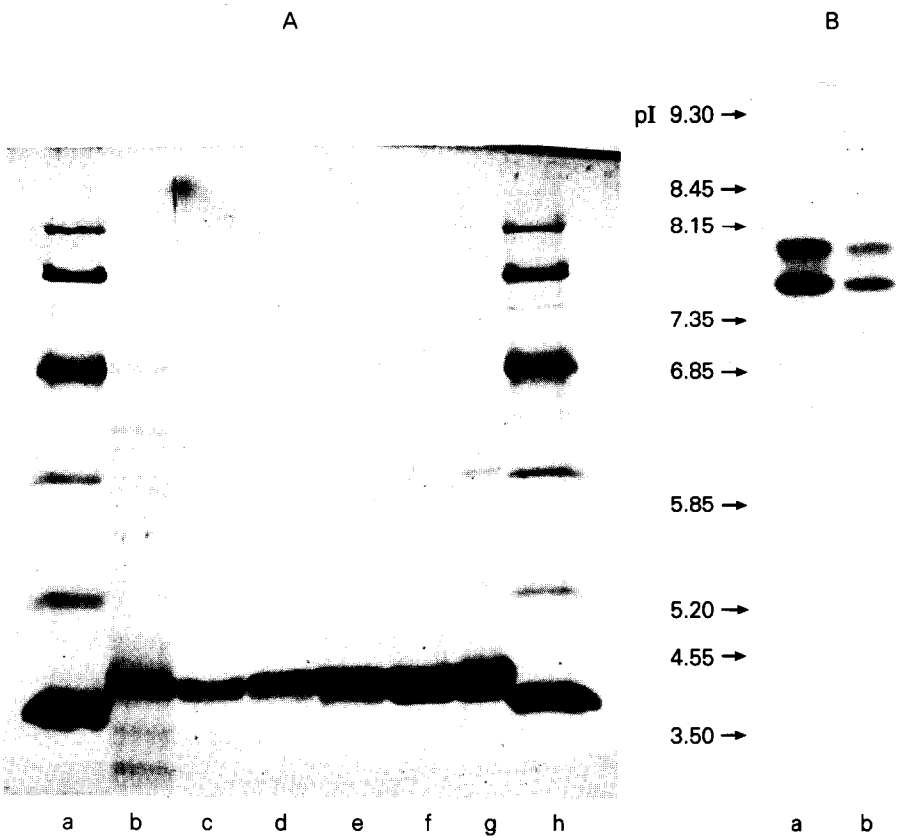


Fig. 4. (A) Reducing SDS-PAGE analysis of fractions from the IL-2 purification procedure. Tracks a and h: molecular weight standards 94000, 67000, 43000, 30000, 20100 and 14400; track b: 1.8 μg GPC purified IL-2; track c: 0.35 μg GPC–IEC purified IL-2; tracks d–g: finally purified IL-2, d, 0.4 μg; e, 2 μg; f, 10 μg; g, 10 μg but unreduced. The gel was silver stained. (B) IEF analysis of pure IL-2. Track a: 15 μg; track b: 5 μg. p*I* values are for marker proteins. The gel was Coomassie R-250 stained.

from other proteins on RP-HPLC was very good and would permit a considerable degree of overloading. Reinjection experiments showed IL-2 recovery from Synchropak RP-P to be greater than 95%, and the protein was stable with respect to irreversible on-column or acid–solvent induced unfolding *i.e.* there was no loss of bioactivity, although since bioassays were performed at neutral pH in the presence of other proteins no conclusions can be drawn from this evidence regarding the conformational state of IL-2 during chromatography.

Analytical RP-HPLC of purified IL-2 showed the presence of a minor (2–3%) contaminating form of IL-2 eluting just before the main peak; this was assigned on the basis of retention time as the Met104 sulphoxide species[13]; peak broadening due to the greater loads employed in preparative RP-HPLC (3–4 mg IL-2 per run per 25 × 1 cm column) precluded resolution of these species on a preparative basis. SDS-PAGE (Fig. 4A) showed only one contaminating non-IL-2 species (mol. wt. 27000), accounting for 0.1% total protein; a small amount of dimer (0.2%) was also present.

TABLE I

PURIFICATION OF RECOMBINANT IL-2

Cell pellet was prepared as described in the text from 12 g *E. coli* cell paste. This stage gave approximately 4-fold purification with < 10% losses of IL-2.

	Total protein (mg)	*Volume (ml)*	*IL-2 (mg)*	*Purity (%)*	*Overall purification*	*Recovery (%)*
GuHCl extract* of cell pellet	134	20	17.5	13	1	100
Sepharose CL-6B* GPC	42	180	14.0	33	2.5	80
Renaturation*	42	4500	4.9	–	–	28
S-Sepharose IEC**	4.4	220	3.7	85	6.5	21
RP-HPLC**	3.5	7	3.5	99.5	7.7	20

* IL-2 was in reduced/unfolded form, and was determined by densitometry from SDS-PAGE gels using purified IL-2 standards to calibrate the quantitation.

** Recovery figures for these rows refer to native IL-2. Renaturation recovery was 35% of total unfolded IL-2, as determined by analytical RP-HPLC.

IEF (Fig. 4B) showed two major species in approximately 4:1 ratio respectively of p*I* 7.7 and 8.0, a band of p*I* 7.5 (2–3% total protein) and a very faint band of p*I* 7.4; elution from gels and bioasay showed the major forms to be fully active IL-2 species, probably N-Met IL-2, p*I* 7.7, and N-Ala IL-2, p*I* 8.0[14]. The p*I* 7.5 form may be Met104 sulphoxide IL-2. Finally, the specific bioactivity of the IL-2 preparation when measured by a cell proliferation assay[15] was the same within error as native IL-2 from the JURKAT human cell line. Hence it appears that this procedure produces fully active recombinant human IL-2 that is over 99.5% pure; a summary of this method is given in Table I.

In conclusion, IL-2 can be successfully recovered from *E. coli* cell pellet by GuHCl extraction in reduced form and controlled oxidation. This method contrasts with the gram-scale process of Battig *et al.*[16] who allowed the IL-2 to oxidize on breakage of the cells and exposure to air, and then recovered that proportion which was correctly folded by acid–propanol extraction. The main drawback with GuHCl extraction and renaturation as described here with respect to gram-scale production is the high dilution needed to avoid aggregation of IL-2 and/or IL-2 folding intermediates, since the molecule is poorly soluble at the neutral pH range needed for rapid thiol oxidation. However, we have found that this molecule can be refolded at around 10 μg/ml with recoveries of 10%; given the efficient loading onto S-Sepharose fast flow, this makes preparation on the gram scale perfectly feasible even without the use of detergents to inhibit aggregation.

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