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Preparation and characterization of human interleukin-5 expressed in recombinant *Escherichia coli*

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The gene coding for human interleukin-5 was synthesized and expressed in *Escherichia coli* under control of a heat-inducible promoter. High-level expression, 10–15% of total cellular protein, was achieved in *E. coli*. The protein was produced in an insoluble state. A simple extraction, renaturation and purification scheme is described. The recombinant protein was found to be a homodimer, similar to the natural murine-derived protein. Despite the lack of glycosylation, high specific activities were obtained in three '*in vitro*' biological assays. Physical characterization of the protein showed it to be mostly α-helical, supporting the hypothesis that a conformational similarity exists among certain cytokines.

INTRODUCTION

Interleukin-5 (IL-5) is a specific haematopoietic growth factor responsible for eosinophil differentiation. Murine IL-5 has also been shown to have B-cell-growth-factor [1] and T-cell-replacing-factor activities [2]. These activities were originally thought to be derived from separate proteins, namely eosinophil differentiating factor (EDF), B-cell growth factor (BCGF-II) and T-cell replacing factor (TRF). The availability of recombinant-derived murine protein showed that these activities were derived from a single protein, namely IL-5.

Natural IL-5 has been isolated only from murine T-cell supernatants and shown to be a glycoprotein which exists as a homodimer (M_r 45000) [2,3]. The cDNA sequence of the murine species predicts an M_r of 13000 for the polypeptide monomer [5].

Human IL-5 cDNA [5] and genomic [6] genes have been cloned using the murine IL-5 cDNA [4] as a probe. The murine and human proteins show 70% sequence similarity and have species cross-reactivity in EDF activity. The natural hIL-5 has not been purified and characterized, but recombinant hIL-5 has been expressed in several eukaryotic systems [7,8] and shown to be a glycosylated, disulphide-linked homodimer of M_r 40000–50000.

Although hIL-5 has BCGF activity on murine B-lymphocytic cell lines and on selected human B-CLL cells [8], this activity has not been detected using human BCGF bioassays [9]. The presence of mRNA coding for hIL-5 has been detected in a human T-cell clone [10], the supernatant of which was shown to have EDF activity. Recently, human T-cell clones have been shown to have IL-5 activity which is blocked with an anti-IL-5 monoclonal antibody [11]. In order to obtain the human protein in relatively large amounts to investigate further its biological properties and perform physicochemical and structural studies, we have produced the recombinant protein in *Escherichia coli*. The present study describes the expression, isolation and characterization of recombinant hIL-5.

MATERIALS AND METHODS

Reagents

Polynucleotide kinase was kindly given by Dr. N. Murray, (Department of Molecular Biology, University of Edinburgh, Edinburgh, Scotland, U.K.). All other enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). Protein standards for gel filtration and for electrofocusing, DEAE-Sepharose, Sephacryl S-200 and Sephadex G-25 resins were from Pharmacia (Uppsala, Sweden). Ultrogel AcA54 was from IBF Biotechnics (Villeneuve-La-Garenne, France).

Buffer compositions

The buffers used for the extraction of hIL-5 were as follows: Buffer A, 0.1 M-Tris/HCl/5 mM-benzamidine/HCl/0.5 mM-phenylmethanesulphonyl fluoride/5 mM-EDTA/10% (w/v) sucrose, pH 7.8; Buffer B, 0.1 M-Tris/HCl/5 mM-benzamidine/HCl/0.5 mM-phenylmethanesulphonyl fluoride/1 mM-EDTA/1 mM-dithiothreitol (DTT), pH 7.8; Buffer C, as Buffer B, but containing 2 M-urea and 2% (w/v) Triton X-100; Buffer D, 20 mM-Tris/HCl/8 M-urea/5 mM-EDTA/1 mM-DTT, pH 8.5; Buffer E, 0.1 M-Tris/HCl/6 M-guanidinium chloride (Gdm·HCl)/5 mM-EDTA/1 mM-DTT, pH 8.0; Buffer F, 0.1 M-Tris/HCl/6 M-urea/1 mM-DTT, pH 8.5; Buffer G, 0.1 M-Tris/HCl/1.4 M-urea, pH 8.5; Buffer H, 0.1 M-Tris/HCl, pH 8.5. All buffers were adjusted to the indicated pH with HCl.

Gene synthesis

The synthetic gene for human IL-5 was constructed as previously described [8]. The oligonucleotide synthesis was carried out on an Applied Biosystems 380A DNA synthesizer. The synthetic oligodeoxynucleotides were purified by PAGE run under denaturating conditions. Pure oligonucleotides were phosphorylated with polynucleotide kinase, ligated and sub-

Abbreviations used: IL-2, interleukin 2; IL-5, interleukin-5; hIL-5, human interleukin-5; EDF, eosinophil differentiating factor; BCGF, B-cell growth factor; TRF, T-cell replacing factor; DDT, dithiothreitol; Gdm·HCl, guanidinium chloride; T4-r.b.s., ribosome-binding site from bacteriophage-T4 gene-32 protein; pL promoter, leftward promoter/operator of bacteriophage λ.

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cloned into pUC9. DNA sequence analysis of the cloned gene was carried out by the Sanger chain termination method [12].

Expression

The gene for the mature position of hIL-5 was placed in a pL promoter/T4-r.b.s. (ribosome-binding site from bacteriophage-gene-32 protein) expression system. The expression of the synthetic gene was initially optimized by fusion of the first 12 amino acids of mature hIL-5 to the *lacZ* α-complementation peptide behind the T4-r.b.s. in a pUC18 vector [13]. A synthetic oligonucleotide linker with 128 silent coding possibilities was fused between the T4-r.b.s. and the *lacZ* α-peptide in plasmid pT4S. The plasmids were then transformed into *E. coli* JM83, plated on Luria-broth agar plates (containing 90 μg of ampicillin/ml and 0.1% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), and blue colonies were selected. Two linker sequences that contained the T4-r.b.s. and the optimized coding sequence of the first 12 amino acids of hIL-5 were selected from the fusion experiment and were combined together with the remainder of the synthetic gene into a pL expression plasmid [14].

Fermentation

The pL expression plasmid pLT4 hIL-5 was transformed into *E. coli* strain W3110. The cells were grown at 30 °C in a 1.5-litre fermenter (MBR BioReactor AG, Wetzikon, Switzerland) in complex media containing casein hydrolysate (20 g/litre), yeast extract (3 g/litre) and glycerol (40 g/litre) in basal-salts solution and 5 μg of tetracycline/ml. Fermentations were maintained at pH 7.0 and aerated at 1 vol/min per vessel vol. When cultures had reached an absorbance of about 30 at 650 nm, the temperature was raised to 42 °C. After 5 h at 42 °C the cells were harvested. The cell yield was about 80 g wet weight/litre.

Protein purification

All steps were carried out at 0–4 °C, except where stated, hIL-5 was monitored in all steps by SDS/PAGE.

E. coli cells (50 g wet wt.) were suspended in 200 ml of buffer A and broken by two passages through an Amicon French pressure cell (Kontron, Basel, Switzerland). To reduce the viscosity, the suspension was sonicated for 30 s after each passage. The solution was centrifuged at 10000 *g* for 30 min and the pellet washed by suspension and re-centrifugation once with 100 ml of Buffer B and once with 100 ml of Buffer C. The final washed pellet was suspended in 500 ml of Buffer D and stirred overnight at 4 °C. The suspension was centrifuged at 100000 *g* for 30 min. The conductivity of the clarified supernatant was adjusted to 1.2 $mS \cdot cm^{-1}$ by dilution with deionized 8 M-urea, and the solution passed through a DEAE-Sepharose CL column (4.4 cm diam. × 20 cm) equilibrated with Buffer D. The column was washed with buffer until the A_{280} reached a base-line value. The flow-through fractions containing hIL-5 were pooled and concentrated by ultrafiltration on an Amicon YM5 filter to 100 ml. Solid $(NH_4)_2SO_4$ was added to 25% saturation, the precipitate collected by centrifugation, dissolved in 30 ml of Buffer E, and heated for 1 h at 60 °C. After cooling to room temperature, the solution was applied to a Sephacryl S-200 column (4.4 cm diam. × 150 cm) equilibrated in Buffer E. Fractions containing monomeric hIL-5 were pooled and concentrated by ultrafiltration to a protein concentration of about 1 mg/ml. The concentrate was applied to a Sephadex G-25 column (4.4 diam. × 30 cm) equilibrated in buffer F, thereby exchanging guanidine for urea. The IL-5-containing fractions were pooled, then diluted with 9 vol. of Buffer G so that the final urea concentration was 2 M. The solution was stirred for 24 h at 4 °C, and then dialysed at 4 °C against 10 litres of Buffer H with two buffer changes over 24 h. The solution was concentrated by ultrafiltration, centrifuged to remove any precipitated protein, and chromatographed on an Ultrogel AcA54 column (4.4 cm diam. × 100 cm) equilibrated in the dialysis buffer. Fractions containing hIL-5 elected at a position corresponding to dimeric protein (M_r 25000) were pooled and concentrated to about 1 mg/ml.

Analytical methods

SDS/PAGE was carried out on 10–15% gradient gels using the PHAST System (Pharmacia) according to the manufacturer's instructions. Protein was stained with Coomassie Blue. Urea-gradient electrophoresis was performed as described by Goldenberg & Creighton [15]. Urea gradients (0–9 M) were buffered with 0.05 M-glycine/NaOH, pH 9.0. Electrophoresis was performed at 30 mA for 16 h at 20 °C. Electrofocusing and pH-titration-curve analysis (combined electrofocusing and electrophoresis) were performed on the PHAST System according to manufacturer's instructions.

C.d. measurements

Spectra were recorded on a Jasco J-600 spectropolarimeter. The mean residue molecular mass was calculated as 114.5. All solutions were filtered with a 0.22-μm-pore-size filter (Millipore) before use.

Protein determination

Concentrations of purified protein were determined by u.v. absorbance using a calculated value for $A^{0.1\%}_{1\,cm}$ of 0.66 at 280 nm. Protein concentrations in crude extracts were determined as described by Bradford [16]. Relative amounts of hIL-5 present at each stage of purification were determined by densitometric scanning of Coomassie Blue-stained SDS/PAGE gels.

Analytical ultracentrifugation

Protein (0.45 mg/ml) was dialysed against 50 mM-Tris/HCl, pH 7.5, containing 1% (w/v) KCl. Measurements were made using a Beckman LB-70 preparative ultracentrifuge equipped with a Prep UV Scanner. For sedimentation-equilibrium measurements, the centrifuge was operated at 15000 rev./min at 20 °C. Measurements were taken after 15 h and a baseline established by centrifugation at 45000 rev./min for 3 h. Sedimentation-velocity measurements were made at 40000 rev./min at 20 °C and were recorded every 10 min for 90 min. Molecular masses and sedimentation coefficients were calculated from the least-squares fits of plots of $\ln A_{280}$ versus r^2 and $\ln r$ versus t respectively.

Reduction and alkylation

Protein, dissolved in 0.1 M-Tris/HCl, pH 8.6 (1–2 mg/ml), containing 1 mM-EDTA and 4 M-Gdm·HCl, was purged with N_2, DTT was added to 10 mM, and the solution was incubated for 15 min at 37 °C. Iodoacetamide (50 mM) was added, followed by incubation in the dark for 30 min at 37 °C. The solution was then desalted on a P10 column (1 cm diam. × 9 cm; Pharmacia), equilibrated in 50 mM-NH_4HCO_3, followed by freeze-drying.

Amino acid analysis

S-Carboxymethylated protein samples were hydrolysed in the vapour phase using constant-boiling HCl at 112 °C for 20 h. N_2-purged evacuated hydrolysis chambers were used. Hydrolysates were analysed on a Beckman 6300 amino acid analyser equipped with a SICA integrator.

N- and *C*-terminal sequence analysis

Edman degradation was performed with a Model 477A protein sequencer (Applied Biosystems). Identification and quantification of amino acid phenylthiohydantoin derivatives was by on-line h.p.l.c. analysis using a Model 120A PHT-Analyzer (Applied Biosystems). The *C*-terminal sequence was analysed by fast-atom-bombardment m.s. on a peptide obtained by CNBr digestion. hIL-5 (10 nmol) was incubated in 70% formic acid with a 100-fold molar excess of CNBr. Before preparative reverse-phase h.p.l.c., a large peptide containing residues 1–105, dimerized by intermolecular disulphide bridges and precipitated on dilution with water, was removed by filtration. M.s. was performed on the peaks separated by h.p.l.c. as previously described [17].

Thiol and disulphide analysis

Thiol groups were measured using 5,5′-dithiobis-(2-nitrobenzoic acid) as previously described [18].

Biological activity

The BCGF assay utilizes a mouse pre-B-cell line, LyH7.B13, which responds to murine IL-3 and hIL-5 [19], kindly provided by Dr. R. Palacios (Basel Institute for Immunology, Basel, Switzerland). B13 cells (10^4/well) were cultured in triplicate together with dilutions of sample hIL-5 in 96-well flat-bottom plates (Costar, Cambridge, MA, U.S.A.), using Dulbecco's modified medium (Gibco, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 15% (v/v) Myoclone foetal-calf serum (Gibco). After 48 h culture at 37 °C [with air/CO_2 (19:1)], 0.5 μCi of [^{3}H-*methyl*]thymidine (5 Ci/mmol; Amersham, International) was added to each well, the cells incubated a further 16–18 h, harvested and counted for radioactivity. The dilution of sample giving 50% maximal proliferative response is equal to 1 unit of hIL-5 activity.

Eosinophil differentiation was assayed on both mouse and human bone marrow. The mouse bone-marrow assay was performed as described by Strath *et al.* [20]. Differentiation was estimated indirectly by measuring peroxidase activity after 5 days of culture. The assay with human bone marrow was performed as described by Clutterbuck & Sanderson [21]. Differentiation after 21 days of culture was measured directly by counting the number of eosinophils after staining with Luxol Fast Blue.

Analytical chromatography

The Pharmacia f.p.l.c. system was used. Anion-exchange chromatography was performed with 1 mg of protein, using a MonoQ 5/5 column (Pharmacia). The column was equilibrated with 20 mM-Tris/HCl, pH 7.0. Bound protein was eluted with a gradient of 0–0.5 M-NaCl.

Chromatofocusing was with a MonoP 5/20 column (Pharmacia). pH gradients of 3–9 and 6.5–4.5 were generated according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Protein purification

Recombinant hIL-5 accumulated in an insoluble form in *E. coli* cells. Although hIL-5 could be solubilized with urea, aggregates of mixed molecular masses were observed. The protein was recovered from the flow-through of a DEAE column and concentrated by precipitation with $(NH_4)_2SO_4$. The resulting precipitate was dissolved in Gdm·HCl, which ensured monomerization of the partly aggregated protein. As hIL-5 was the major low-molecular-mass species present at this stage, subsequent gel filtration on Sephacryl S-200 equilibrated in Gdm·HCl resulted in the separation of a discrete peak of nearly pure monomeric hIL-5.

To obtain good yields of refolded protein from the denatured monomeric protein in Gdm·HCl, it was found useful to exchange the Gdm·HCl for urea and remove the latter slowly by dialysis. Direct removal of the Gdm·HCl resulted in extensive aggregation of the protein. Similarly, direct removal of urea from protein solubilized in urea also resulted in most of the protein aggregating.

The overall recovery of protein was about 0.72 mg of hIL-5/g wet wt. of cells, which represents a final yield of about 6.5%. The purification is summarized in Table 1, and the SDS/polyacrylamide gel shown in Fig. 1 (lanes a–e) indicates the purity of the protein at various stages.

Table 1. Purification of recombinant hIL-5

Purification stage	Total protein (mg)*	IL-5 content (as % of total protein)†	Total hIL-5 (mg)	Yield (%)
Cells (50 g wet wt.)	3700	15	555	100
Washed pellet	735	63	463	83
DEAE-Sepharose ('flow through')	450	68	305	55
Sephacryl S-200 (pooled fractions)	153	98	150	27
Ultrogel AcA54 (pooled fractions)	37	98	36	6.5

* Protein concentrations were determined by using the method of Bradford [20].

† hIL-5 contents were estimated by densitometric scanning of Coomassie Blue-stained SDS/polyacrylamide gels.

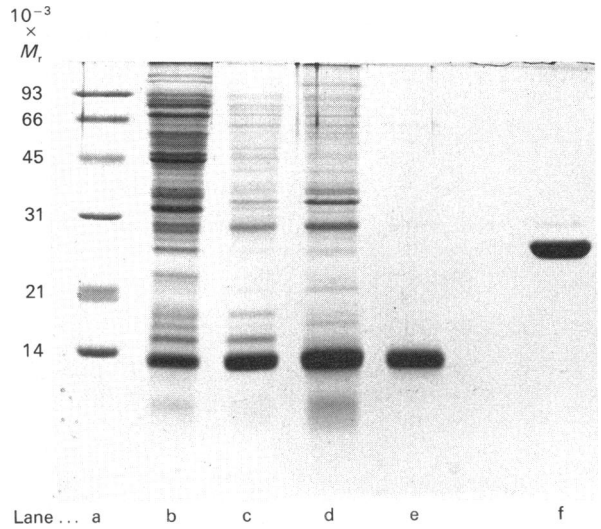

Fig. 1. SDS/PAGE of hIL-5 purification

SDS/PAGE was carried out on 15%-(w/v)-polyacrylamide gels as described in the text and stained with Coomassie Blue. Lane a, standards (M_r indicated); lane b, *E. coli* cell extract; lane c, washed pellet; lane d, pooled fractions from DEAE-Sepharose flow-through; lane e, pooled fraction from Sephacryl S-200; lane f, pooled fractions from Ultrogel AcA54 (in absence of reductant).

Table 2. Amino acid analysis of recombinant hIL-5

Tryptophan contents were not determined (nd). Theoretical values are derived from the cDNA sequence [8]. The values given are the averages of three separate analyses of 20 h acid hydrolysates. Cysteine contents was estimated as *S*-carboxymethylcysteine (CmCys) derived from protein reduced with DTT and alkylated with iodoacetamide.

Amino acid	Composition (mol of amino acid/mol of protein)	
	Theoretical	rhIL-5
CmCys	2	1.7
Asx	7	7.6
Thr	11	10.8
Ser	5	4.8
Glx	19	19.8
Pro	3	3.3
Gly	7	7.9
Ala	3	3.6
Cys	1	1.2
Val	7	7.3
Met	1	1.9
Ile	9	8.5
Leu	15	15.8
Tyr	2	1.8
Phe	4	4.1
His	3	3.1
Lys	8	8.1
Arg	6	6.3
Trp	1	nd

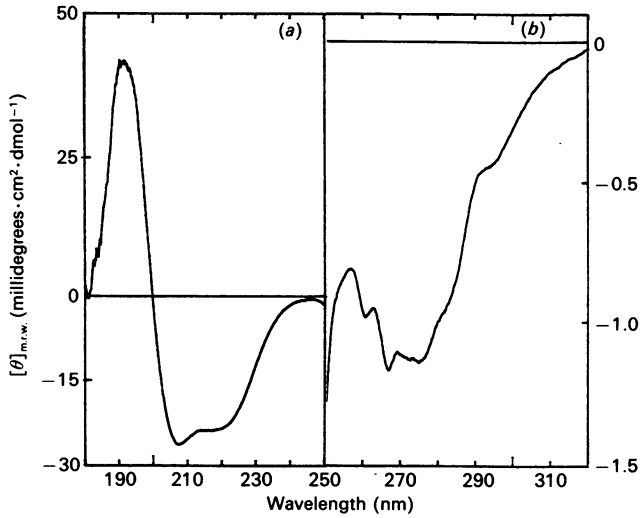

Fig. 2. C.d. spectra hIL-5

(*a*) Far-u.v. region; (*b*) near-u.v. region. Spectra are the average of four scans with baseline subtracted. The protein was 2.0 mg/ml in 50 mM-Tris/HCl, pH 7.5. Spectra were recorded at 20 °C with either a 0.01-cm-pathlength cell and 2 nm bandwidth (far-u.v. region) or 1.0-cm-pathlength cell and 1 nm bandwidth (near-u.v. region). The units for the ordinate are the same for (*a*) and (*b*), namely, mean residue ellipticity ($[\theta]_{m.r.w.}$) in millidegrees·cm²·dmol⁻¹.

Molecular mass and hydrodynamic properties

SDS/PAGE of hIL-5 pretreated with DTT showed a single band of M_r 13000 (Fig. 1, lane e), which is close to the value of 12291 predicted from the cDNA sequence [8]. Analysis of the protein under non-reducing conditions gave a single bond of M_r of 27000 (Fig. 1, lane f), indicating that the protein is a disulphide-linked dimer.

The M_r of the protein under native conditions was measured by sedimentation equilibrium. An M_r of 24000 was determined, indicating that hIL-5 is a homodimer under native conditions. This result was confirmed by gel filtration, where a single symmetrical peak was observed corresponding to an M_r of about 27000.

Physical homogeneity of the hIL-5 preparation was indicated by a single boundary during sedimentation-velocity analysis ($s_{20,w}$ 2.5S) and by the linearity of the plot of protein distribution at sedimentation equilibrium.

Natural glycosylated murine IL-5 [2,3] and glycosylated recombinant human and murine IL-5 species produced in mammalian cells [7,8] all appear to be homodimers. The lack of glycosylation in the *E. coli*-derived protein described here does not affect subunit structure.

Thiol and disulphide analysis

No free thiol groups were detected by either titration with 5,5′-dithiobis-(2-nitrobenzoic acid) or by amino acid analysis of protein pretreated with iodoacetate. However, after reduction of the protein with DTT, about two thiol groups/molecule of monomeric protein were detected (Table 2). This result, with those of the previous section, indicates that there are two intermolecular disulphide bonds connecting the subunits of the IL-5 dimer. It is noteworthy that hIL-5 contains a leucine-zipper motif ([22] and references cited therein). This region (residues 8–29) may provide the dimerization interface for the protein, the dimer being further stabilized by the two intermolecular disulphide bonds.

Amino acid composition and sequence analysis

The amino acid composition was close to that predicted from the cDNA sequence (Table 1). The first cycle of *N*-terminal sequencing indicated that the protein retained methionine derived from initiating *N*-formylmethionine. Further sequencing (20 cycles) confirmed the sequence predicted for the first 20 residues of the mature protein [8].

The sequence of the *C*-terminal region was confirmed by m.s. of protein digested with CNBr [17]. The major peak obtained after h.p.l.c. gave a signal at $m/z = 991.4$, corresponding to the peptide NTEWIIES (theoretical $m/z = 991.0$), as expected for the *C*-terminal peptide resulting from cleavage at Met-105.

Charge heterogeneity

Major species of pI values 5.9 and 5.6 and minor species of pI 6.4 and 5.4 were observed after electrofocusing (results not shown). Titration-curve analysis indicated that the charge differences were probably due to deamidation (results not shown). Separation of the various charge isomers was achieved by either chromatofocusing or anion-exchange chromatography at pH 7.0. All the separated species had similar specific biological activities, similar to that for unfractionated protein (see below).

Conformational analysis; c.d. and urea-gradient electrophoresis

The far-u.v. dichroic spectrum (Fig. 2*a*) indicated two minima at 207 nm and 221 nm, typical of proteins with substantial α-helical content. Analysis of the spectrum by the Contrin method [23] and by the method of Chang *et al.* [24] indicated 76% helix/24% remainder and 60% helix/40% remainder respectively. The protein thus appears to be mostly α-helical, with little or no contribution by β-sheet to the overall secondary structure. Similar results have been reported for the cytokine interleukin-2 (IL-2), which contains no β-sheet structure [25].

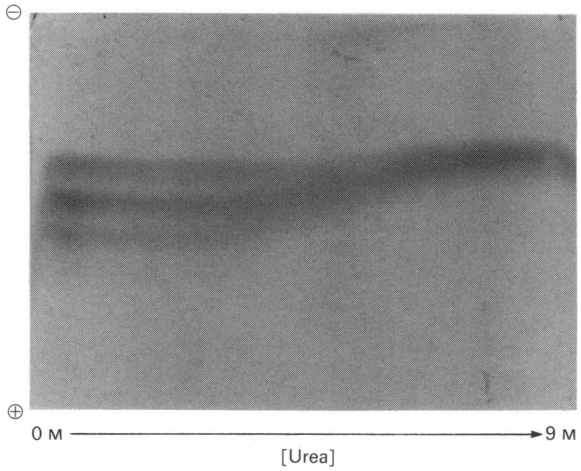

Fig. 3. Urea-gradient electrophoresis of hIL-5

The buffer for electrophoresis and sample application was 50 mM-glycine/NaOH, pH 9.0. Electrophoresis in the direction indicated was towards the anode.

It has been suggested that hIL-5 contains a four-α-helical motif [26], similar to that determined for IL-2 by X-ray crystallography [27]. This motif has been proposed for other cytokines as well, suggesting a conformational similarity between them [26]. The α-helical content estimated here for hIL-5 is certainly consistent with this type of structure.

The near-u.v. c.d. spectrum (Fig. 2*b*) shows weak negative ellipticity, with several minima in the region 260–280 nm, presumably due to contributions from tyrosine residues. The near-u.v. c.d. signal was lost upon treating the protein with 4 M-Gdm·HCl, indicating protein unfolding.

Urea-gradient electrophoresis also supports the view that the hIL-5 preparation has a native-like conformation. A single unfolding transition was observed at about 5 M-urea (Fig. 3), typical of proteins with high rates of folding and refolding relative to the rate of electrophoresis [15]. Although only one transition zone was observed, several distinct bands were observed in the region of the gel corresponding to native protein (0–5 M-urea). These various species, not clearly resolved in unfolded protein, most likely correspond to the charge isomers observed upon electrofocusing analysis (see above).

The charge differences did not affect the thermodynamic stability of the protein and, as mentioned above, did not affect biological activity.

Biological activity

The specific activities using the B-13, murine EDF and human EDF assays were 0.75×10^7 (± 0.085, $n = 5$), 1.1×10^7 and 2.5×10^7 units/mg respectively. The EDF assay value is similar to that reported for recombinant hIL-5 derived from yeast [8]. Glycosylation is clearly not essential for either biological activity or subunit assembly of the protein.

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