

# Human interleukin-5 expressed in *Escherichia coli*: assignment of the disulfide bridges of the purified unglycosylated protein

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Human interleukin-5 is a homodimer; each subunit contains two cysteine residues that form two inter-subunit disulfide bonds. The topology of the disulfides in recombinant human interleukin-5 produced in *Escherichia coli* was studied by proteolytic digestion and peptide mapping. Disulfide linked peptides containing cysteine 42 linked to cysteine 84 were isolated. This indicated that cysteines 42 and 84 of one subunit were linked in an antiparallel manner to cysteines 84 and 42 of the other subunit.

Interleukin-5; Recombinant DNA technology; Disulfide bond; Proteolytic digestion; Peptide mapping; Homodimeric subunit structure

## 1. INTRODUCTION

Natural IL-5 has been purified from murine T-cells and shown to be a disulfide-linked homodimer [1]. Both murine and human IL-5 have been cloned [2,3] and the latter expressed in several eucaryotic systems [4,5]. Human IL-5 has also been produced in *E. coli* [6]. The various recombinant IL-5s have all been shown to be disulfide-linked homodimers. The dimer structure appears to be essential for biological activity as the monomer is inactive [5,7].

Natural IL-5 is glycosylated [1] as is recombinant IL-5 expressed in eucaryotic systems. Glycosylation neither affects dimer formation nor biological activity as unglycosylated IL-5 produced in *E. coli* is both homodimeric and biologically active [6].

Human IL-5 expressed in a eucaryotic system was shown to contain two inter-subunit disulfide bonds in an antiparallel arrangement [7]. In this report, we have studied the disulfide linkage pattern of human IL-5 produced in *E. coli*. The protein expressed in bacteria is produced in an aggregated state. To prepare biologically active protein, the protein is first extracted and purified in a denatured state in which the cysteine residues are in the reduced form. This is followed by protein renaturation and reoxidation [6]. In protein exposed to a denaturation/renaturation cycle, it was considered most important to establish the disulfide bond topology and to confirm that the protein did not contain mismatched disulfides.

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## 2. EXPERIMENTAL

### 2.1. Protein purification

Human IL-5 expressed in *E. coli* was purified as previously described [6]. The reduced protein exhibited a single band of  $M_r = 13\ 000$  on SDS-PAGE. The protein was, however, heterogeneous with respect to pI, probably due to specific deamidations (A. Proudfoot, unpublished observations). As this heterogeneity caused multiplication of peaks during HPLC peptide mapping (see below), separation of the charge isomers was carried out. The protein (10 mg) was applied to a Mono Q HR 10/10 anion-exchange column equilibrated with 10 mM Tris-HCl, pH 8.0. The protein was eluted with a gradient of 0–0.1 M NaCl and the column fractions analysed by isoelectric focusing using a Phast system (Pharmacia).

### 2.2. Tryptic digestion

Porcine trypsin (Sigma) was treated with TPCK to remove any residual chymotryptic activity [8]. hIL-5 (800  $\mu$ l of 0.21 mg/ml in 10 mM Tris-HCl, pH 8.0) was mixed with 1.33 ml of 8 M urea, 0.123 ml 1 M Bis-Tris, pH 6.0 (final conditions: 5 M urea and pH 6.0). Trypsin was added (20 ml of 1 mg/ml in water) followed by incubation at 37°C for 2 h. The reaction was monitored by HPLC using a C-18 reverse phase (4  $\times$  250 mm) column (Machery-Nagel). After complete digestion of the protein, 1 ml of the reaction mixture was applied to a Mono Q HR 10/10 column equilibrated in 20 mM Tris-HCl, pH 7.4. A gradient of 0–0.18 M Na<sub>2</sub>SO<sub>4</sub> was applied. The major eluted peak was further purified on an Ultrapore C-3 RP (4.6  $\times$  75 mm) column (Beckman). The column was washed for 10 min with 0.1% (w/v) TFA and a 15 ml gradient of 0–50% acetonitrile was applied. The flow rate was 1 ml/min. All separations were monitored at 214 nm and were carried out using a Beckman Gold HPLC system.

### 2.3. Peptic digestion

Separations were made on an Aquapore C-8 RP (2.1  $\times$  220 mm) column (Applied Biosystems) connected to a HP1090 HPLC system (Hewlett Packard). Detection was at 214 nm. hIL-5 (1 ml of 0.14 mg/ml in 3.5% v/v formic acid, pH 1.8) was digested with 2% w/w pepsin (Boehringer) at 25°C for 2 h. A second addition of 1% w/w pepsin was added with incubation for a further 3 h. Digestion was stopped by lyophilization; the digest reconstituted with 0.1% TFA (w/v) and then fractionated by reverse phase HPLC.

Peptides containing disulfide bonds were identified by comparison of peptide maps of oxidized and reduced protein. Digests were reduced with 2 mM dithiothreitol in 6 M guanidine-HCl, 50 mM Tris-HCl, pH 7.8. The elution position of peptides containing disulfide linkages shifted after reduction. Separated disulfide-containing peptides were rechromatographed either directly or after reduction. Purified peptides were subjected to N-terminal sequencing.

#### 2.4. N-Terminal sequencing

N-Terminal amino acid sequence analysis was carried out on a Model 477A pulse-liquid sequencer (Applied Biosystems). Samples were loaded on filters precycled with polybrene. PTH-amino acid derivatives were analysed on-line by a Model 120 PTH-analyser (Applied Biosystems).

### 3. RESULTS

#### 3.1. Protein characterization

Recombinant hIL-5 exhibits charge heterogeneity; this is true for the glycosylated protein [5], the unglycosylated *E. coli*-derived protein [6] and the eucaryotic-derived protein expressed in the presence of tunicamycin [9]. Hence, the charge heterogeneity is not solely due to carbohydrate, deamidation of Asn and Gln residues being a likely cause in both eucaryotic and procaryotic-derived proteins.

We attempted to resolve the charge isomers using anion-exchange chromatography at pH 8.0. Three main peaks were obtained (Fig. 1). Isoelectric focusing (Fig. 1, insert) indicated that although the peak functions were not homogeneous, the band patterns were simplified relative to the starting material. The starting material migrated as a single band on SDS-PAGE with  $M_r = 13\ 000$  under reducing conditions and  $M_r = 27\ 000$  in the absence of reductant (Fig. 1, insert). SDS-PAGE of the fractions separated by anion-exchange chromatography indicated single bands migrating at the same molecular weight as the starting material (results not shown). Fractions from peak A were pooled as indicated and used for the peptide mapping studies described below.

Separation of the charge isomers was also attempted by chromatofocusing but poor separations were obtained with low yields, which we attributed to the poor solubility of hIL-5 at pH values below 6 (results not shown).

#### 3.2. Assignment of disulfide bonds: tryptic digestion

As protein disulfide-sulphydryl exchange reactions occur more rapidly under basic conditions [10], we performed enzymic digestions at pH values below 7.0. Tryptic digestions were carried out at pH 6.0 in the presence of 5 M urea using porcine trypsin. The urea was necessary to maintain solubility of hIL-5 and thus ensure efficient digestion. The porcine enzyme has the same specificity as the more commonly used bovine enzyme but is more stable against denaturation by chaotropic agents (A. Proudfoot, unpublished observations) and denaturation by organic solvents [11,12].

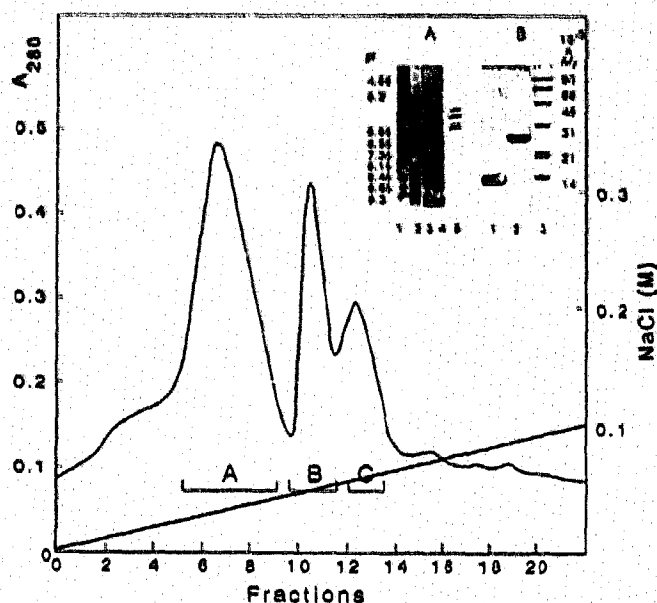


Fig. 1. Anion-exchange chromatography of hIL-5. The elution profile is from a Mono Q column. Fractions were pooled as indicated. Fraction A was used for peptide mapping. Insert A shows a polyacrylamide isoelectric focusing gel. (Lane 1) pI standards, pH 3–9; (lane 2) Pool A; (lane 3) Pool B; (lane 4) Pool C; and (lane 5) column starting material. Insert B shows an SDS-PAGE gel. (Lane 1) starting material in the presence of reductant; (lane 2) starting material in the absence of reductant; (lane 3) molecular weight markers.

Anion-exchange chromatography of tryptic digests resolved a major peptide that was eluted with 0.8 M  $\text{Na}_2\text{SO}_4$  (Fig. 2). This peptide was further purified by reverse phase HPLC and then sequenced. Two in-phase N-terminal sequences were recorded, corresponding to

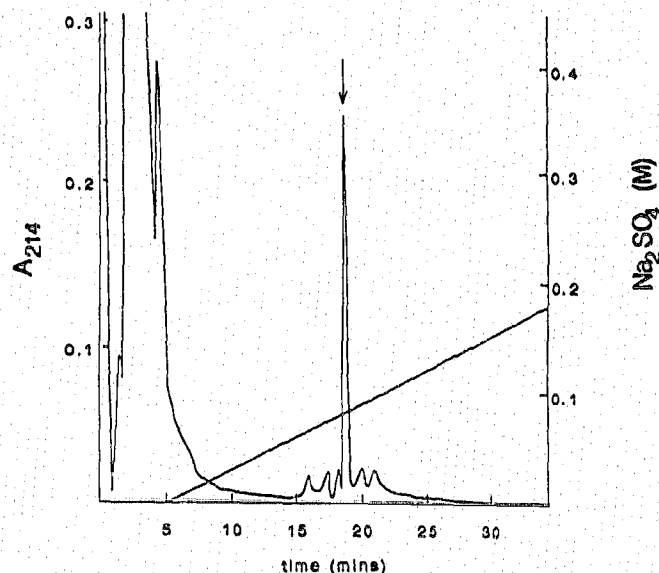


Fig. 2. Anion-exchange chromatography of hIL-5 digested with trypsin. The peak indicated was used for further analysis.

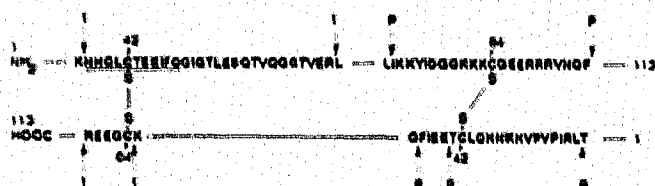


Fig. 3. Reverse phase HPLC of hIL-5 digested with pepsin. Elution profiles from: (A) oxidized protein digest; and (B) protein digest pretreated with dithiothreitol. The peaks (P1 and P2) that shifted after treatment with reductant are indicated with arrows in (A).

residues 84-88 and 38-46 of the IL-5 sequence (Fig. 4). This result indicated inter-subunit linkage between cysteines-42 and -84. (The cysteines were not derived from an intra-molecular linkage as dimeric IL-5 only contains inter-molecular disulfides [6].)

### 3.3. Assignment of disulfide bonds: peptic digestion

IL-5 was digested with pepsin and fractionated by reverse phase HPLC. Two peptides changed elution position upon reduction (Fig. 3, peptides P1 and P2). These peptides were rechromatographed before and after reduction with dithiothreitol. Before reduction the peptides eluted in their original positions (Fig. 3)

whereas after reduction each peptide generated two new peaks (results not shown). Sequence analysis of the oxidized peptides P1 and P2 and their constituent disulfide-linked peptide pairs indicated that peptide P1 contained residues 73-94 disulfide linked to residues 29-43. Peptic cleavages occurred on one polypeptide chain between Thr-28-Leu-29 and Thr-43-Glu-44 and on the other chain between Leu-72-Ile-73 and Phe-94-Leu-95. The peptide P2 contained residues 73-95 disulfide linked to residues 29-47. Peptide P2 thus contained the same disulfide pairing (Cys-42-Cys-84) as P1 except peptic cleavage had occurred between Phe-47-Gln-48 instead of Thr-43-Glu-44. The results of the tryptic and peptic digestion studies are summarized in Fig. 4.

### 4. DISCUSSION

While the manuscript was in preparation, the assignment of the disulfide linkages of glycosylated human IL-5 produced in CHO cells was published [7]. These authors described the same anti-parallel disulfide linkage configuration as we have described here. Thus, the same disulfide linkage pattern exists in bacterial produced protein that has undergone denatura-

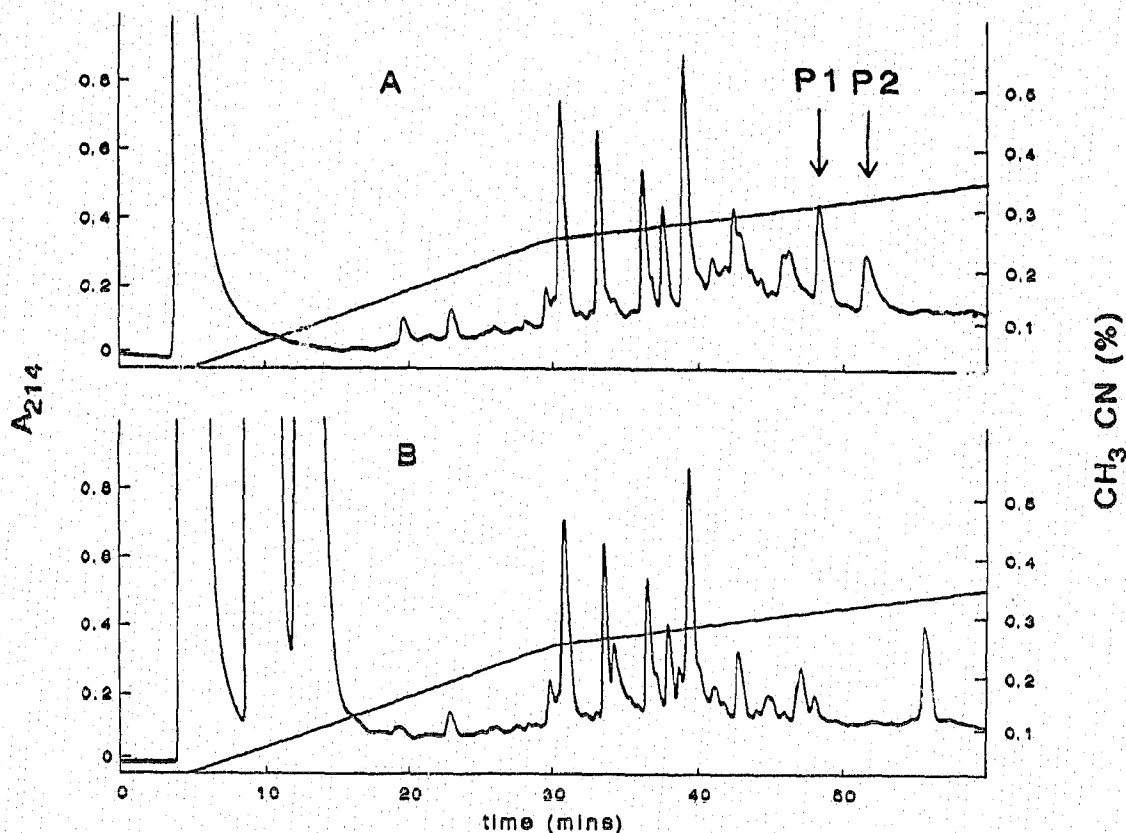


Fig. 4. Disulfide linkage pattern in hIL-5. The proteolytic cleavage sites are indicated by arrows: t, refers to tryptic cleavage and p refers to peptic cleavage. The peptides after the indicated cleavage sites were identified by N-terminal sequencing. The large tryptic peptide (residues 38-66) was only partially sequenced. The sequence determined (residues 38-48) is underlined. The complete amino acid sequence of hIL-5 is given in [2].

tion/renaturation and disulphide bond reduction/oxidation cycles. Based on the high molar yields of the disulfide containing peptides (>90%), there is little or no mismatched disulfide linked protein in the protein fractions analysed.

Disulfide-linked dimers are not uncommon and are found in proteins such as immunoglobulins and membrane receptors such as the insulin and transferrin receptors. However, cytoplasmic proteins containing intermolecular disulfide-linked homodimeric topologies are not common. Some secreted and cytoplasmic proteins have been shown to be homodimers. Most of these proteins are not disulfide-linked, although glutathione reductase contains a single covalent disulfide bridge between cysteine-90 of each monomer [13] and ribonuclease has been shown to exist as a disulfide-linked dimer in bovine seminal fluid [14]. The steroid binding protein uteroglobin is also homodimeric containing two antiparallel intermolecular disulfide linkages analogous to hIL-5 [15].

Mason et al. [16] have proposed a family of hormonal proteins having the disulfide-linked homodimeric motif, including transforming growth factor beta, Mullerian inhibiting substance and inhibin. Another growth factor, platelet-derived growth factor, adopts homo- and heterodimeric configurations, with the monomer and dimers having different activities [16,17]. IL-5 may belong to this structural class of protein hormones.

## 5. CONCLUSIONS

Human IL-5 is a homodimer which contains two inter-molecular disulfide bonds. The arrangement of the disulfides was determined by peptide mapping. The linkages occurred between cysteines-42 and -84 on opposite subunits. This anti-parallel arrangement is similar to that found in glycosylated mammalian cell produced protein. As cysteines-42 and -84 are conserv-

ed in murine IL-5 [5], the disulfide bond topology is probably the same as in the human protein.

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