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Expression, purification and crystallization of fully active, glycosylated human interleukin-5

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Recombinant human interleukin-5 (hIL-5) has been expressed at high levels and produced in large quantities in baculovirus infected Sf9 insect cells. The glycosylated protein was purified using immuno-affinity chromatography and gel filtration. Purified hIL-5 has been crystallized using standard vapour diffusion techniques with PEG as a coprecipitant. The crystals belong to the C2 space group and diffract to 2 Å.

Large scale production; Recombinant hIL-5; Sf9 insect cell; Baculovirus; Immuno-affinity chromatography; Preliminary crystallographic analysis

1. INTRODUCTION

IL-5 is a cytokine secreted mainly by T_{H2} cells during an allergic immune response [1]. It has been shown to be essential for both growth and differentiation of eosinophils in humans and mice. In the same context IL-5 is responsible for eosinophilia observed in parasitaemia and allergic diseases such as chronic asthma. Since eosinophils are known to cause damage to bronchial tissue, it is believed that the reduced lung function in asthma is related to the number of infiltrated eosinophils. Therefore the development of an IL-5 antagonist might have a therapeutic value. Knowledge of the structure of hIL-5 and of the receptor binding site in particular, would facilitate the design of such an antagonist. A detailed review of IL-5, its role in eosinophilia and related disease was published by Sanderson [2].

The IL-5 gene has been cloned by several groups [3–6]. The hIL-5 cDNA codes for a mature polypeptide of 115 amino acids [7], corresponding to a calculated M_r of 13,149. A larger apparent M_r range is predominantly due to the heterogeneous addition of carbohydrate (*O*- and *N*-linked glycosylation in recombinant hIL-5 produced by CHO cells [8]). Enzymatic removal of these sugar moieties leaves a fully active molecule, demonstrating that glycosylation is not necessary for receptor binding [9,10].

In solution, hIL-5 forms an antiparallel homodimer via two disulfide bonds [8,11] which is essential for the biological activity of the protein [6,8,10,12,13].

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Recombinant hIL-5 can be produced in substantial quantities by transformed *E. coli* [14], *S. cerevisiae* [6,10], Sf9 cells [6,10] and CHO cells [8].

This paper describes the large scale production of recombinant hIL-5 using the baculovirus expression system (allowing for the attachment of native-like carbohydrate structures), its purification, and crystallization.

2. MATERIALS AND METHODS

2.1. *High level expression of hIL-5 in Sf9 cells*

A synthetic hIL-5 gene [6] was inserted into the *Bam*HI site of the pVL941 vector using *Bam*HI linkers and was integrated into the AcMNPV genome by cotransfection and in vivo homologous recombination in Sf9 cells as described [15]. Recombinant virus was selected by testing serial dilutions of the supernatants in the B13 cell proliferation assay [16]. Recombinant plaques were picked after visual screening.

2.2. *Large scale production*

Sf9 cells were grown in spinner flasks using the low cost SF-1 medium [17] supplemented with 1.5% (v/v) fetal calf serum and lipid–Pluronic F68 mixture (Sigma) according to Inlow et al. [18]. Scale up fermentation and production of recombinant hIL-5 were performed in airlift fermentors (Chemap AG, Switzerland) with 23 liter working volume according to Maiorella et al. [19]. After inoculation to an initial titer of 2×10^5 cells/ml, the cells were grown in the reactor (pO_2 30%, 27°C, pH 6.0–6.3) for 3–4 days to a density of $3–4 \times 10^6$ cells/ml and infected with the recombinant virus preparation at a multiplicity of infection (moi) of 1–2. The production was terminated 3–4 days post-infection by cooling. The harvested volume was centrifuged and the culture supernatant concentrated 10- to 15-fold, using the Amicon SP20 ultrafiltration system (membrane cut off: 10 kDa)

2.3. *Preparation of the mAb and the affinity matrix*

A non-neutralizing mAb (designated H30) was obtained by immunizing Wistar rats with purified baculovirus-derived hIL-5 emulsified

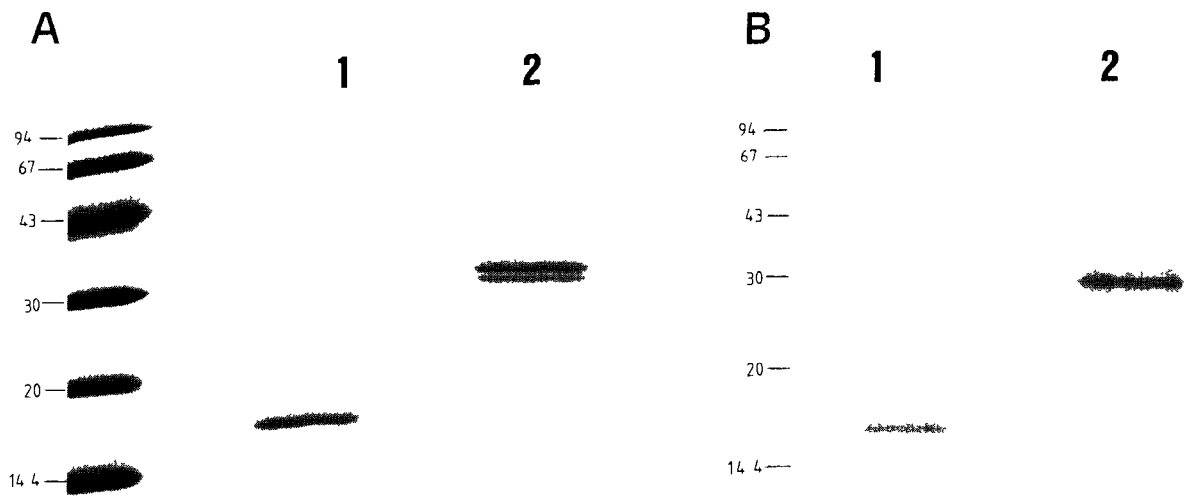

Fig. 1. SDS-(15%)-PAGE analysis of purified hIL-5. Panel A: hIL-5 (6 μg/lane) was separated and stained with Coomassie brilliant blue. Prior to analysis the sample was treated with 5 mM (lane 1; predominantly monomer), resp. 1 mM (lane 2; disulfide bonds connecting the two monomers preserved) dithiothreitol. The M_r of the standard proteins are indicated on the left. Panel B. Western analysis after SDS-(15%)-PAGE of hIL-5 (1.5 μg/lane) in reducing (lane 1) and non-reducing (lane 2) conditions

in Freund's adjuvant (Gibco). Lymph node cells were fused with sp2/0-Ag14 mouse myeloma cells using PEG 5000 (Boehringer) as previously described [20,21]. Specific mAbs were selected for their ability to bind ^{125}I-labeled hIL-5 (Iodogen, Pierce). H30 ascites was induced in pristan-primed nude mice after i.p. injection of the hybridomas.

Most of the contaminants were removed from the ascites fluid by caprylic acid precipitation [22] and low speed centrifugation. The supernatant was dialysed versus PBS, passed over a Protein G-Sepharose column (Pharmacia) and eluted with 100 mM Tris-glycine pH 2.7. Fractions containing the H30 mAb were pooled and brought to neutral pH. Alternatively, H30 was directly purified on Protein G-Sepharose from conditioned hybridoma medium.

120 mg H30 was coupled to Hydrazide AvidGel (BioProbe International) according to the manufacturer's instructions

2.4. *Purification*

The hIL-5 containing medium was applied on the mAb column (0.25 ml/min), previously equilibrated with 20 mM KP_i pH 6. Subsequently the column was washed with the following successively used solutions: 20 mM KP_i pH 6; 20 mM KP_i pH 6, 0.05% (v/v) Triton X-100; 20 mM KP_i pH 6, 200 mM KCl, 50 mM Tris-HCl pH 7.4. The adsorbed material was eluted with 2 M $MgCl_2$, the pooled peak fractions extensively dialysed against 50 mM Tris-HCl pH 7.4 and clarified from precipitates by ultracentrifugation. The protein solution was concentrated and further purified by gel filtration in 50 mM Tris-HCl pH 7.4, 0.05% (w/v) NaN_3 at 1 ml/min on a TSK-G2000 SWG column (Ultropac 21.5 × 600 mm, TosoHaas).

2.5. *Analytical methods*

SDS-PAGE was performed according to Laemmli [23]. IEF was performed on a Phastsystem (Pharmacia).

Western blot analysis: proteins separated via SDS-PAGE were transferred to an Immobilon-P filter (Millipore). The membrane was incubated with polyclonal rabbit antiserum raised against yeast derived recombinant hIL-5, followed by an incubation with a sheep anti-rabbit IgG tagged with alkaline phosphatase (Seralab).

Deglycosylation experiments were performed using *N*-Glycosidase F (Boehringer), according to the manufacturer's instructions.

hIL-5 was assayed for biological activity in the CTLL/CA1 proliferation assay (Cornelis et al., in preparation). For this purpose a hIL-5-dependent cell line was established by transfection of murine IL-2-dependent CTLL-2 cells with the expression plasmid pCAGGS [25] containing the hIL-5Rα cDNA. Selection was performed in the presence of hIL-5. From several selected clones one, CTLL1/CA1, was further analyzed. The functional high affinity IL-5 receptor complex on the transfectant is composed of the transfected human α and the endogenous mouse β receptor chain (AIC2B).

hIL-5 protein concentrations were determined using optical density measurements at 280 nm and a calculated $\varepsilon^{1\ mg/ml}_{1\ cm}$ of 0.631, or measured according to Bradford [26], using a Bio-Rad kit with BSA as a standard.

N-terminal amino acid sequence analysis was carried out as previously described [27].

2.6. *Crystallization procedures*

Prior to crystallization, the protein was examined in solution using laser light scattering techniques to determine their suitability for crystallization, as described by Zulauf and D'Arcy [28].

The purified protein was concentrated to 5–6 mg/ml with an Amicon 8MC unit using YM 10 membranes. A sparse matrix similar to that described by Jancarik and Kim [29] was set up at room temperature using the hanging drop vapour diffusion method [30].

3. RESULTS AND DISCUSSION

3.1. *Expression, production and purification*

We have previously described the efficient expression of mature, biologically active hIL-5 in a number of eukaryotic expression systems [6]. The highest production, however, was obtained in Sf9 cells using a baculovirus vector: 3.6×10^4 U/ml culture medium 72 h post-infection, when assayed on CTLL/CA1 cells, or 12 to 18 μg hIL-5/ml assuming a specific biological activity of $2–3 \times 10^6$ U/mg.

The non-neutralizing mAb, H30 (isotype IgG2b), was found to facilitate the purification of large quantities of hIL-5 (± 10 mg/run/50 ml column bed) when coupled via its oxidized carbohydrate chains to Hydrazide AvidGel.

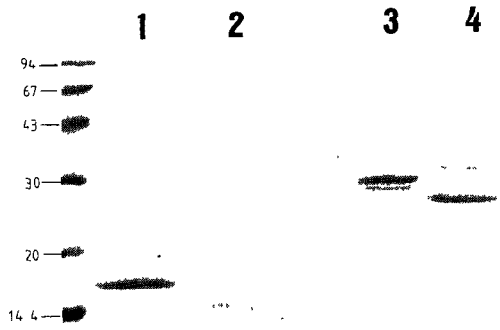

Fig. 2. Deglycosylation of recombinant hIL-5. 7 μg protein was treated (37°C; 21 h) with 1 U *N*-glycosidase F under denaturing condition. After enzymatic digestion, the sample was subjected to SDS-(15%)-PAGE analysis in the presence (lane 2) or absence (lane 4) of 2-mercaptoethanol and stained with Coomassie brilliant blue. As a control non-treated hIL-5 was run (lane 1 and 3).

Although the sample purity on silver-stained gels was estimated to be > 95% after immuno-affinity chromatography, further purification by gel filtration was employed to remove high molecular weight- and immuno-complexes. Heterogeneity of the recombinant protein was observed on reducing and non-reducing SDS-PAA gels as well as on Western blot (Fig. 1): several bands were recognized well by a polyclonal rabbit antiserum, proving that they represent different isoforms of the same protein. Upon SDS-PAGE, deglycosylated hIL-5 migrated as one single band (14.5 kDa under reducing and 27.5 kDa under non-reducing conditions in Fig. 2), which indicates that the several bands represent different glycosylation states. Analytical IEF gels showed 6–7 discrete bands (data not shown) indicating a range of pI's between 5.10 and 8.45. The major band corresponds to an IEP of 7.25 ± 0.1 which is close to the theoretical value of 7.4 and also to the pI of 7.2 determined by Ingley et al. [10] for yeast as well as for baculovirus derived recombinant hIL-5.

N-terminal analysis of the baculo-produced hIL-5 revealed the unique sequence Ile-Pro-Thr-Glu-Ile-, as predicted from the cDNA sequence.

3.2. *Light scattering measurements*

Initial light scattering data with the protein solution (0.5–2.5 mg/ml) showed a bimodal distribution with hydrodynamic radii between 22 Å and 700 Å. A radius of 22 Å would correspond to a hIL-5 dimer, while 700 Å indicated that the sample still contained high molecular weight aggregates. These aggregates were removed by centrifugation in an airfuge (20 min 100,000 × *g*). The supernatant was reexamined and shown to be monodisperse. We believe that the large aggregates originate from denatured molecules, resulting from the 2 M $MgCl_2$, used to elute hIL-5 from the antibody column.

3.3. *Crystallization*

A sparse matrix screen, used for initial crystallization conditions, produced only one drop containing microcrystals viz. in 25% PEG 4000, 100 mM $MgCl_2$, 100 mM cacodylate pH 6.5. The drop was touched with a tungsten needle and the next day long needle shaped crystals had grown. It has been possible to reproducibly grow larger needles, suitable for X-ray diffraction studies using crystal seeding techniques. For macro seeding, small crystals were isolated and washed in 27.5% PEG 8000, 100 mM $MgCl_2$, 100 mM cacodylate pH 6.5 and placed in drops with 3 μl of protein and 3 μl of reservoir solution containing 25% PEG 8000, 100 mM $MgCl_2$, 100 mM cacodylate pH 6.5. Seeded crystals grew in size and also caused secondary nucleation for further crystals to grow. The crystal seeding techniques used are similar to those described in detail by Stura and Wilson [31]. Crystals reached a maximum size of up to 0.8 mm × 0.12 mm × 0.12 mm after 2–3 weeks. They diffract to 2 Å resolution and belong to the *C*2 space group with cell parameters a = 118.6 Å, b = 24.4 Å, c = 45.5 Å, β = 109.2°. The crystals thus grew under similar conditions and belong to the same space group as those described recently for *E. coli* derived recombinant hIL-5 [32,33]; however the cell parameters are different and (in contrast to what was found for the bacterial material) the asymmetric unit contains only one monomer. These differences can be explained by alternative packing, probably due to the carbohydrate moiety which is included upon crystallization.

It is particularly important to establish that the crystallized protein contains a biologically active form. We have demonstrated this by washing a number of hIL-5 crystals in stabilising buffer to remove soluble protein

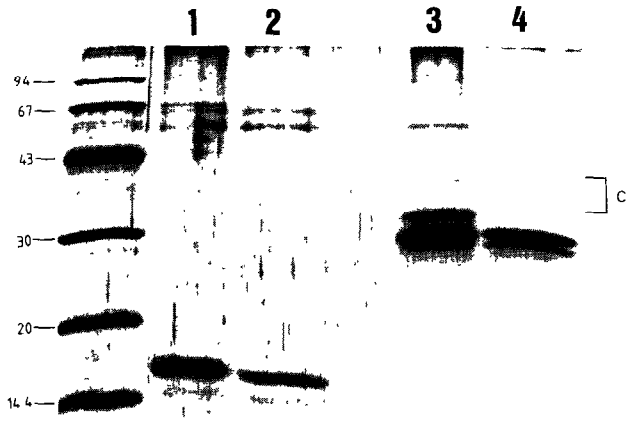

Fig. 3. Comparison of purified recombinant hIL-5 before and after crystallization. The protein samples recovered from washed crystals (lanes 2 and 4) were separated on an SDS-(15%)-PAA gel in reducing (lanes 1 and 2) as well as in non-reducing conditions (lanes 3 and 4) and silver stained following the method of Morrissey [24]. A solution of crystallized protein seems not to contain minor contaminants (c) which are seen in the starting material (lanes 1 and 3; 0.75 μg protein/lane). Bands ranging from 50 kDa to 68 kDa correspond with artifacts [34].

and testing the biological activity compared to a sample prior to crystallization. The recovered protein from the crystals proved to have a slightly higher specific biological activity than the starting material which may be explained by the fact that minor contaminants in the starting material were not included in the crystal as demonstrated in Fig. 3. From the same SDS-PAGE patterns, it can also be concluded that the different glycosylated hIL-5 forms were included into the crystals.

Whilst this paper was in preparation, Milburn et al. [35] published the crystal structure of *E. coli* derived recombinant hIL-5. Further analysis is needed to verify that this structure fits with the one for glycosylated hIL-5.

4. CONCLUSIONS

With the availability of sufficient quantities of purified, highly biologically active, glycosylated recombinant hIL-5, it has been possible to grow crystals suitable for X-ray diffraction studies. Complete native data sets have been collected and suitable heavy atom derivatives prepared. Resolution of this structure and of the receptor binding site in particular would facilitate the design of a low molecular weight antagonist as a potential pharmaceutical drug in preventing eosinophilia.

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