ELSEVIER

Process Biochemistry 41 (2006) 1343–1346

Process Biochemistry

www.elsevier.com/locate/procbio

Improved periplasmic production of biologically active murine interleukin-2 in *Escherichia coli* through a single amino acid change at the cleavage site

Johan Robbens [a,b,*], Wim De Coen [b], Walter Fiers [a], Erik Remaut [a]

[a] *Department of Molecular Biomedical Research (DMBR), Flanders Interuniversity Institute for Biotechnology, Ghent University, Technologiepark 927, 9052 Gent, Belgium*

[b] *Laboratory Ecophysiology, Biochemistry and Toxicology, University Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium*

Received 6 May 2005; received in revised form 27 December 2005; accepted 17 January 2006

Abstract

We fused the mature murine Interleukin-2 (mIL-2) gene to the signal peptide of the Outer membrane protein A (OmpA). We generated mutants mimicking different cleavage sites. A hybrid protein consisting of the OmpA signal peptide fused precisely to mature mIL-2, thereby mimicking the cleavage site of the OmpA native protein, was very poorly secreted into the periplasm of *Escherichia coli* (200 U/ml). Insertion of a serine residue between the OmpA signal peptide and the mIL-2 mature sequence, thus mimicking the mIL-2 natural cleavage site, increased the secretion by a factor of 40,000 (8×10^6 U/ml). The specific biological activity of secreted mIL-2 equaled that of natural mIL-2 and was about five times higher than that of mIL-2 refolded from inclusion bodies. We also show that the temperature at which the culture is grown has a major impact on the secretion level.

Keywords: Interleukin-2; *E. coli*; High-level production; Periplasm; Signal peptide; Recombinant

1. Introduction

Interleukin-2 (IL-2) is a lymphokine synthesized and secreted primarily by T helper lymphocytes that have been activated by stimulation with certain mitogens or by interaction of the T cell receptor with antigen/MHC complexes on the surface of antigen presenting cells [1]. IL-2 displays a variety of biological activities. Of particular interest is its role in clinical applications, such as treatment of renal cancer [2,3], HIV [4] and potentially several other diseases as reviewed recently [5].

Different production routes for adventitious proteins have been developed in *Escherichia coli* (for review [6]). Although cytoplasmic accumulation allows the production of high level of proteins, often up to 40–50% of the total cellular protein [7], it suffers from several drawbacks. Often overexpressed proteins end up in inclusion bodies in which proteins are present in a biologically inactive form. In addition, the reducing environment of the cytoplasm prevents the formation of intra- and intermolecular disulfide bridges, and hampers the production of these proteins where disulfide bridges are essential for its structure and function. Periplasmic secretion is a production route that may overcome these drawbacks [8], (for review [9]), and in addition offers the advantage of ease of purification as the periplasm contains only 5% of total cellular proteins [10]. An important drawback, however, is the low amount of secreted protein [11].

Murine IL-2 (mIL-2) is a protein of 169 amino acids which contains a signal peptide of 20 amino acids. It contains three cysteines, two of which form a disulfide bridge that is required for its biological activity [12]. This, together with the fact that cytoplasmic production of mIL-2 leads to inclusion body formation [13] hampers the cytoplasmic production of biologically active protein. Refolding of mIL-2 from inclusion bodies is a low efficiency process and refolded protein has a specific biological activity five times lower than natural IL-2 [13].

In this paper, we describe the high level periplasmic secretion of fully biologically active mIL-2. By optimizing the sequence surrounding the cleavage site we were able to drastically increase the production and secretion level. To the best of our knowledge has this secretion strategy never been

* Corresponding author. Fax: +32 3 265 3497.
E-mail address: Johan.Robbens@ua.ac.be (J. Robbens).

1359-5113/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2006.01.009

explored. We fused the Outer membrane protein A (OmpA) signal peptide to the gene of mature mIL-2. The secreted protein has a specific biological activity equal to that of natural mIL-2, which is 10 times higher than mIL-2 purified from denaturant- and detergent-treated inclusion bodies (12). The results clearly demonstrate the importance of these amino acid residues for production and periplasmic secretion of mIL-2 and in general indicate that optimization of the amino acid sequence around the cleavage site can dramatically increase the level of secretion. In addition, we demonstrated temperature to be another important factor in the optimization strategy for augmented secretion and protein production. We expect this approach to be one of the possible routes for the optimization of production of fully biologically active recombinant proteins.

2. Materials and methods

2.1. Bacterial strain and growth medium

E. coli strain RRlΔM15 (RR1, *lacZΔM15/F′ lacI^q lacZΔM15proA*) was used for cloning procedures and induction experiments. Cells were grown on Luria–Bertani medium containing 1% tryptone (LabM, Bury, England), 0.5% yeast extract (LabM, Bury, England) and 0.5% NaCl. The antibiotics triacilline and chloramphenicol were added at concentrations of 100 μg/ml and 25 μg/ml, respectively.

2.2. Expression plasmids

All expression plasmids make use of the *tac*-promoter as present on pMc519. The OmpA signal sequence was from pMaHB2Mmls. The sequence encoding mIL-2 was from pSP65mIL2m. The final interleukin-2 expression plasmids, pNIL2, pSIL2 and pAIL2 (see Fig. 1A for relevant features), were obtained through several intermediate constructs (details are available from the authors). Source plasmids were obtained from BCCM™/LMBP - Plasmid Collection, Ghent, Belgium (http://www.belspo.be/bccm/db/plasmid_search.asp).

Oligonucleotides, used for mutagenesis, were from Eurogentec- Belgium. The mIL2A primer, d[5′GGAGCTTGAAGTGGGTGCGGCCTGCGCTACGG 3′] lacks the TCA-triplet coding for serine; the mIL2B primer, d[5′GAAGTGGGTGCTG-ACTGCGCTACGGTAGCG3′] lacks the GCC-triplet coding for alanine. All mutations were confirmed by DNA sequence analysis (data not shown).

2.3. Induction of protein synthesis, cell-fractionation and analysis of protein fraction

Bacterial cultures containing a mIL-2 plasmid were grown overnight at 28 °C in selective Luria–Bertani (LB) medium, diluted 100-fold in fresh selective medium and incubated at 28 °C or 37 °C to a density of 5×10^8 cells/ml. Synthesis of mIL-2 was initiated by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM. The cells were collected by centrifugation at 5 h post-induction.

Plasmid	Amino acid sequence: Signal peptide	Mature protein
Wt-mIL2	**MYSMQLASCVTLTLVLLVNS▼APTS**	
Wt-OmpA	**MKKTAIAIAVALAGFATVAQA▼APKD**	
pNIL2	**MKKTAIAIAVALAGFATVAQA▼APTS**	
pSIL2	**MKKTAIAIAVALAGFATVAQA▼SAPT**	
pAIL2	**MKKTAIAIAVALAGFATVAQS▼APTS**	

Fig. 1. Amino acid sequence of the signal peptide and cleavage site of the different constructs. The triangle (▼ T) indicates the cleavage site.

2.4. Subcellulular fractions

Periplasmic proteins, cytoplasmic soluble and insoluble material were prepared as follows: 1.5 ml of cells were pelleted (8000 × *g*, 2 min) resuspended in 200 μl shock buffer (100mM Tris–HCl, pH 7.4, 20% sucrose (w/v) and 10 mM EDTA) and incubated on ice for 5 min. After centrifugation (8000 × g, 2 min) the supernatant was removed, and the pellet was rapidly resuspended in 200 μl water with vigorous shaking and incubated on ice for on additional 5 min. The periplasmic fraction was collected following centrifugation (16,000 × *g*, 2 min). The pelletted cells were resuspended in 200 μl water and sonicated on ice for 2 min with a Vibra Cell 500 W sonicator (Sonics and Materials, Danbury, CT) with the output control set at 4 and at 40% duty cycle. Finally the supernatant (the soluble fraction) was collected (16,000 × *g*, 2 min) and the pellet was washed and resuspended in 200 μl distilled water (insoluble fraction). Protein analysis was performed on 15% SDS-PAGE according to Laemmli et al. [14]

2.5. Biological assay

The biological titer of mIL-2 was determined by a proliferation assay using a continuous IL-2-dependent line of cytotoxic T cells [15]. Serial three- or fivefold dilutions of the test samples were incubated with CTLL-2 cells in microtiter plates (2×10^4 cells/well) for 24 h at 37 °C in the presence of [^{3}H]-thymidine. One unit of IL-2 is defined as the amount in 1 ml that induces CTLL-2 cells to incorporate [^{3}H]-thymidine at 50% of their maximum level. All values were referred to a human IL-2 (Jurkat) standard. The specific biological activity of this pure Biological Response Modifiers Program (BRMP) reference reagent was 1.31×10^6 U/mg. The specific activity of mIL-2 is known to be approximately 50 times higher, i.e. 5×10^7 U/mg [16]

2.6. DNA manipulations

Unless otherwise stated, the techniques and conditions used for DNA manipulation were as compiled in Sambrook [17]. Restriction endonucleases and all other enzymes were purchased from New England Biolabs (Beverly, MA 01915-5599, USA) and were used according to the manufacturers' instructions.

2.7. mIL-2 purification

mIL-2 was purified to determine its specific biological activity. Purification was performed as described previously [19]. In brief, periplasmic proteins were precipitated by addition of ammonium sulphate to 70% saturation. After centrifugation (30 min, 13,000 × *g*), the pellet was solubilized in 20 mM ethanolamine (pH 9) and applied on a Q sepharose FF (AmershamPharmacia, Roosendaal, The Netherlands). After elution with a linear 0–1 M NaCl gradient, the mIL-2 containing fractions, which eluted around 200 mM NaCl, were more than 95% pure. Finally these fractions were pooled, dialyzed against PBS (10mM sodium phosphate, pH 8.0; 150 mM NaCl) for 12 h at 4 °C, and loaded on a MonoQ HR 16/10 column (AmershamPharmacia, Roosendaal, The Netherlands). Elution at a NaCl concentration of about 300 mM in PBS buffer, yielded mIL-2 that was more than 99% pure.

2.8. NH2-terminal amino acid sequence analysis

After 15% SDS-PAGE gelelectrophoresis, proteins were electroblotted onto poly(4-vinyl *N*-methylpyridinium iodide) coated glass fiber sheet [18]. The appropriate band was cut out and the NH_2-terminal amino acid sequence was determined by Edman degradation on an A470 gas-phase sequenator equipped with a 120A on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Foster City, CA).