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Improved Strategy for Production and Purification of the Human Tumor Necrosis Factor α from Inclusion Bodies

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Summary

Production of human tumor necrosis factor α (hTNF-α) in the expression system **E. coli/pMAX** *resulted in high expression of the 25 kDa fusion protein appearing as an insoluble fraction in inclusion bodies. Several isolation procedures, cleavage methods and renaturation procedures are described, which have been investigated in order to obtain a high amount of active hTNF-α. Electroelution from semipreparative SDS-PAGE yielded a relatively small amount of the fusion protein, while cation exchange chromatography turned out to be a convenient method for purification of large amounts of hTNF-α protein. Two methods for the cleavage of fusion protein were checked: chemical by CNBr and enzymatic by the blood coagulation factor Xa. CNBr treatment of the fusion protein ADK-Xa-TNF completely cleaved the fusion protein even at pH of 6.8, whereas cleavage by the factor Xa was only partial. We succeeded in increasing the efficiency of this cleavage by introducing the collagen linker in front of the factor Xa cleavage site. It was shown that thus obtained fusion protein ADK-CL-Xa-TNF was advantageous relative to the fusion protein ADK-Xa-TNF also because its degree of precipitation during the dialysis against buffer solution, used for cleavage with factor Xa, was smaller. After the cleavage by CNBr, hTNF-α was isolated by electroelution from the semipreparative gel, whereas after the cleavage by the factor Xa, the cation exchange column was used. In both cases dialysis was used for renaturation of the protein. The second procedure involving cation exchange chromatography turned out to be more efficient; the quantity of hTNF-α was lower, however, the percentage of active protein was higher.*

Keywords: human tumor necrosis factor α, inclusion bodies, transformation and expression studies

Introduction

Human tumor necrosis factor α (hTNF-α in further text) is classified together with hTNF-β, interferons, interleukins and growth factors into the group of hormone-like substances called cytokines. hTNF-α was first observed by Carswell *et al. (1)* in the serum of endotoxin-treated mice and rabbits that had previously been sensitized with Bacillus Calmette-Guerin. This factor caused hemorrhagic necrosis of various transplanted tumors in mice. From this – its first known property – originates its name. Today it is known that besides its cytotoxic effects upon tumor cells, hTNF-α has a broad spectrum of activities upon hematopoetic and nonhematopoetic cells. A complete register of processes, in which hTNF-α takes part directly or indirectly, is very difficult to elaborate, since this pluripotent cytokine is secreted by different cells and participates in a large number of processes in the organism. Some of its most important activities are: growth inhibition of some tumor cells, stimulation of human fibroblasts, B cell and thymocyte proliferation, activation of phagocytic and endothelial cells, induction of prostaglandin synthesis as well as regulation of oncogenes, transcription factors and major histocompatibility complex antigen expression. hTNF-α has (besides the above cited activities) also quite a few severe side effects. Excessive production of hTNF-α can be very harmful to the organism, the most frequent side effects being fever, anorexia, diarrhea, nausea and hypotension *(2–4)*.

Mature hTNF-α is a nonglycosylated protein, with a sequence consisting of 157 amino acid residues. It contains a single intramolecular disulfide bond, which is not important for biological activity and lacks any methionine residue. It is biologically active as compact trimer *(4)*. It's molecular weight is about 17 000.

hTNF-α is an interesting subject to study, becuse potentially it may become useful as an antitumor agent, and on the other hand, it represents the basis for studying pathological states due to its increased amounts in the body. Therefore, for biochemical, pre-clinical and clinical

studies production of larger amounts of hTNF-a is necessary, which is today possible by recombinant DNA technology.

hTNF-α was first successfully cloned in *E. coli* in the year 1984 *(5)* and afterwards by several other research groups *(6–8)*. Most of them have chosen *E. coli* expression systems in which hTNF-α is expressed in the cytoplasm in the soluble form. Expression of hTNF-α was relatively low in most of these cases, and in addition it was necessary to isolate the protein from the complex mixture of all host cell proteins usually by multistep purification procedure including gel chromatography, ion exchange chromatography and adsorption chromatography. We have chosen a different approach by exploring the applicability of an expression plasmid pMAX for the laboratory production of hTNF-α. We expected to get the high production of hTNF-α in the form of a fusion protein in the insoluble fraction of the cell lysate which could be cleaved *in vitro* and easily separated from majority of the cell proteins.

Materials and Methods

Organism and plasmids

Escherichia coli, strain JM 109, was used for transformation and expression studies. Plasmid BBG-14 bearing synthetic hTNF-α gene with codons optimized for *E. coli* was supplied by British – Biotechnology. Plasmid pAX5+ bearing sequence for collagen linker was supplied by Mo-Bi-Tec. Plasmid pMAX was provided by Prof. T. Samejima (Tokyo, Japan). It is a construct of plasmids pKK-223 and pUC18 with a partial sequence of adenylate kinase (ADK) and synthetic linker, coding for recognition sequence for cleavage of the fusion protein by blood coagulation factor Xa. Expression of fusion proteins is under control of the *tac* promoter.

Cloning and expression of hTNF-α

A synthetic gene coding for hTNF-α was subcloned into an expression vector pMAX in the reading frame on the C-terminal end of the sequence for adenylate kinase and synthetic linker Xa. Collagen linker was inserted between the sequence for adenylate kinase and synthetic linker Xa in the reading frame. For all manipulations of plasmids standard procedures were used using enzymes supplied by Boehringer (Mannheim) or Pharmacia.

Prior to each fermentation, *E. coli* JM109 cells were transformed with pMAX/TNF and then grown as shaken cultures (250 r.p.m.) for 8 hours at 37 °C in LB medium containing ampicilin (50 μg/mL) without IPTG induction.

hTNF-α preparation

E. coli cells containing fusion proteins with hTNF-α were disrupted by combination of lysis with lysozyme and sonification. After centrifugation the pellet fraction of cell lysate (which contained inclusion bodies) was washed with the following solutions: 20 mM Tris/HCL (pH = 8.0), 5 mM EDTA, 0.2 mg/mL lysozyme; 20 mM Tris/HCl (pH = 8.0), 5 mM EDTA, 20 mg/mL sodium deoxycholate, and water respectively, in order to remove contaminating cellular components *(9)*. Inclusion bodies were dissolved in 8 M urea, 50 mM Na-phosphate buffer, 5 mM DTT pH = 7.5 or in SDS-sample buffer (4% SDS, 62.5 mM Tris HCl, 20% glicerol 5% 2-mercaptoethanol pH = 6.8). Fusion proteins were cleaved chemically with CNBr *(10)* or enzymaticaly with the factor Xa *(11)*. For purification of fusion proteins and hTNF-α we applied preparative SDS-PAGE, which was followed by electroelution of the appropriate protein band from the gel, or ion-exchange chromatography. hTNF-α was renaturated by dialysis or by dilution.

Analysis of proteins

Protein concentration was estimated by Bradford procedure using bovine serum albumin (BSA) as a standard *(12)*. SDS-PAGE was performed according to the method of Laemmli using 4% stacking gel and 15% separation gel in a vertical electrophoresis system of Pharmacia. Proteins were visualized by partially modified procedures described for silver staining and for staining with Coommassie Brilliant Blue R 350 as described in the manufacturer instruction guide for the PhastSystem (Pharmacia). Western blot analysis was performed according to the general procedure from the laboratory manual of Sambrook *et al. (13)*. The bioactivity of hTNF-α in bacterial extracts and fractions from the purification procedures was measured by an *in vitro* cytotoxicity assay using murine L929 cells as the target cells *(14)*. Densitometric scanning was performed by BIO-RAD imaging densitometer.

Results and Discussion

Cloning and expression of hTNF–α

Synthetic hTNF-α gene with codons optimized for bacteria *E. coli* was properly inserted into expression

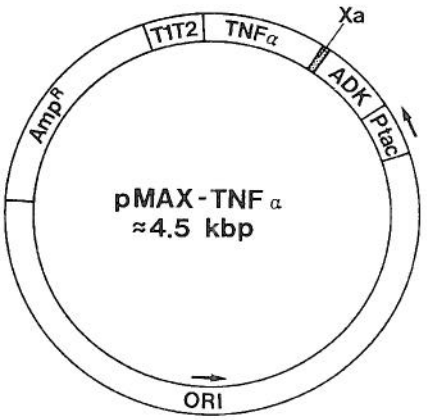

Fig. 1. Expression plasmid pMAX/TNF

ORI: origin of replication

Amp^R: resistance to ampicillin

P_{tac}: tac promoter

TIT2: transcriptional terminator

ADK: about one half of the adenylate kinasse gene (N-terminal part) which is highly expressed under the control of tac promoter

Xa: a recognition sequence (Ile-Glu-Gly-Arg) inserted between ADK and TNF for specific cleavage by coagulation factor Xa

TNF-α: human tumor necrosis factor α gene is inserted so that the following fusion protein results: ADK-Xa-TNF

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      Asp Pro Gly Pro Val Gly Pro Val Gly Ala
5'  1 GAT CCT GGT CCT GTT GGT CCT GTT GGT GCT
      CTA GGA CCA GGA CAA CCA GGA CAA CCA CGA

      Phe Gly Pro Arg Gly Leu Ala Gly Pro Gln
   31 TTT GGC CCA AGA GGT CTC GCT GGC CCA CAA
      AAA CCG GGT TCT CCA GAG CGA CCG GGT GTT

      Gly Pro Arg Gly Glu Lys Gly Glu Pro Gly
   61 GGT CCA CGT GGT GAG AAA GGT GAA CCT GGT
      CCA GGT GCA CCA CTC TTT CCA CTT GGA CCA

      Asp Lys Gly His Arg Gly Leu Pro Gly Leu
   91 GAT AAG GGA CAT AGA GGT CTG CCT GGC CTG
      CTA TTC CCT GTA TCT CCA GAC GGA CCG GAC

      Lys Ala His Asn Gly Leu Gln Gly Leu Pro
  121 AAG GCA CAC AAT GGA TTG CAG GGT CTT CCT
      TTC CGT GTG TTA CCT AAC GTC CCA GAA GGA

      Gly Leu Ala Gly Gln His Gly Asp Pro Pro
  151 GGT CTT GCT GGC CAA CAT GGT GAT CCG CCT
      CCA GAA CGA CCG GTT GTA CCA CTA GGC GGA

      Ala
  181 GCA
      CGT
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Fig. 2. Nucleotide and amino acid sequence of collagen linker

plasmid pMAX so that the following fusion protein resulted: ADK-Xa-TNF (Fig. 1). In order to obtain better accessibility of the recognition site for the proteolytic factor Xa, we introduced the collagen linker (CL) (Fig. 2) sequence before the cleavage site for the factor Xa which gave ADK-CL-Xa-TNF fusion protein sequence.

Both fusion proteins were highly expressed in *E. coli* intracellularly in the form of insoluble inclusion bodies. Densitometric scanning of the gel lanes representin pellet fraction of the cell extracts revealed that the fusion protein ADK-Xa-TNF represents up to 42%, and fusion protein ADK-CL-Xa-TNF up to 28% of the stained protein (Fig. 3). From one liter of cell culture we got up to

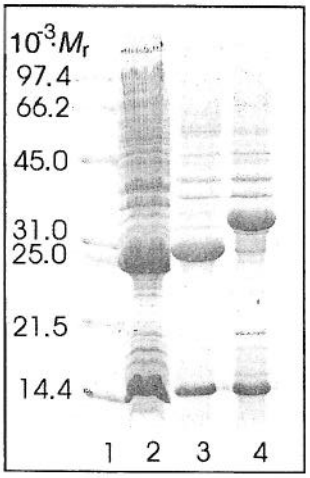

Fig. 3. Expression of fusion proteins ADK-Xa-TNF and ADK-CL-Xa-TNF in LB medium in shake cultures without IPTG induction. 15% SDS-PAGE, stained with Commassie blue.
Lane 1: molecular weight markers; lane 2: ADK-Xa-TNF in the whole cell extract; lane 3: ADK-Xa-TNF in the pellet fraction of cell extract; lane 4: ADK-CL-Xa-TNF in the pellet fraction of cell extract.

76 mg of fusion protein ADK-Xa-TNF and up to 37 mg of fusion protein ADK-CL-Xa-TNF.

Soluble fraction of the cell extracts showed low but measurable cytotoxic activity on L929 cells, which meant that small amounts of fusion proteins were present also in the soluble fraction. Presence of minor amount of soluble fusion proteins was determined also by Western blot technique.

IPTG, which is a relatively expensive compound, was not needed as inducer in the pMAX/TNF system. The mechanism of leaky expression in this system is not clear. One reason might be in too high copy number of the expression vector, since the replication origin of the vector originates from a high copy plasmid pUC18. In such a case lack of sufficient repressor to control the transcription initiation may cause leaky expression *(15)*. Another possibility is that DNA supercoil in the promoter and surrounding region is not proper, so that it interferes with the binding of repressors *(16)*.

Isolation of hTNF–α

Inclusion bodies containing the fusion protein were obtained by combination of lysis with lysozyme and sonification, followed by centrifugation. After washing with detergents, the fusion proteins represented up to 60% of the total protein. Such a high amount of recombinant protein in the inclusion bodies is one of the main advantages of isolation of the protein from the inclusion bodies. In order to obtain also a high quality of the final biologically active hTNF-α we applied two different isolation procedures, which are shown in Fig. 4. According to the first scheme, after solubilization of inclusion bodies in the SDS-sample buffer, all the dissolved proteins were treated by CNBr, which resulted in the complex mixture of cleaved proteins. On one hand, cleavage by CNBr represents a method of choice for hTNF-α fusion proteins since hTNF-α molecule contains no methionine residue and should remain intact after this procedure. On the other hand, cleavage by CNBr is usually performed in acidic pH *(17)*, which is known to be harmful to hTNF-α. Therefore we have chosen milder reaction conditions, at higher pH values approaching to pH = 7.0. It turned out that solubilization of the fusion protein in the sample buffer for electrophoresis, which contains 2% SDS, provides sufficient milieu for complete cleavage of the fusion protein by CNBr even at pH = 6.8 (Fig. 5).

After cleavage by CNBr, electrophoretically pure hTNF-α (9 mg/L cell culture) was isolated by means of semipreparative SDS-PAGE and by electroelution of the appropriate protein band from the gel. Renaturation was done by dialysis, and the final product exhibited the specific activity between 1.10^6 and 2.10^6 U/mg, which is less than 10% of cytotoxic activity of the native hTNF-α.

Assuming that SDS and CNBr treatments could probably be responsible for low percentage of biologically active protein, we also tried another way of isolation of hTNF-α from inclusion bodies. This procedure included cleavage with enzyme factor Xa, which is performed at milder, more natural conditions. Before cleavage the inclusion bodies were dissolved in 8 M urea, 50 mM Na-phosphate buffer, pH = 7.5, and the fusion protein was isolated by cation exchange chromatog-

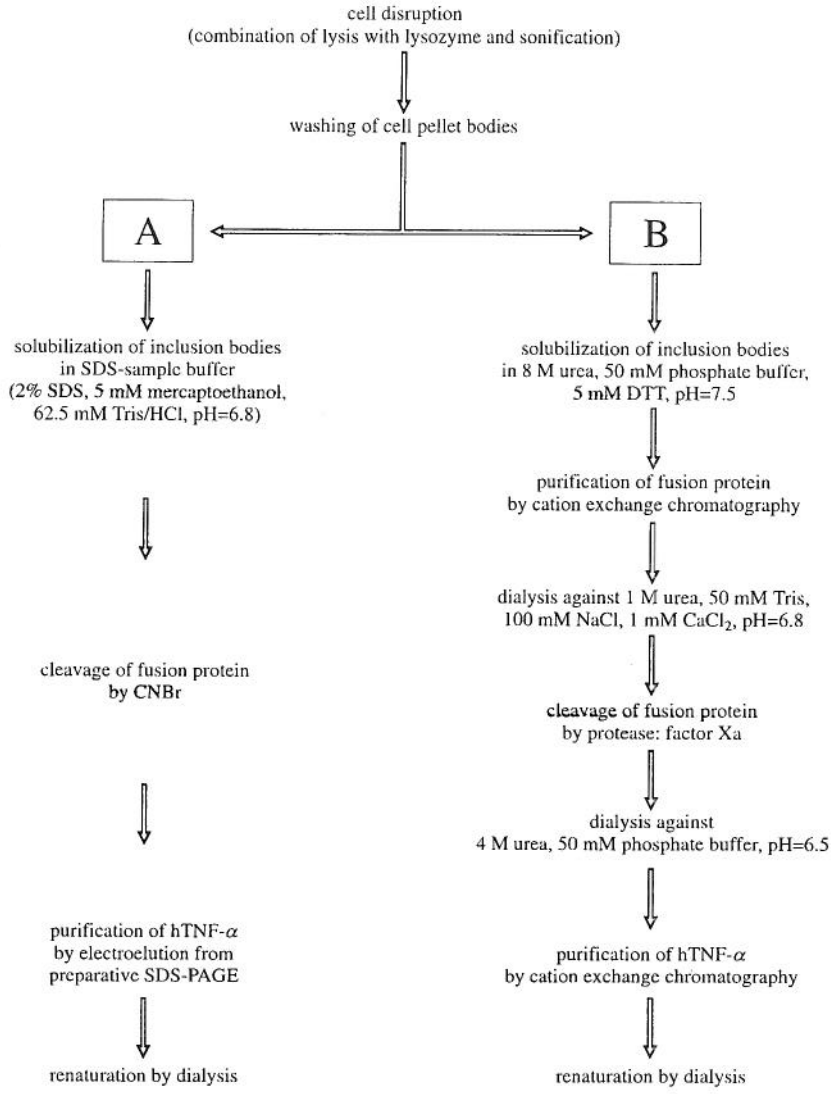

Fig. 4. Scheme of isolation of hTNF-α

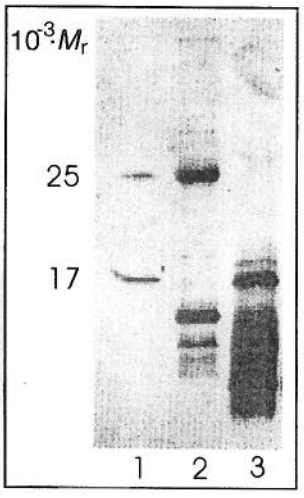

Fig. 5. Cleavate of fusion protein ADK-Xa-TNF with CNBr. 15% SDS-PAGE stained with Commasic blue.
Lane 1: molecular weight markers; lane 2: fusion protein ADK--Xa-TNF before cleavage with CNBr; lane 3: hTNF-α after cleavage with CNBr.

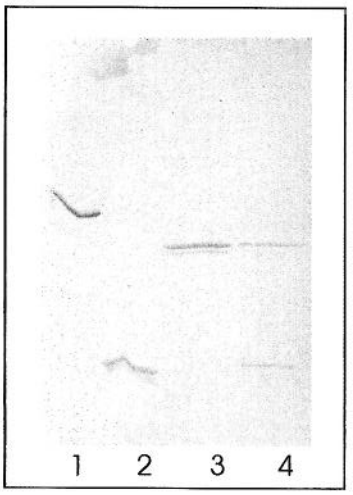

Fig. 6. Cleavage of fusion proteins with blood coagulation factor Xa. 15% SDS-PAGE, stained with Commasie blue.
Lane 1: fusion protein ADK-CL-Xa-TNF; lane 2: fusion protein ADK-CL-Xa-TNF after cleavage; lane 3: fusion protein ADK-Xa--TNF; lane 4: fusion protein ADK-Xa-TNF after cleavage.

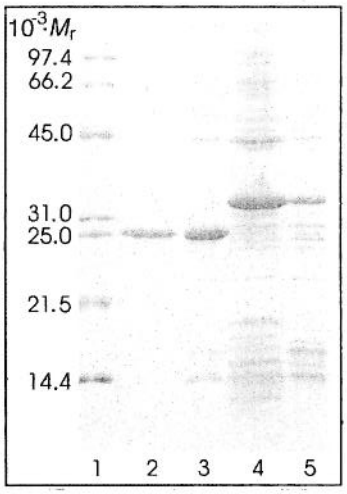

Fig. 7. Renaturation of fusion proteins. 15% SDS-PAGE, stained with Commassie blue.
Lane 1: molecular weight markers; lane 2: soluble ADK-Xa-TNF; lane 3: unsoluble ADK-Xa-TNF; lane 4: soluble ADK-CL-Xa-TNF; lane 5: unsoluble ADK-CL-Xa-TNF.

raphy. The method was chosen because of a high isoelectric point of the fusion protein which is due to high amounts of basic amino acids in ADK and collagen linker. We obtained 76 mg/L cell culture of the fusion protein ADK-Xa-TNF, and 37 mg/L cell culture of the fusion protein ADK-CL-Xa-TNF. In the next step only a partial cleavage of the fusion protein ADK-Xa-TNF by factor Xa was achieved despite various cleavage conditions tried. We succeeded in increasing the efficiency of this cleavage by modifying structure of the fusion protein: before the cleavage site for the factor Xa the collagen linker was introduced (Fig. 6). Thus, the obtained fusion protein ADK-CL-Xa-TNF was advantageous relative to the fusion protein ADK-Xa-TNF also because of its lower degree of precipitation during the dialysis against buffer solution, used for cleavage with factor Xa (Fig. 7). It seems that collagen linker, inserted between proteins ADK and hTNF-α, enables them to fold independently into their native structures, decreases exposure of hydrofobic surfaces and by that decreases the degree of aggregation of protein.

After the cleavage by factor Xa, the protein solution was applied to cation exchange column in the following buffer: 4 M urea, 50 mM Na-phosphate buffer, pH = 6.5. Proteins were eluted using gradient of the same buffer containing 0.5 M NaCl. Fractions containing hTNF-α (6 mg/L cell culture) were dialysed against renaturation buffer, and the final product exhibited the specific activity of $1.2 \cdot 10^7$ U/mg, which is approximately 50% of the cytotoxic activity of native hTNF-α.

To conclude, our experiments with an expression plasmid pMAX resulted in a very high production of hTNF-α in the form of two different fusion proteins which represent the main constituent of the insoluble fraction of cell lysate. The second fusion construct, with the sequence for collagen linker included between the ADK and hTNF-α part of the protein proved to enable high yields of biologically active protein. With some optimization, the separation could possibly be used for producing hTNF-α at larger scale.

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Poboljšani postupak za dobivanje i čišćenje ljudskoga tumorskoga nekroznoga faktora α iz inkluzijskih tijela

Sažetak

Proizvodnjom ljudskoga tumorskoga nekroznoga faktora α (hTNF-α) u ekspresijskom sustavu **E. coli/pMAX** *dobivena je velika ekspresija 25 kDa fuzijskoga proteina u obliku netopljive frakcije u inkluzijskim tijelima. Autori prikazuju više načina izolacije, cijepanja i renaturacijskih postupaka istraživanih s namjerom da bi se dobila veća*

količina aktivnoga hTNF-α. Elektroelucija sa semipreparativne SDS-PAGE dala je relativno malu količinu fuzijskoga proteina u usporedbi s kromatografskom kationskom izmjenom koja se pokazala pogodnom pri purifikaciji veće količine proteina hTNF-α. Istražena su dva postupaka za cijepanje fuzijskoga proteina: kemijski sa CNBr i enzimski s koagulacijskim faktorom krvi Xa. Postupak sa CNBr potpuno cijepa fuzijski protein ADK-Xa-TNF čak i pri pH = 6.8, dok je cijepanje s pomoću Xa-faktora bilo samo djelomično. Autori su uspjeli povećati djelotvornost cijepanja tako da su ispred mjesta za odcjepljenje faktora Xa ugradili kolagenski »linker«. Tako dobiven fuzijski protein ADK-CL-Xa-TNF ima prednosti pred fuzijskim proteinom ADK-Xa-TNF i zbog manjeg stupnja precipitacije pri dijalizi prema puferskoj otopini upotrijebljenoj prilikom cijepanja s faktorom Xa. Nakon cijepanja sa CNBr, hTNF-α izoliran je elektroelucijom iz semipreparativnoga gela, dok je nakon cijepanja s faktorom Xa upotrijebljena kationska izmjena na koloni. U oba slučaja provedena je dijaliza za renaturaciju proteina. Kromatografija kationskom izmjenom pokazala se djelotvornijom; iako je količina hTNF-α bila manja, udjel je aktivnoga proteina bio veći.