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Histidines in affinity tags and surface clusters for immobilized metal-ion affinity chromatography of trimeric tumor necrosis factor α

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Abstract

In order to achieve efficient IMAC (immobilized metal-ion affinity chromatography) purification of tumor necrosis factor α (TNF-α) and its analogs by a common chromatographic procedure, we tested four histidine-rich affinity tags attached to the N-termini of the trimeric TNF-α molecule. Using low cultivation temperature and appropriate protease deficient *E. coli* strains, it was possible to obtain intact, full-length proteins with NHis2Xa and HisArg tags, which could be purified to over 95% purity in a single step. However, in comparison to model proteins bearing a surface histidine cluster, accumulation of the histidine-tagged proteins in *E. coli* was significantly reduced, even in protease deficient strains. In addition, the histidine tagged TNF-α proteins never displayed good chromatographic behavior, which was otherwise easily achieved with model proteins. Although the most popular hexa-histidine tag is generally recognized as very convenient for single step isolation of monomeric proteins, our results with trimeric TNF-α indicate that oligomeric proteins may require further optimization of the tag, with respect to its length, composition, and location. Histidines, relatively rigidly inserted in the structure, as in our model proteins, display superior chromatographic characteristics vis a vis flexible tags with the same total number of histidines.

Keywords: Immobilized metal affinity chromatography; Tumor necrosis factor α; Histidine; Proteins

1. Introduction

Purification of recombinant proteins to the highly purified, homogeneous and biologically active products required for pharmaceutical use could avail itself of the power of recombinant DNA technology. In recent years, many purification approaches have been introduced among which histidine affinity tags have become quite popular [1–3]. Very frequently, the resulting metal-chelate binding proteins can be easily purified from crude mixtures by means of the immobilized metal-ion affinity chromatography (IMAC), that is based on the affinity of specific amino acid residues for immobilized transition metal ions. This chromatography has proved to be simple, highly selective, inexpensive and amenable to scaling up, and enables one step separation of the protein of interest from the bulk of other proteins and biological contaminants. New types of chromatographic supports have further increased the applicability of this technique [4,5]. However, there are only a few reports on the use of IMAC for the isolation of proteins for human therapy [6–8] under

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the very stringent and validated conditions required [9].

In recent years, we have been involved in the design and development of pharmaceutically valuable analogs of tumor necrosis factor α (TNF-α) with improved biologic properties, which make them potential candidates for future cancer therapy [10]. To this end, numerous analogs have been isolated in highly purified form for pre-clinical testing in animal models. Purification procedures for isolating different analogs with different characteristics and specific chromatographic behavior typically involve numerous pre-chromatographic and chromatographic steps to meet the criteria set for proteins in parentheral application. Therefore, it would be very convenient to develop a common purification protocol based on an optimized affinity tag, which could be fused to different analogs. It would appear that histidine affinity tags could fulfill these demands, especially if appropriate cleavage sites are engineered between the tag and the protein. After the appearance of Hochuli's papers [11,12], the hexa-histidine tag has become the most frequently used tool for the rapid purification and analysis of various recombinant proteins, especially in combination with the use of Ni-NTA (nickel-nitrilotriacetic acid) matrix [3,13–16]. However, TNF-α is an oligomeric protein composed of three almost symmetrically arranged subunits, which makes the design of the histidine tag more complicated. An ideal affinity tag should enable effective but not too strong binding, and allow elution of the desired protein under mild conditions, both for the protein and chromatographic column. Taking into account that many proteins from the *E. coli* also adhere to the IMAC matrices, it would be advantageous to construct an affinity tag that could be recovered in the ''contaminant-free window''. For monomeric proteins, a rational approach of selecting specific peptide ligands for IMAC by a phage displayed library showed that affinity tags with two histidine residues possessed chromatographic characteristics superior to those of the most common hexa-histidine tag [17,18]. However, binding depends to a great extent also on the nature of neighboring residues, especially hydrophobic ones. Unfortunately, in the case of TNF-α due to its trimeric structure, such a rational approach can not be directly employed. Therefore, we chose a more intuitive approach to design a useful affinity tag, based on the known three-dimensional (3D) structure of TNF-α. Some years ago we prepared a TNF-α analog LK-801 with a double histidine mutation in the polypeptide chain, which exhibited excellent chromatographic behavior [19]. We have used this analog and two related ones as models for the comparison with four newly designed histidine-tagged TNF-α proteins. To achieve expression and accumulation of non-truncated forms of these histidine-tagged proteins, we also tested their behavior in different *E. coli* strains at different cultivation temperatures.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade and were purchased from Sigma, Deisenhofen, Germany.

2.2. Model proteins and histidine tagged TNF-α proteins

TNF-α analogs LK-801 (Glu107His, Gly108His), LK-802 (Ser95Cys, Gly148Cys, Glu107His, Gly108His) and LK-817 (Ser95Cys, Glu107His, Gly108His), were used as model proteins.

Four different histidine affinity tags were designed and attached to the N-terminus of TNF-α. They are listed in Table 1.

3D structure coordinates of TNF-α were obtained from Brookhaven Protein Data Bank.

2.3. Expression of model proteins and histidine tagged TNF-α proteins in E. coli

For the expression of model proteins, as well as of NHis2Xa, HisArg and NHis5 tagged TNF-α proteins, an expression plasmid pCYTEXP1 (Medac, Hamburg, Germany), modified to constitutively express foreign proteins, driven by strong promoters from phage lambda, was used.

Synthetic TNF-α gene with *E. coli* optimized codons was supplied by British Biotechnology (Oxford, UK). After insertion of the gene into the expression vector pCYTEXP1, the DNA sequences

Table 1
Characteristics of different histidine affinity tags attached to the N-terminus of TNF-α

His-tagged TNF-α	Tag sequence	Tag length (No. of aa)	Spacer length (No. of aa)	Cleavage
NHis2Xa	*Met(**His**)$_2$IleGluGlyArg–TNF-α*	7	4	Xa
HisArg	*MetArg**His**(Arg)$_3$**His**Met–TNF-α*	8	1	CNBr
NHis5	*MetArg(**His**)$_5$Met–TNF-α*	8	1	CNBr
His10	*MetGly(**His**)$_{10}$(Ser)$_2$Gly**His**Ile (Asp)$_4$Lys**His**Met–TNF-α*	24	12	CNBr, enterokinase

encoding histidine tags were inserted using oligonucleotide-directed mutagenesis, based on single stranded DNA. For the expression of His10 tagged TNF-α, original expression plasmid pET19b (Novagen, Madison, WI, USA) was used. Production of foreign protein was induced by IPTG according to supplier's protocol.

All genetic manipulations with plasmid DNA were done according to standard molecular biology methods [20].

2.4. Bacterial strains and growth conditions

In addition to *E. coli* NM522 strain (Invitrogen, Groningen, The Netherlands) with normal proteolytic activity, the following protease deficient *E. coli* strains were used: SF103, SG935, SG927, SF110, SF120 (ATCC, Rockville, MD, USA), and BL21(DE3) (Novagen). Details on proteins and genes affected, are given in the text.

Model proteins, and histidine tagged TNF-α proteins, were expressed intracellularly as soluble, biologically active proteins in the above mentioned *E. coli* strains. For production, the LB medium, supplemented with 0.1 mg/ml of ampicillin was used. In the case of the model protein LK-801, an expression level of approximately 20% of total proteins was usually achieved at 37°C, while TNF-α analogs LK-802 and LK-817 accumulated up to 10% only, if produced at 30°C or below. The expression levels of histidine tagged proteins were only up to 5% of total proteins, even if produced at 30°C.

2.5. Preparation of crude extracts

The *E. coli* cells were collected by centrifugation at 5000 rpm for 5 min. The pellet was harvested, washed, and resuspended in 50 m*M* Tris–HCl, 30 m*M* NaCl, 5 m*M* EDTA, 0.05% polyethyleneimine, pH 8.0. Cells were disrupted using a W-358 sonicator (Heat SystemsUltrasonics, NY, USA). After 30 min-centrifugation at 15 000 rpm, nucleic acids in the supernatant were removed by further precipitation with 0.1% polyethyleneimine. After centrifugation at 15 000 rpm, the protein fraction containing model or histidine tagged TNF-α proteins, was precipitated at 65% saturated ammonium sulfate.

All pre-chromatographic operations were performed at +4°C.

2.6. IMAC of model and histidine tagged proteins

Chromatography was done on a fast protein liquid chromatography (FPLC; (Pharmacia, Uppsala, Sweden) system, consisting of two P-500 pumps, MV-7 valve, UV-1 monitor, strip chart recorder and Frac-100 fraction collector. The system was controlled by a LCC-500-Plus controller.

Three different metal chelate affinity matrices were used: Chelating Superose (Pharmacia, Uppsala, Sweden), Ni-NTA Agarose (Qiagen, Hilden, Germany), and TALON (Co) (Clontech, Palo Alto, CA, USA), packed in 20 mm long glass columns with a diameter of 10 mm. Chelated Superose with covalently bound iminodiacetic acid (IDA) was charged with Cu^{2+} ions by flushing the column with ten column volumes of 0.2 *M* cupric sulfate aqueous solution and washing with ten volumes of Milli Q water prior to chromatography. Chelating Superose has a loading capacity of 35 μmol Cu^{2+}/ml gel. The other two matrices, Ni-NTA Agarose and TALON (Co), were used without additional metal loading, as supplied. Ni-NTA Agarose contains 8–12 μmol Ni^{2+}/ml gel, and TALON has a minimum loading

capacity of 200 μmoles Co^{2+}/g dry weight. Binding capacities for all matrices are 5–10 mg of protein/ml resin. With all three stationary phases, the same adsorption buffer (0.02 *M* potassium phosphate, 0.2 *M* NaCl, pH 7.1) was used, and the elution was accomplished by an increasing gradient of imidazole in the same buffer. Prior to chromatography, the columns were saturated with imidazole by flushing with at least five column volumes of the adsorption buffer containing 50 m*M* imidazole. One or two blank runs were performed before first sample injection. The flow rate was 1 ml/min. Proteins were detected at 280 nm and 1 ml fractions were collected. All chromatographic runs were done at room temperature.

2.7. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Fractions from FPLC runs were analyzed by SDS–PAGE, using a Mini-Protean II electrophoresis Cell (Bio-Rad, Munich, Germany) on 15% separation gel with 1 cm of the 4% stacking gel. Protein bands were detected by silver staining procedure according to the manufacturer's instructions for the PhastSystem (Pharmacia).

2.8. Western blots

Western blots were used to determine the ratio of intact and truncated forms of histidine tagged proteins in crude protein extracts. After SDS–PAGE, electrophoretic transfer to the nitrocellulose membrane in the NovaBlot system (Pharmacia) and blocking of the non-specific membrane binding sites, the histidine tagged TNF-α proteins were immunodetected by reaction with polyclonal rabbit anti TNF-α antibodies (Blood Transfusion Center, Ljubljana, Slovenia), followed by the incubation with goat anti-rabbit IgG (Bio-Rad). These secondary antibodies were coupled to horseradish peroxidase and final detection was accomplished by reaction with 4-chloronaphthol.

Western blots were evaluated densitometrically on an Imaging Densitometer, Model GS-670 (Bio-Rad).

3. Results and discussion

3.1. Model proteins

Native TNF-α is a non-glycosylated M_r 17 000 protein, existing in solution in the form of a compact homotrimer, which is also its biologically active form. TNF-α contains three histidine residues per each subunit, one of them being partially exposed, which accounts for a weak retention on Cu-IDA [21], which is not enough to separate recombinant TNF-α from *E. coli* proteins.

Model proteins LK-801, LK-802 and LK-817 were designed on the basis of the known 3D structure of TNF-α molecule in such a way that mutations of Glu107 and Gly108 residues to histidines resulted in a surface cluster of six histidines, symmetrically arranged on the top of the trimeric, bell-shaped molecule. In the case of LK-801 (Fig. 1a) and LK-802, this structural feature led to excellent chromatographic isolation on different IMAC matrices (Fig. 1b) and resulted in one-step procedures, yielding proteins of over 95% purity [19,22] and high biologic activity [10]. Elution was effected by an increasing gradient of imidazole since exposure to low pH, which is frequently used for elution in IMAC, can damage the biological properties of TNF-α. Using imidazole elution, in fractions containing TNF-α analogs, pH never dropped under 7. Most probably, due to presaturation of columns with imidazole and performance of blank runs, the effect of imidazole proton pump [23,24] was negligible.

Analog LK-801 possesses only the double histidine mutations, while the analog LK-802 contains additional double cysteine mutations, which results in covalent linkage between the TNF-α subunits (Fig. 2a). On IMAC, analog LK-802 exhibits the same chromatographic behavior as the analog LK-801 (Fig. 2b). This indicates that the locations of cysteine mutations at the inner subunit binding surfaces do not interfere with the binding between TNF-α subunits. The structural topography of the protein, at least in the region of its histidine cluster, was not affected.

By comparison, the chromatographic behavior of TNF-α analog LK-817 on Cu-IDA matrix was

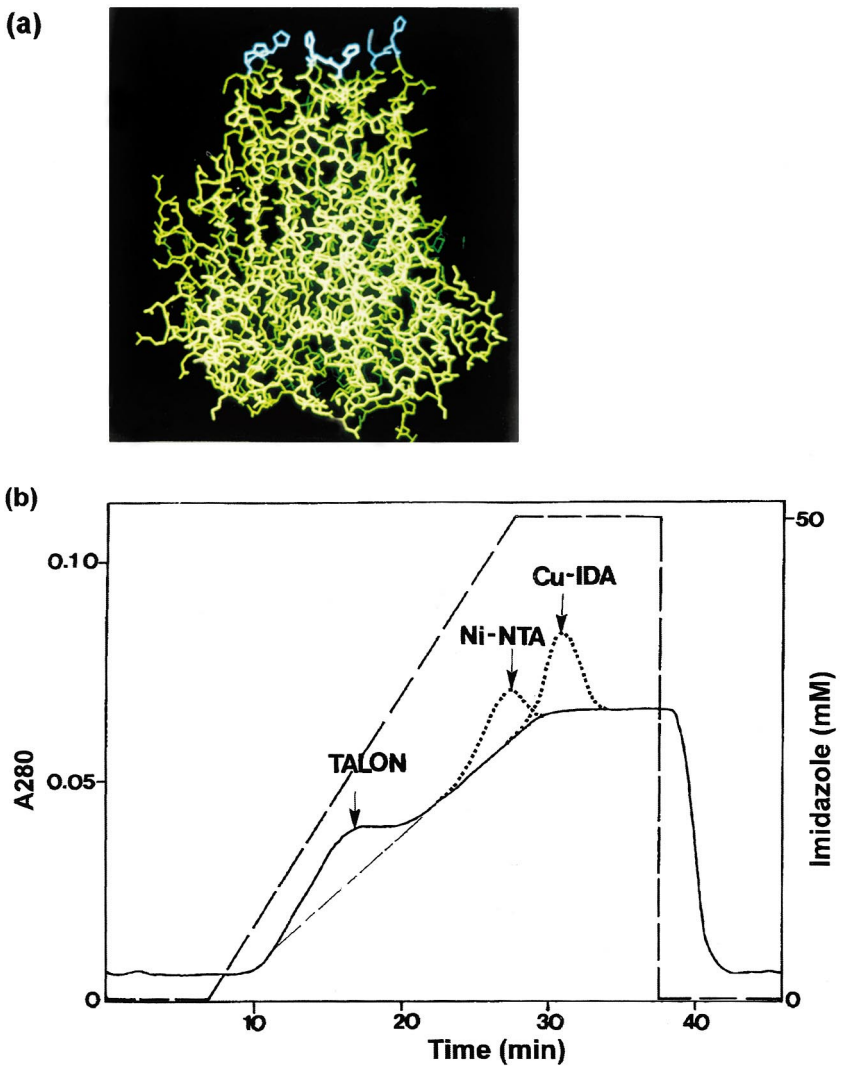

Fig. 1. (a) 3D structure of TNF-α analog LK-801; the newly introduced histidine residues are in blue. (b) Elution profiles of TNF-α analog LK-801 on three different IMAC matrices: Cu-IDA, Ni-NTA, and TALON. In each case, 100 μg of the purified analog was applied to a freshly prepared and equilibrated affinity column.

significantly different from that of LK-801 or LK-802 (Fig. 2). This analog, which contains a single serine to cysteine mutation localized at the inner natural binding surfaces between the subunits, reproducibly displayed higher elution times and a broader chromatographic peak, suggesting some minor changes in the surface histidine cluster and/or its neighborhood. Presumably, this mutation slightly modified the natural binding of subunits, leading to a less compact trimeric molecule. Consequently, histidines in the loops are probably more flexible and can better accommodate the matrix-bound ligand.

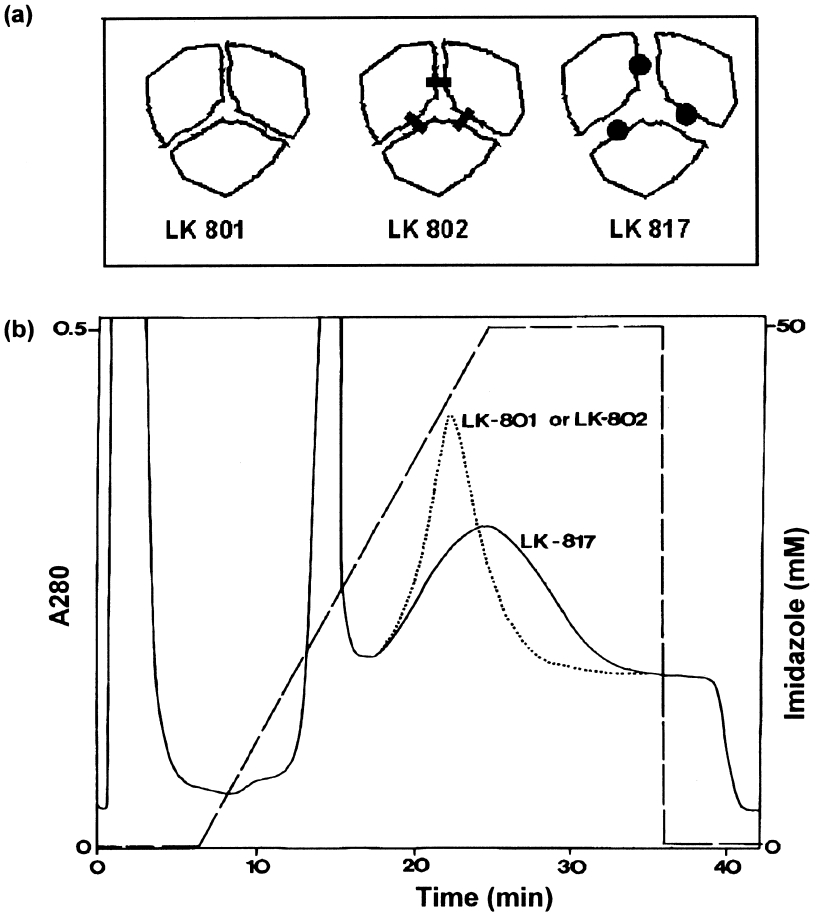

Fig. 2. (a) Schematic representation of model proteins: TNF-α analogs LK-801, LK-802, and LK-817 (cross-section in the plane perpendicular to the three-fold axis of symmetry). ■ Represents a disulfide bond connecting TNF-α subunits (due to introduced cysteine residues); ● represents -SH group of the introduced cysteine residue. (b) Elution profiles resulting from alternating application of crude *E. coli* NM522 extracts containing LK-801 or LK-817 analogs, to the Cu-charged Chelating Superose (Pharmacia). TNF-α analog LK-802 shows essentially the same chromatographic behavior as analog LK-801.

3.2. Design of histidine tags attached to the N-terminus of TNF-α

In general, histidine tags can be attached to N- or C-termini of a protein. Due to the basic structural and functional features of TNF-α, the histidine tags, which should be removed after an IMAC purification step, can only be attached to the N-terminus. The C-terminus of TNF-α is not surface accessible and, also, very susceptible to every change in sequence [25]. Only one example of the histidine tag attached to the C-terminus of TNF-α has been reported, but the fusion protein expressed either in *E. coli* or *P. pastoris* was insoluble and was not further investigated [26].

In contrast, the N-termini of the subunits in the trimeric TNF-α molecule are surface exposed, completely flexible and available for the attachment of histidine tags. A hexa-histidine tagged TNF-α was mentioned as an example for the assay of histidine tagged proteins on Ni-NTA HisSorb Strips [27], but we have not found any reference for its use by IMAC. Oligo-histidine tags have also been applied successfully by Unizyme Laboratories, Hørsholm, Denmark [28]: an affinity tag, consisting of histidines interrupted by another amino acid residue,

was used for efficient isolation of TNF-α from *E. coli* lysates. The tag was removed by commercially available enzymes (TAGZyme System,) but detailed information on tag sequences has not yet been revealed.

In order to achieve efficient purification of pharmaceutically valuable TNF-α and its various analogs, using a common chromatographic procedure, we designed our own affinity tags and attached them to the N-terminus of the molecule. Four different tags have been presented, containing different number of histidine residues in various arrangements. The length and structure of these tags, together with cleavage sites to allow production of the authentic TNF-α, are shown in Table 1.

Two different approaches were used to construct these tags. Firstly, we explored oligo-histidine tags. The most frequently used affinity tags for IMAC, especially for Ni-NTA and TALON matrices, contain six consecutive histidines [3,29], and thus our NHis5 construct is very similar. In the case of trimeric TNF-α, this means 15 histidine residues per molecule. Another frequently utilized tag contains 10 histidines, which is also the case with our His10 construct, resulting in 30 histidine residues per trimer.

The other approach in designing histidine tags was partly intuitive and partly based on the known 3D structure of TNF-α. Having had good experience with model protein LK-801 (surface cluster of 6 histidines, composed of two histidines in the flexible surface loop of each TNF-α subunit), we extended this approach to the affinity tags. Similarity is based on the assumption that the simultaneous interaction of all three tags with immobilized metal ions is possible. Two of the constructs, NHis2Xa and HisArg tagged proteins, contain two histidine residues in each affinity tag. With two consecutive histidine residues in each tag, NHis2Xa tagged TNF-α can be considered as a direct analog of the model protein LK-801, the only difference being that the histidine residues in tags are probably completely mobile, while in the loops, all the histidines are forced to face the exterior. In HisArg tagged TNF-α, the two histidines are separated by three polar and bulky arginine residues, which results in co-lateral arrangement of histidines if the peptide segment assumes the helical structure as has often been suggested [1,30]. This would presumably lead to increased probability for cooperative interaction of histidines with the matrix.

For research purposes, the attached affinity tags often do not have to be removed. However, when the authentic structure is necessary, as in the case of pharmaceutical proteins for human therapy, histidine tags must be cleaved off after the purification is accomplished. In the case of NHis2Xa and His10 tagged proteins, we introduced specific amino acid sequences to allow proteolytic cleavage by factor Xa and enterokinase, respectively. Due to the lack of methionine residues within the polypeptide chain of TNF-α, chemical cleavage by CNBr will also be possible if a methionine residue is introduced between the affinity tag and the protein, as in the case of HisArg, NHis5 and His10 tagged proteins (Table 1).

3.3. Chromatography of oligo-histidine tagged TNF-α

His10 tagged TNF-α, probably due to resident multiple histidines, exhibited very strong affinity for Cu-IDA, Ni-NTA and TALON (Co) matrices (data not shown). Elution was only possible under very harsh conditions. These were either 0.5 *M* imidazole, which can deteriorate the column performance, or 50 m*M* EDTA, which strips off the immobilized metal ion. As a result, the affinity column has to be freshly prepared prior to each separation. In addition, His10 tagged TNF-α monomer is always present in different forms, representing the full-length affinity tag and several truncated forms, contributing further to the heterogeneity of the trimer. We were not able to separate the different forms of His10 tagged TNF-α, by either linear or step gradients of imidazole.

The affinity of NHis5 tagged protein is also very high. We tried different metal affinity supports, and found that at least 100 m*M* imidazole was needed for the elution even from TALON (Co) support, where the retention is lowest compared with Cu-IDA and Ni-NTA. Separation of NHis5 tagged TNF-α from *E. coli* proteins was easily achieved, using the linear gradient of imidazole although it was eluted in a large volume, which could be inconvenient for a scale up. Application of the step gradient resulted in somewhat better chromatographic behavior (Fig. 3)

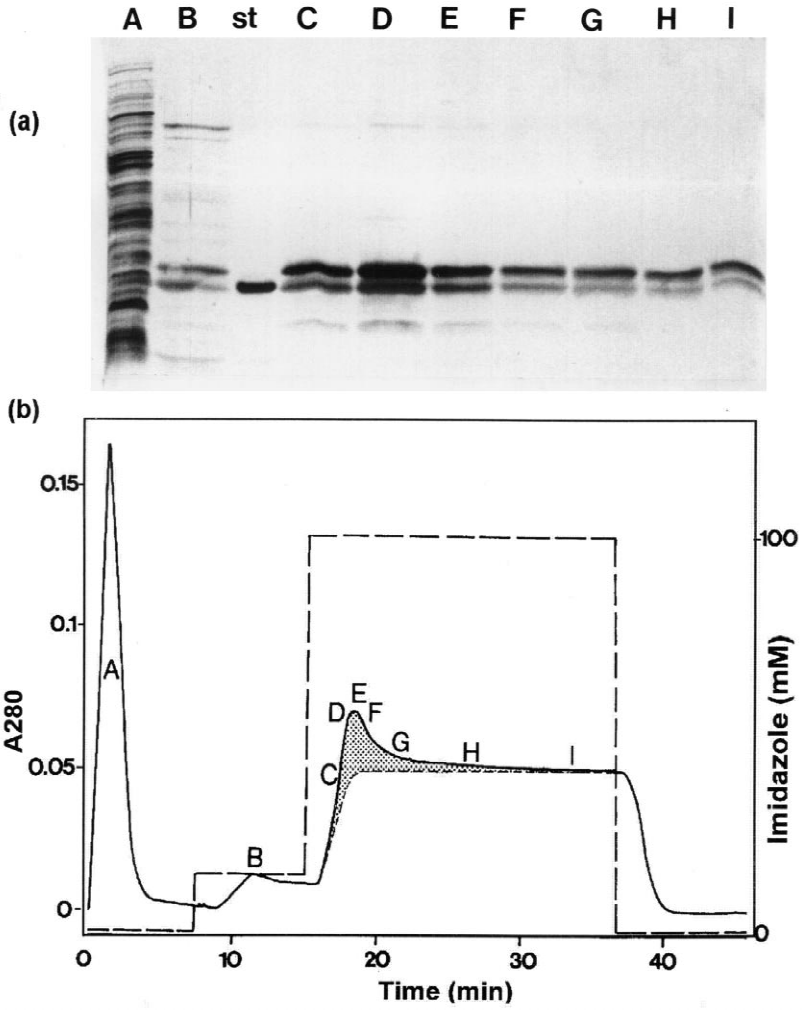

Fig. 3. (a) SDS-PAGE analysis of fractions from the chromatogram below; st represents 0.25 μg of TNF-α standard. (b) Chromatography of crude *E. coli* NM522 extract containing NHis5 tagged TNF-α, on TALON matrix using a step gradient of imidazole.

but SDS–PAGE analysis of the fractions showed the heterogeneous nature of NHis5 tagged protein with intact and partially truncated forms. The proportion of intact, full-length form increases with elution time. A clear separation of different forms cannot be achieved since different length tags are attached to the same trimeric molecule. Although NHis5 tag offers the possibility of simple, one step purification from the bulk of *E. coli* proteins, the truncated forms might be disturbing if the tag is to be enzymatically removed. NHis5 tagged TNF-α was originally designed for CNBr cleavage (Table 1), which proved somehow inconvenient for TNF-α since it is normally performed under strongly acidic conditions. Therefore, cleavage at pH 6 was also tried, but in this case, cross-linked products were formed in addition to properly processed TNF-α (unpublished results). It seems that introduction of sequences for enzymatic removal of affinity tag could be advantageous for TNF-α. However, in this case the full length tags are necessary. After IMAC isolation of NHis5 tagged TNF-α, the shortest forms observed on SDS-PAGE (Fig. 3a) resemble the properly cleaved protein but resolution of SDS-PAGE analysis is not

high enough to exclude also the presence of slightly shorter or longer forms. In some cases, the sequences necessary for enzymatic cleavage could be lost, which would result in heterogeneous N-termini. Further chromatographic separation of proteins, differing only in differently processed N-termini, is not easily achievable, leading to additional cost and reduced yield. On the other hand, heterogeneity of the N-terminus is not acceptable for pharmaceutical quality proteins.

3.4. Chromatography of double histidine tagged TNF-α

As already indicated, the second group of histidine tagged proteins, comprising the NHis2Xa and HisArg tags, was designed on the assumption of analogy with the model proteins. All contain 6 additional histidine residues per trimeric molecule of TNF-α. Although IMAC separations of crude *E. coli* extracts containing NHis2Xa (Fig. 4) or HisArg

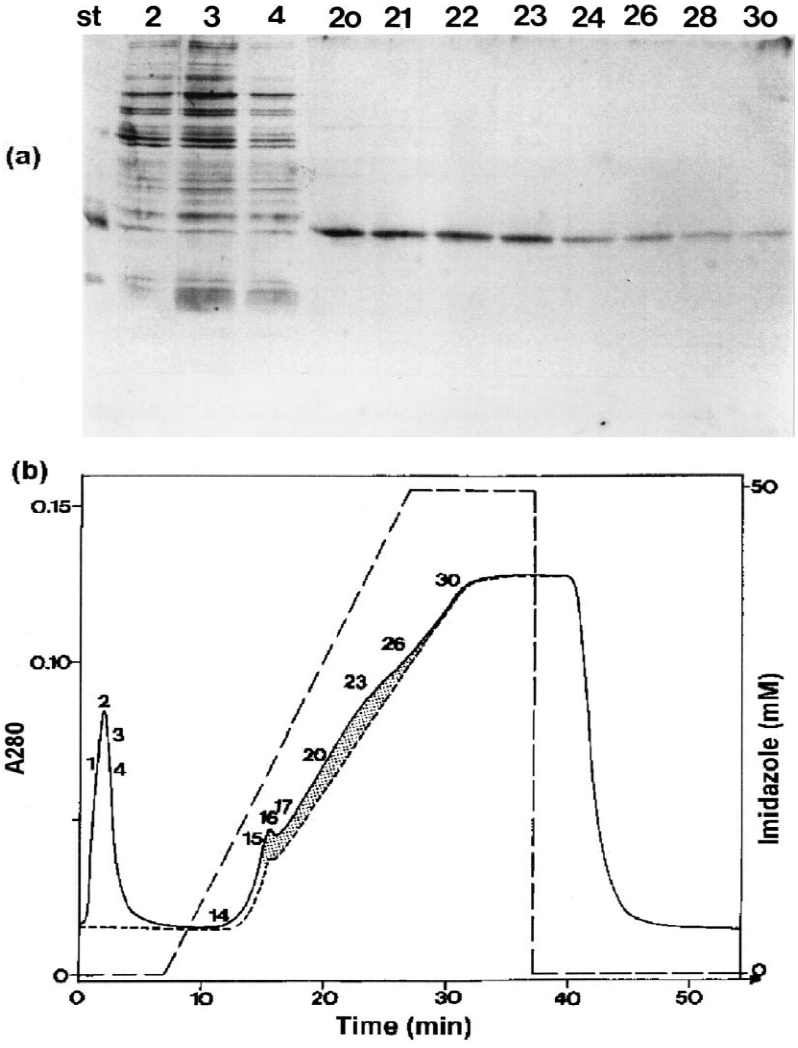

Fig. 4. (a) SDS-PAGE analysis of fractions from the chromatogram below; st represents 0.25 μg of TNF-α standard. (b) Chromatography of crude *E. coli* BL21 (DE3) extract containing NHis2Xa tagged TNF-α, on Ni-NTA matrix using a linear gradient of imidazole.

(data not shown) tagged TNF-α result in one step isolation of the respective proteins, the chromatographic behavior of the histidine-tagged proteins is significantly different from the model proteins. The sharp and symmetrical chromatographic peaks, characteristic of the model proteins, are not obtained. As seen in Fig. 4, on Ni-NTA the NHis2Xa-tagged TNF-α elutes over a very broad range. On the same matrix, the model protein LK-801 elutes later (Fig. 1). It is known that affinity on IMAC matrices generally correlates with the number and arrangement of exposed histidines [31], and the same is true for TNF-α analogues, bearing different histidine clusters [19]. The lower affinity of NHis2Xa tagged TNF-α compared with the model protein LK-801, suggests that the six histidines in the tags are not simultaneously available for interaction with immobilized metal ions.

3.5. Proteolytic degradation of histidine tagged TNF-α proteins

Compared to native TNF-α and model analog LK-801, there is a much reduced accumulation of histidine tagged proteins even if produced at lower temperatures and in protease deficient strains. This could be partly due to enhanced proteolytic cleavage of exposed affinity tags. It is known that sterically easily accessible peptide bonds, especially the flexible protein terminals, are prone to proteolytic attack [32]. Alternatively, newly engineered histidine tags could interfere with protein folding, leading to lower folding rates and thus higher availability of proteolytically susceptible intermediates.

To find conditions for efficient production of non-truncated forms, we tested different *E. coli* strains and different incubation temperatures. When *E. coli* NM522 strain with normal proteolytic activity was used to express different histidine tagged TNF-α proteins, it was evident that higher temperatures promoted proteolytic cleavage (Table 2). Increased temperature means stress for bacterial cell, which reacts by synthesizing numerous proteases, including the highly active cytoplasmic Lon protease (La) [33]. Nevertheless, the accumulation of intact histidine tagged TNF-α depends on the composition of the tag, NHis5 tag being the most and NHis2Xa being the least affected. According to the N-end rule [34], some amino acids, especially arginine and lysine destabilize the protein and make it more prone to proteolytic attack. This could explain the extreme susceptibility of the HisArg tag, which had also been designed as a poly-arginine tag for purification by cation exchange chromatography. In addition, many proteases, including the outer membrane protease, cleave the peptide bond between two basic amino acids [35], consistent with our results that the HisArg and NHis5 tags, containing several basic amino acid residues, are more susceptible to proteolysis.

The accumulation of relatively susceptible NHis5 tagged TNF-α was tested in different protease deficient *E. coli* strains. The highest percentage of intact protein accumulated in BL21(DE3), SF110 and SF120 strains (Table 3), which are all deficient in outer membrane protease (OmpT). Clearly, the initial purification steps, involving disruption of the *E. coli* cells, are critical for the action of this protease. Intracellular expression of TNF-α tagged proteins in the soluble form prevents contacts with this protease prior to cell disruption. However, the contribution of this outer membrane protease seems quite important. Expression of different histidine tagged TNF-α proteins in *E. coli* strain BL21(DE3), which is OmpT and La deficient, leads to a high

Table 2
Accumulation of different His-tagged TNF-α proteins in *E. coli* strain NM522 with normal proteolytic activity at different cultivation temperatures

E. coli strain NM522 Cultivation temperature	Percentage of intact His-tagged TNF-α		
	NHis2Xa	HisArg	NHis5
30°C	94	61	36
37°C	95	13	28
42°C	86	8	16

Table 3
Accumulation of NHis5-tagged TNF-α in different protease-deficient *E. coli* strains compared to *E. coli* NM522 strain at 30°C. (Data on *E. coli* strains were obtained from the suppliers' product sheets)

E. coli strain	Protein(s) affected	Gene(s) affected	Percentage of intact NHis5-tagged TNF-α
NM522			34
SF103 (ATCC)	protease III	Δptr32::Ω*cat*^{r}	47
SG935 (ATCC)	La, htpR (rpoH)	Δ lon, *htpR*	54
SG927 (ATCC)	La, htpR (rpoH)	Δ lon, *htpR*	70
BL21(DE3) (Invitrogen)	OmpT, La	Δ ompT, Δ lon	74
SF110 (ATCC)	OmpT, DegP	Δ ompT, Δ degP41 (ΔPsI):: Δ*kan*^{r}	80
SF120 (ATCC)	OmpT, DegP, protease III	Δ ompT, ΔdegP41 (ΔPsI):: Δ*kan*^{r}, Δptr32::Ω*cat*^{r}	84

percentage of intact proteins, almost regardless of the tag composition and cultivation temperature (Table 4).

4. Conclusions

With the aim of achieving efficient purification of oligomeric proteins by IMAC, we have compared properties of the trimeric cytokine TNF-α with four different histidine affinity tags attached to the N-termini, to those of model TNF-α proteins with two histidine mutations in each subunit. The latter form a cluster of surface exposed histidines at one end of the trimer and exhibit good chromatographic behavior on different IMAC matrices, enabling them to be isolated from crude cell extracts in a single step. By contrast, proteins with histidine affinity tags exhibit less ideal chromatographic behavior and are, in addition, prone to proteolytic attack. Heterogeneous truncation of the tagged proteins results in broad elution patterns leading to less reproducible purification procedures. Due to the trimeric structure, the longer tags with 10 histidines show very high affinities and molecules can only be eluted under very harsh conditions. When comparing the model proteins with 6 histidines in the surface cluster with proteins having two histidines in different arrangements for each affinity tag, which also results in 6 histidines per molecule, we observed stronger binding and superior chromatographic features in the case of model proteins. Our results show that applicability of histidine tags for isolation of oligomeric proteins leads to particular problems, especially in the case of pharmaceutical grade proteins.

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Table 4
Accumulation of different His-tagged TNF-α proteins in protease deficient *E. coli* strain BL21 (DE3) at various cultivation temperatures

E. coli strain BL21(DE3) Cultivation temperature	Percentage of intact His-tagged TNF-α			
	NHis2Xa	HisArg	NHis5	His10
30°C	100	100	96	97
37°C	100	100	87	95
42°C	98	100	86	91

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References

[1] F.H. Arnold, Bio/Technology 9 (1991) 150.
[2] J. Schmitt, H. Hess, H.G. Stunnenberg, Mol. Biol. Rep. 18 (1993) 223.
[3] K.A. Henry, C. Zwieb, H.M. Fried, Protein Express. Purif. 9 (1997) 15.
[4] J. Liesiene, K. Racaityte, M. Morkeviciene, P. Valancius, V. Bumelis, J. Chromatogr. A 764 (1997) 27.
[5] S.M. O'Brien, R.P. Sloane, O.R. Thomas, P. Dunnill, J. Biotechnol. 54 (1997) 53.
[6] P.A. Feldman, P.I. Bradbury, J.D. Williams, G.E. Sims, J.W. Mcphee, M.A. Pinnell, L. Harris, G.I. Crombie, D.R. Evans, Blood Coagulat. Fibrinol. 5 (1994) 939.
[7] D.C. Kaslow, J. Shiloach, Biotechnology 12 (1994) 494.
[8] J.L. Casey, P.A. Keep, K.A. Chester, L. Robson, R.E. Hawkins, R.H. Begent, J. Immunol. Methods 179 (1995) 105.
[9] A. Jungbauer, E. Boschetti, J. Chromatogr. B 662 (1994) 143.
[10] S. Novakovic, V. Menart, V. GabercPorekar, A. Stalc, G. Sersa, M. Cemazar, B. Jezersek, Cytokine. 9 (1997) 597.
[11] E. Hochuli, H. Doebeli, A. Schacher, J. Chromatogr. 411 (1987) 177.
[12] E. Hochuli, W. Bannwarth, H. Doebeli, R. Gentz, D. Stueber, Bio/Technology (1988) 1321.
[13] Y. Hidaka, H. Park, M. Inouye, FEBS Lett. 400 (1997) 238.
[14] L. Nieba, S.E. Nieba-Axmann, A. Persson, M. Hämäläinen, F. Edebratt, A. Hansson, J. Lidholm, K. Magnusson, A.F. Karlsson, A. Pluckthun, Anal. Biochem. 252 (1997) 217.
[15] R. Grisshammer, J. Tucker, Protein Express. Purif. 11 (1997) 53.
[16] S. Mukhija, B. Erni, J. Biol. Chem. 271 (1996) 14819.
[17] G.N. Goud, A.V. Patwardhan, E.J. Beckman, M.M. Ataai, R.R. Koepsel, IJBC 3 (1997) 123.
[18] A.V. Patwardhan, G.N. Goud, R.R. Koepsel, M.M. Ataai, J. Chromatogr. A 787 (1997) 91.
[19] V. Menart, V. Gaberc-Porekar, V. Harb, in: D.L. Pyle (Ed.), Separations for Biotechnology 3, The Royal Society of Chemistry, Cambridge, 1994, p. 308.
[20] J. Sambrook, E.F. Fritsch, T. Maniatis, in: C. Nolan (Ed.), Molecular Cloning, a Laboratory Manual, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[21] E. Sulkowski, in: R. Burgess (Ed.), Protein Purification, Micro to Macro, Allan R. Liss, New York, 1987, p. 149.
[22] V. Menart, B. Kus, S. Novakovic, G. Sersa, V. Gaberc-Porekar, V. Harb, S. Milicic, A. Stalc, Pflugers Arch.-Eur. J. Phisiol. 431 (Suppl) (1996) R233.
[23] E. Sulkowski, J. Mol. Recogn. 9 (1996) 389.
[24] E. Sulkowski, J. Mol. Recogn. 9 (1996) 494.
[25] X. VanOstade, J. Tavernier, W. Fiers, Protein Eng. 7 (1994) 5.
[26] K. Sreekrishna, R.H.B. Potenz, J.A. Cruze, W.R. McCombie, K.A. Parker, L. Nelles, P.K. Mazzaferro, K.A. Holden, R.G. Harrison, P.J. Wood, D.A. Phelps, C.E. Hubbard, M. Fuke, J. Basic Microbiol. 28 (1988) 265.
[27] K. Steinert, A. Eichenlaub, J. Ribbe, Qiagen News, No. 2, 1997, pp. 12–15.
[28] J. Pederson, personal communication.
[29] K.D. Pryor, B. Leiting, Protein Express. Purif. 10 (1997) 309.
[30] M. Olin, J. Carlsson, L. Bullow, Anal. Lett. 28 (1995) 1159.
[31] R.D. Johnson, F.H. Arnold, Biotechnol. Bioeng. 48 (1995) 437.
[32] G.B. Kresze, in: R. Seetharam, S.K. Sharma (Eds.), Purification and Analysis of Recombinant Proteins, Marcel Dekker, New York, 1991, p. 85.
[33] S. Gottesman, Annu. Rev. Genet. 30 (1996) 465.
[34] A. Varshavsky, Genes to Cells 2 (1997) 13.
[35] M. Murby, M. Uhlen, S. Stahl, Protein Express. Purif. 7 (1996) 129.