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Identification of the heparin-binding domain of TNF-alpha and its use for efficient TNF-alpha purification by heparin–Sepharose affinity chromatography

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ABSTRACT

The N-terminus of the trimeric TNF-alpha molecule comprises two basic arginines within the short amino-acid sequence VRSSSR, which is here shown to be essential for binding of TNF-alpha to heparin–Sepharose. Mixed trimers containing full-length and ΔN6-truncated subunits revealed a single VRSSSR sequence to be sufficient to achieve binding. On the basis of this newly identified heparin-binding domain, a new method for efficient purification of TNF-alpha is described. Affinity chromatography on heparin–Sepharose was introduced as a key step for highly purified TNF-alpha at a high yield. With minor modifications, this procedure can be used for TNF-alpha analogues that have full-length N-termini, as shown for the less toxic analogue LK-805.

1. Introduction

TNF-alpha is a cytokine that shows strong anti-tumour effects and it is currently used for local treatment of inoperable soft tissue sarcoma in combination with melphalan and mild hyperthermia, with the isolated limb perfusion technique [1–4]. The international non-proprietary name of recombinant human TNF-alpha is tasonermin, which has been registered as Beromun® (Boehringer Ingelheim). Native TNF-alpha is too toxic for systemic use; instead, less toxic analogues appear to be good candidates for future anti-tumour treatments [5–11]. In a tumour-bearing mouse model, the TNF-alpha analogue LK-805, which was designed in our laboratory, had almost the same anti-tumour activity as TNF-alpha itself, which was accompanied by lower systemic toxicity, thus making LK-805 an interesting candidate for further studies [12].

Human TNF-alpha was initially purified from the human HL-60 cell line following induction with the phorbol ester 4β-phorbol 12-myristate 13-acetate [13]. This procedure comprised four chromatographic steps that were combined with two dialysis and concentrating steps and one ultrafiltration step; this resulted in a final yield of approximately 6%. The first expression of human TNF-alpha in *Escherichia coli* was introduced by Pennica et al. in 1984, which enabled more in-depth research of this protein [14]; however, the full purification details were not provided. There have also been some further reports of expression of recombinant human TNF-alpha in *E. coli*, which was then purified to various levels of purity at generally relatively low yields [15,16].

Sreekrishna et al. [17] produced recombinant TNF-alpha in *Pichia pastoris*, where a three-step chromatographic procedure provided purified TNF-alpha at a very high yield (75%). This isolate was highly active; however, it appears that the TNF-alpha was predominantly in an N-terminally truncated and/or deamidated form, as judged from the isoelectric points [17]. The marketed form of TNF-alpha (tasonermin) is produced in *E. coli* and purified by a five-chromatographic-step procedure, although the full details have not been disclosed [4].

There have also been reports of the purification of TNF-alpha from other species: sheep [18], woodchuck [19], rat [20], guinea pig [21], rabbit [22] and mouse [23], either in a recombinant form or from natural sources. The yields are again usually low, and details of the final purities are not usually provided. Recombinant sheep TNF-alpha was expressed as a glutathione *S*-transferase (GST)-fusion protein in *E. coli*, with the GST cleaved with thrombin after affinity purification [18]. The cleaved GST was removed by rechromatography using the same affinity matrix; however, no data were provided as to if and how the cleaving enzyme was removed. Recombinant woodchuck TNF-alpha was produced as a histidine-tagged protein expressed in *E. coli*, which was then purified via two-step immobilized metal ion affinity chromatography (IMAC) using a Ni-nitrilotriacetic acid (NTA) matrix. The tag was not cleaved off after this purification, resulting in very low biological activity of the purified protein [19]. Recently, the purification of a TNF-alpha analogue,

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RGD4C-rmhTNF, was reported, as a procedure involving two chromatographic steps that produces a highly purified protein with a final yield of around 40% [24].

Purification of recombinant proteins still represents a demanding and time-consuming process, and consequently this represents the most expensive step in their production, as especially in the case of therapeutic proteins. In general, affinity chromatography is one of the most effective methods for obtaining highly purified recombinant proteins, especially through the use of immobilized monoclonal antibodies. However, these procedures are usually too expensive for the production of large quantities of recombinant proteins on an industrial scale. The introduction of well-defined, easily accessible, cost-effective affinity or pseudo-affinity matrices is therefore needed, such as the use of IMAC, immobilized heparin, cellulose, streptavidine, chitin, glutathione or cross-linked amylose.

Heparin–Sepharose is one of the cheapest affinity matrices on the market, and therefore its use should be considered whenever a protein of interest is known to have a potentially high affinity for heparin. To the best of our knowledge, heparin–Sepharose has not been used for the purification of either human or animal TNF-alpha from a complex mixture of proteins (e.g. from *E. coli* proteins). However, in studies of complexes of pure TNF-alpha and its soluble receptor, an interaction between pure TNF-alpha and heparin–Sepharose was reported by Lantz et al. [25], although they did not use this interaction for purification purposes. The amino-acid sequence that is responsible for this binding was also not identified.

In our previous study of the mechanisms of increased *in-vitro* cytotoxicity of the TNF-alpha analogue LK-805 [26], we obtained interesting results that indicated that the N-terminus of LK-805 might provide a decisive contribution to the interaction of TNF-alpha with heparin. We have therefore now further investigated the role of the N-terminus of TNF-alpha in this interaction with heparin–Sepharose. Furthermore, chromatography with heparin–Sepharose was used to develop an efficient method for the purification of TNF-alpha from a complex mixture of *E. coli* proteins.

To date, the isolation procedures for TNF-alpha reported in the literature have not considered the criteria necessary for the production of the high-purity TNF-alpha that is needed for pharmaceutical applications. This is defined by the absence of polymeric impurities, such as host-cell proteins, DNA and lipopolysaccharides, and includes the need for quantitative analyses to determine the precise content, product-related substances and oligomeric forms of TNF-alpha. The aim of this study was thus to develop an efficient and economic purification procedure for TNF-alpha that provides high-quality TNF-alpha for use as a pharmaceutical preparation.

2. Experimental

2.1. Instruments and chemicals

Bacterial cultures were grown in shaking flasks, using an ISF-1-W stackable incubator shaker (Adolf Kühner AG, Birsfelden, Switzerland). The cells were disrupted using a high-pressure EmulsiFlex-C5 homogenizer (Avestin, Ottawa, Canada), and centrifuged with JA-10 and JA-20 fixed angle rotors in a model J2-HS centrifuge (Beckman, Palo Alto, California, USA). The chromatography system comprised two HPLC 64 pumps and a variable UV–vis wavelength monitor (Knauer, Berlin, Germany), with a FRAC-100 fraction collector (Amersham Pharmacia Biotech, Buckinghamshire, England). The system was controlled by Eurochrom 2000 for Windows, version 2.05 (Knauer). All of the chromatographic columns were from Amersham Biosciences, including the PD-10 desalting columns. The SDS-PAGE was run on a Mini-Protean III system (BioRad, Hercules, Californina, USA), with calibration with a low range, LMW, molecular weight standard (BioRad). Coomassie blue stained gels were scanned using a GS 670 densitometer (BioRad), with Molecular Analyst software (BioRad) used for processing this data. The SDS-PAGE gels (Nu-PAGE, Invitrogen, Carlsbad, California, USA) for Western blotting were run on an Xcell Sure-LockTM System, and the transfer from gel to nitrocellulose membrane was performed using an Xcell II™ Blot Module (both from Invitrogen). The SeeBlue Plus2® prestained standard (Invitrogen) was used for calibration. Isoelectric focusing was performed using the CleanGel, Broad p*I* calibration kit (pH 3–10) and a Multiphor II NovaBlot electrophoretic transfer system (both from Amersham Biosciences). Protein concentrations were determined by the Bradford procedure [27] using an 8452A Diode Array Spectrophotometer (Hewlett-Packard, Palo Alto, California, USA) supported by UV-Visible Chemstation software, Rev. A. 08 03 [71] (Agilent Technologies, Palo Alto, California, USA). The absorbance on microplates was measured using a Synergy HT reader (Bio-Tek, Winooski, Vermont USA), with the reader controlled by KC4 Kinetical for Windows, version 3.4rev18. Fluorescence measurements were performed on a C-61 quantitation spectrofluorometer (Photone Technology International, West Sussex, England). All of the chemicals used were of analytical grade and purchased from Sigma (Taufkirchen, Germany), unless otherwise stated.

2.2. Expression of TNF-alpha and its analogues

The synthetic TNF-alpha gene with *E. coli*-optimized codons was obtained from British Biotechnology and subcloned into the pCyΔcI expression plasmid [28]. The BL21(DE3) *E. coli* strain was used as the host organism for the production of the recombinant proteins. To obtain the TNF-alpha analogue LK-805, the E107K mutation was introduced by oligonucleotide-directed mutagenesis on ssDNA, using the following oligonucleotide: 5′-CTGCACCCTTGGGAGTTTCACGCT-3′.

Both TNF-alpha and LK-805 were expressed in BL21(DE3) *E. coli* cultures in 250 mL LB medium supplemented with 100 μg/mL ampicillin, in shaking (150 rpm) 1-L Erlenmeyer flasks at 30 °C. Each flask was inoculated with 15 mL of pre-culture (7 h of cultivation at 37 °C and 150 rpm), and the bacteria were collected after 16 h of cultivation, when they were in a late exponential phase.

2.3. Preparation of mixed trimers and chromatographic separation on heparin–Sepharose

The LK-805 and ΔN6LK-805 TNF-alpha analogues were used as the starting materials for the preparation of the mixed trimers. ΔN6LK-805 is a truncated form of LK-805 that lacks the first six amino acids (VRSSSR). This truncated protein was prepared by enzymatic cleavage of His7-ΔN6LK-805 by dipeptidyl aminopeptidase (DAPase) [29]. Samples of LK-805 and ΔN6LK-805 were desalted using PD-10 columns, with a final buffer of 10 mM potassium phosphate, pH 6.5. Equal amounts of LK-805 and ΔN6LK-805 were mixed and incubated at 4 °C for four days. The formation of mixed trimers was confirmed by isoelectric focusing. This protein mixture was then loaded onto HR5/5 columns packed with ∼1 mL of heparin–Sepharose 6 Fast Flow. A linear gradient of NaCl from 0.0 M to 0.5 M was applied to elute the bound protein. The fractions obtained were analyzed by isoelectric focusing immediately after this chromatographic separation.

2.4. Prechromatographic steps in the protein purification

Bacterial cells were collected by centrifugation at 2700 × *g* for 5 min, washed with 50 mM Tris–HCl buffer containing 30 mM NaCl, and resuspended in the same buffer. The cells were then homogenized in an EmulsiFlex-C5cell disruptor. The homogenate was centrifuged at 17,600 × *g* for 30 min, and the nucleic acids

were removed from the supernatant by precipitation with 0.1% polyethyleneimine. The precipitate was removed by further centrifugation at 17,600 × *g* for 30 min. The protein in the supernatant was precipitated with 65% saturated ammonium sulfate solution. The protein precipitate was used immediately or stored at 4 °C for up to several weeks. These storage conditions were shown to preserve the protein for up to one year without any changes in structure or loss of biological activity.

2.5. Chromatographic purification of TNF-alpha and LK-805

The precipitate from the ammonium sulfate that contained approximately 50 mg total protein was dissolved in 5 mL 10 mM potassium phosphate buffer, pH 8.0, and desalted on PD-10 columns before being diluted to 10 mL with the same buffer. The final protein concentration here was around 5 mg/mL. Samples were loaded onto an HR10/10 column packed with DEAE-Sepharose Fast Flow (Amersham Biosciences) and equilibrated with the same buffer. Different gradients of NaCl were tested to obtain the best purification of TNF-alpha in this chromatographic step, with the optimized gradient used in the final purification procedure shown in Fig. 4a. The flow rate was 2 mL/min and 2-mL fractions were collected. For the purification of the basic TNF-alpha analogue LK-805, the ammonium sulfate precipitate was dissolved in 10 mM potassium phosphate buffer, pH 9.0, and this same buffer was used for column equilibration. Fractions were analyzed by SDS-PAGE, and those containing TNF-alpha were pooled and concentrated to approximately one half of their starting volume, using an Amicon cell with a Millipore YM10 membrane. These samples were loaded onto an HR10/10 column packed with heparin–Sepharose 6 Fast Flow (Amersham Biosciences). With LK-805, this target protein remained in the flow-through fraction of the DEAE-Sepharose separation step (see Section 3), so this fraction was used for the further purification. To achieve better binding to heparin–Sepharose, the pH of the loading solution was carefully adjusted to 6 with 0.1 M *ortho*-phosphoric acid. The column was equilibrated with 10 mM potassium phosphate buffer, pH 6.0. Again, different NaCl gradients were tested, with the optimized gradient illustrated in Fig. 4b.

Finally, size-exclusion chromatography on Superose12 was introduced as a sample 'polishing' (cleaning) step. Phosphate-buffered saline (PBS, Maniatis) containing 0.2 M NaCl was used to equilibrate the column and to elute the protein. Prior to loading on the Superose12, the samples were appropriately concentrated. All of the chromatographic procedures were performed at room temperature.

2.6. Final product analysis

The final purified TNF-alpha and LK-805 products were analyzed by SDS-PAGE, with the gels stained with Coomassie blue. Isoelectric focusing was performed to determine the isoelectric points of the purified proteins. The identities of the TNF-alpha and LK-805 were confirmed through immunodetection using polyclonal rabbit anti-TNF-alpha primary antibodies prepared at the Blood Transfusion Centre of Slovenia. An anti-rabbit IgG (whole molecule) peroxidase conjugate was used as the secondary antibody, and the colour was developed with 4-chloro-1-naphtol. Residual *E. coli* protein in the final products was also revealed by Western blotting, using primary polyclonal rabbit anti-*E. coli* antibodies (DakoCytomation) in combination with anti-rabbit IgG, alkaline-phosphatase-conjugated secondary antibodies. A 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue tetrazolium (NBT) premixed colour development solution was used to detect the bands, with different amounts of *E. coli* protein standards used to estimate the amounts of *E. coli* proteins in samples. The *E. coli* protein standards were precipitated with 100% saturated ammonium sulfate solution from supernatants after sonicating BL21(DE3)/pET3a *E. coli* cultures (with the pET3a expression plasmid without the TNF-alpha and LK-805 coding genes). Traces of bacterial endotoxins were measured using the QCL-1000 *Limulus amebocyte* lysate test (Cambrex, Walkersville, Maryland, USA). Traces of bacterial DNA were measured using the Quant-iT PicoGreen dsDNA kit (Molecular Probes Inc., Eugene, Oregon, USA).

3. Results and discussion

3.1. Identification of the heparin-binding domain of TNF-alpha

There are many proteins that are known to interact with heparin. Basic amino-acid residues are mainly responsible for this binding, with arginines in particular showing high binding affinities towards the sulfate groups of heparin [30]. The heparin-binding sites are very different from protein to protein: in some proteins, just a few basic amino-acid residues are needed to achieve heparin binding, while other proteins have binding domains that include numerous exposed basic amino-acid residues, which might not be close together in the primary protein structure, but which are instead brought together in the three-dimensional protein structure [30–36].

Our previous investigations into the chromatographic properties of heparin–Sepharose towards TNF-alpha and its analogues, including LK-805, were performed to study the mechanisms of increased *in-vitro* cytotoxicity of some TNF-alpha analogues. Chromatographic resins with immobilized heparin served as a model for cell-surface heparin sulfates. On this basis, we would assume that an increased cytotoxicity should be related to the increased basicity of a TNF-alpha analogue, which would lead to more favourable orientation of the molecule for TNF-alpha receptor binding, and thus to an increased concentration of TNF-alpha in the proximity of the receptors. Stronger binding was expected for LK-805 and ΔN6LK-805, the two analogues that have additional lysines introduced into the tip region of the TNF-alpha trimer. However, we saw that as well as LK-805, TNF-alpha efficiently bound to immobilized heparin, while the truncated forms of ΔN6TNF and ΔN6LK-805 remained in the flow-through fraction [26]. Based on these data, we concluded that a conserved N-terminus with an RSSSR amino-acid sequence is crucial for this interaction with heparin. Despite the p*I* values of TNF-alpha and it analogue ΔN6LK-805 being almost identical (6.83 and 6.76, respectively), TNF-alpha binds to immobilized heparin, and ΔN6LK-805 does not. Obviously, the p*I* values, and therefore the overall basicities of these proteins, are not the only factors that affect their binding, as the appropriate arrangement of the basic amino-acid residues will also be very important. Our first binding experiments were performed at pH 7.0; however, later it became apparent that the same results were obtained at pH 6.5 and at pH 6.0.

Primarily we wanted to determine whether all three of the trimer N-termini are needed to promote heparin binding, or whether just one is sufficient. This was investigated using a mixture of trimers (see Fig. 1) that had been prepared from the purified TNF-alpha analogues LK-805 and its truncated form ΔN6LK-805, as described in Section 2.6. The formation of mixed trimers was confirmed by isoelectric focusing (Fig. 2a). The trimer mixtures were separated using a heparin–Sepharose column (Fig. 2b), with selected fractions analyzed by isoelectric focusing (Fig. 2c). Here, the trimers that contained at least one VRSSSR sequence bound to the heparin–Sepharose matrix. The mixed trimers then eluted with a linear NaCl gradient according to the increasing number of VRSSSR sequences. The trimers containing no VRSSSR sequences (the pure ΔN6LK-805 analogue) remained in the flow-through fraction.

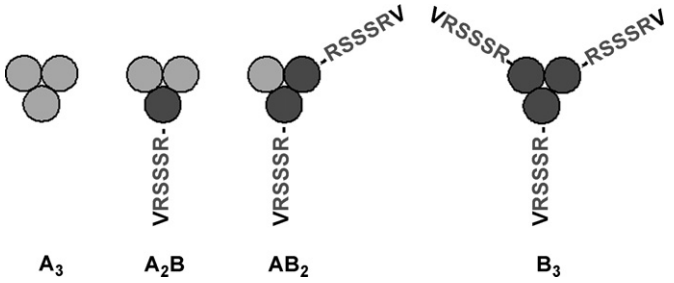

Fig. 1. The mixed trimers. In the incubated mixture of ΔN6LK-805 (A) with LK-805 (with its conserved N-terminus; (B), four different species are possible, containing no N-terminus VRSSSR sequences (A_3), or one (A_2B), two (AB_2) or three (B_3) N-termini VRSSSR sequences.

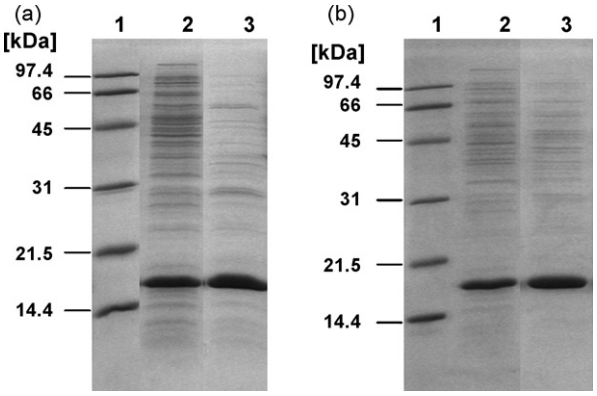

Fig. 3. SDS-PAGE analysis of the crude extracts and ammonium sulphate precipitates for TNF-alpha and LK-805. (a) TNF-alpha: 1 – LMW standards; 2 – supernatant after homogenization; 3 – ammonium sulphate precipitate. (b) Same for LK-805.

The experiments described above unequivocally prove that the arginine residues of the VRSSSR domain naturally present at the N-terminus of each TNF-alpha subunit are responsible for binding to heparin. Although positively charged amino acid residues are in principle appropriate for such binding, it cannot be widely generalized that the recognized sequence will also work in other proteins or on other locations within the TNF-alpha structure. Anyway, to prove wider applicability of the VRSSSR sequence it would be interesting to engineer this domain into various proteins and investigate the chromatographic behaviour of obtained analogues on heparin columns.

3.2. Purification of TNF-alpha and LK-805 using heparin–Sepharose

When the recombinant *E. coli* were cultivated at 30 °C, the high levels of expression of TNF-alpha and LK-805 usually exceeding 40% of the total soluble protein. SDS-PAGE gels from the crude extracts and ammonium sulphate precipitates for TNF-alpha and LK-805 are shown in Fig. 3, and Table 1 summarizes the expression levels of both of these target proteins and their contents in the ammonium sulfate precipitates. Both TNF-alpha and LK-805 were located intracellularly as soluble proteins as well as in the insoluble fraction. The majority of the correctly folded protein was in the soluble form, and this fraction was used for the purification studies.

Many studies have described the isolation of TNF-alpha and its different analogues, but none of these were actually optimized [13–24] in terms of the purities and/or final yields obtained. As we saw a strong enough interaction between TNF-alpha and the heparin–Sepharose, we wanted to include this chromatographic step in our development of an efficient purification method for TNF-alpha and its analogues with conserved N-termini. The first step in this direction was to confirm the binding of TNF-alpha and LK-805 to heparin–Sepharose in the presence of *E. coli* proteins. Both TNF-alpha and LK-805 were bound at pH 7.0, and even better at pH 6.0,

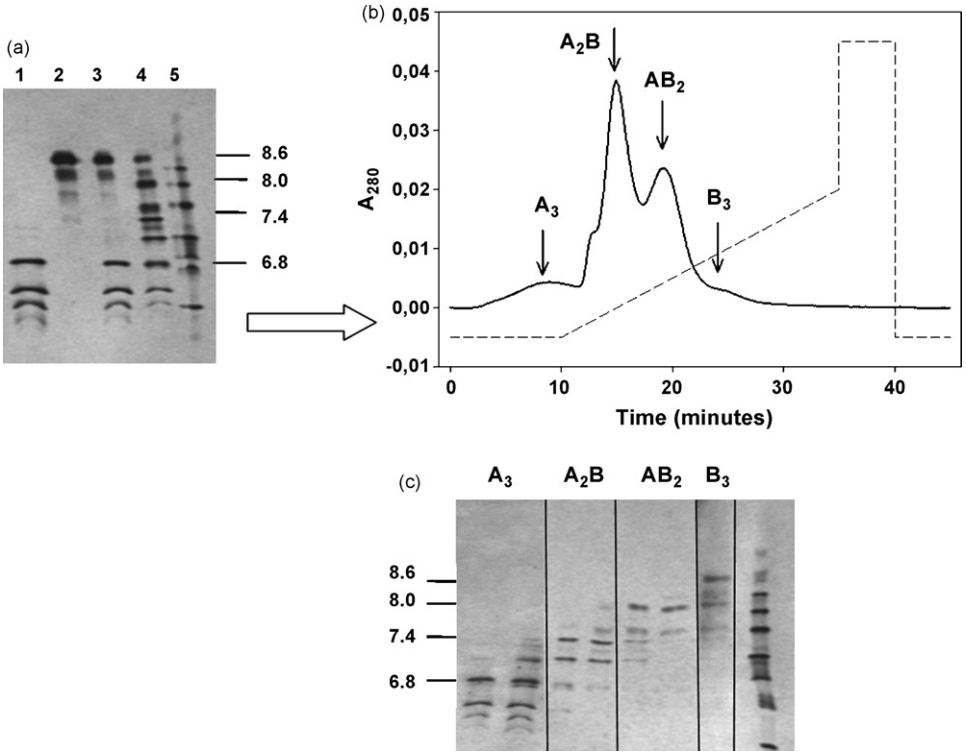

Fig. 2. Purification of the mixed trimers. (a) Isoelectric focusing showing the formation of mixed trimers: 1 – ΔN6LK-805; 2 – LK-805; 3 – ΔN6LK-805 plus LK-805 mixture before incubation; 4 – same mixture after incubation; 5 – standards. (b) Chromatographic separation of the mixed trimers on heparin–Sepharose. (c) Isoelectric focusing of the chromatographic pools from (b), as indicated.

Table 1
Expression of TNF-alpha and LK-805

Target protein	Percentage of the target protein in the soluble fraction of the *E. coli* proteins	Percentage of the target protein in the ammonium sulfate precipitate
TNF-alpha	41	75
LK-805 (E107K)	50	66

where the binding capacity was also greater. Using an increasing NaCl gradient for the elution, some *E. coli* proteins co-eluted with our target proteins, and therefore DEAE-Sepharose was introduced as the first chromatographic step, to remove the majority of the host-cell protein. TNF-alpha bound to DEAE-Sepharose at pH 8.0 and was eluted with increasing NaCl concentrations. A substantial portion of the *E. coli* protein was removed in this anion-exchange chromatography step. On the other hand, with its theoretical p*I* value of 8.56, LK-805 did not bind to the matrix at pH 8.0 or at pH 9.0. Therefore, a negative chromatography approach was used here on the basis that the majority of the *E. coli* protein bound very strongly at pH 8.0, while LK-805 remained in the flow-through fraction.

The use of heparin–Sepharose chromatography provided the key affinity step in our new purification procedure. Under optimized conditions, relatively strong binding of the target proteins was achieved. The first chromatographic separations on heparin–Sepharose were performed at pH 7.0, although it was later discovered that a much more efficient binding of TNF-alpha was achieved at pH 6.0. Of note, the binding capacity of the heparin–Sepharose matrix was also much higher at pH 6.0, and for this reason all of the further separations were carried out at pH 6.0, including those with the TNF-alpha analogues. TNF-alpha itself was eluted with approximately 200 mM NaCl, while its analogues, which all have higher p*I* values, were eluted with approximately 250 mM NaCl.

Size-exclusion chromatography was used as a 'polishing', or cleaning, step, to remove the traces of high molecular weight impurities that were still present after the heparin–Sepharose chromatography (enlarged chromatogram in Fig. 4c). In the case of LK-805, this step was omitted, as the required purity had already been achieved by the two-step procedure involving ion-exchange chromatography and affinity chromatography on heparin–Sepharose.

Representative chromatograms of all three of the purification steps for TNF-alpha are shown in Fig. 4: anion-exchange chromatography on DEAE-Sepharose; affinity chromatography on heparin–Sepharose; and size-exclusion chromatography on Superose12. Similar chromatograms were obtained with LK-805 (data not shown), except for the first step, where LK-805 remained in the flow-through fraction. SDS-PAGE and Western blotting of the final products are shown in Fig. 5.

It is not common practice to introduce ion-exchange chromatography prior to affinity chromatography, but in our case this is the optimal solution. The binding capacities for TNF-alpha and LK-805 of the heparin–Sepharose is much higher when some of the *E. coli* protein is already removed. Another reason is the simple transfer of the fractions containing the target proteins from DEAE-Sepharose to heparin–Sepharose. The target proteins were eluted from DEAE-Sepharose at low ionic strengths (TNF-alpha at ~90 mM NaCl, and LK-805 in the flow-through fraction without NaCl). Consequently, there were no problems with binding to heparin–Sepharose that would be caused by a high-salt content. If these two steps were to be inverted, a desalting step would have to be introduced after the heparin–Sepharose to allow binding to DEAE-Sepharose.

The so-called 'negative purification' is even less frequently used in similar systems; here, the target protein does not bind to the chromatographic matrix, while other proteins are bound and efficiently removed. This type of chromatography is appropriate when the expression levels of a target protein are very high, as in our case; otherwise, problems with the capacity of the column would occur. A large amount of the *E. coli* protein binds tightly to the DEAE-Sepharose under the conditions used here, and therefore it is efficiently removed. These procedures that comprise three steps for the purification of TNF-alpha and two steps for the purification of its analogue LK-805 are very efficient and result in very high

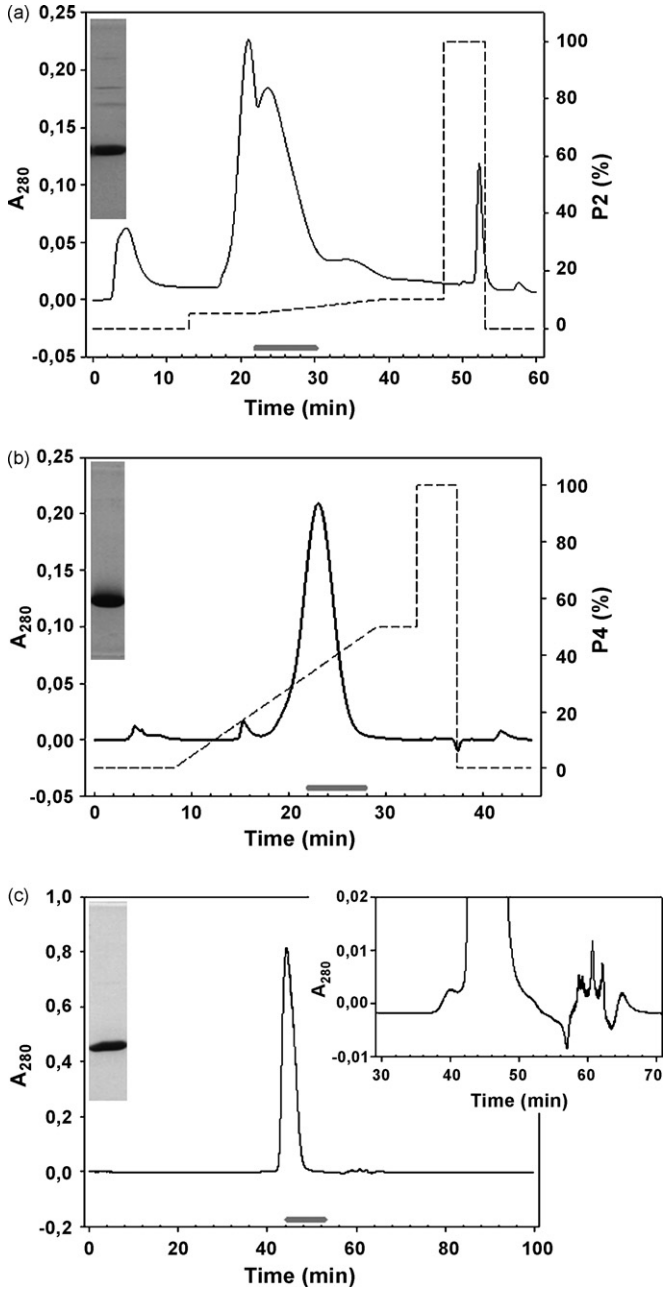

Fig. 4. Chromatograms of the three-step purification of TNF-alpha. (a) Anion-exchange chromatography on DEAE-Sepharose. (b) Affinity chromatography on heparin–Sepharose. (c) Size-exclusion chromatography on Superose12. For each chromatogram, the fractions containing TNF-alpha are marked with a grey line near the X-axis. Inset: SDS-PAGE analysis of each of the main peak pools for TNF-alpha.

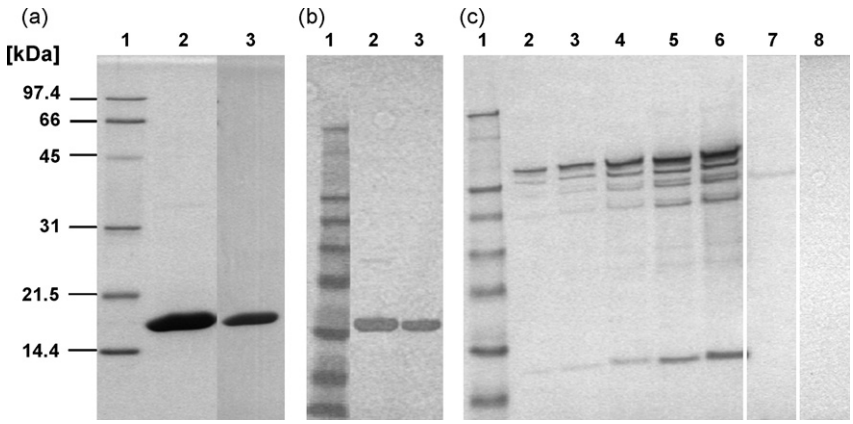

Fig. 5. Final analysis. (a) SDS-PAGE analysis, Coomassie blue staining: 1 – LMW standards; 2 – purified TNF-alpha; 3 – purified LK-805. (b) Western blotting, anti-TNF-alpha: 1 – See Blue Plus 2 MW-ST; 2 – purified TNF-alpha; 3 – purified LK-805. (c) Western blotting, anti-*E. coli*: 1 – See Blue Plus 2 MW-ST; 2–6 – *E. coli* proteins at 5 ng, 10 ng, 25 ng, 50 ng, 100 ng; 7 – purified TNF-alpha: 20 μg. 8 – purified LK-805: 20 μg.

Table 2
Summary of TNF-alpha and LK-805 purification

Target protein	Purification step	Percentage of target protein	Yield (%)	Total yield (%)
TNF-alpha	Ammonium sulfate precipitate	75	100	31% (7.2 mg/g cell paste)
	DEAE-Sepharose	94	87[b]	
	Heparin–Sepharose	99	65	
	Size-exclusion chromatography	99.9[a]	56[b]	
LK-805	Ammonium sulfate precipitate	66	100	57% (11.3 mg/g cell paste)
	DEAE-Sepharose	85	95	
	Heparin–Sepharose	99.9[a]	60	

[a] See Table 3.
[b] Including the yield of sample concentration.

Table 3
Final analysis – traces of major polymeric impurities and biological activities of the purified target proteins

Target protein activity	*E. coli* protein (ppm)	Bacterial endotoxins (ng/mg)	Host cell DNA (ng/mg)	Biological (EU/mg)
TNF-alpha	<50	0.2	5	3.8 E07
LK-805	<50	0.3	8	7.2 E07

yields. A summary of the purification steps and final yields is given in Table 2.

To confirm the high purity of the final products, the residual *E. coli* protein and DNA, and the traces of bacterial endotoxins were measured. The residual *E. coli* protein is below 50 ppm for both TNF-alpha and LK-805, as estimated from the blots illustrated in Fig. 5c and as summarized in Table 3, which also includes the other residuals specifically investigated. At the same time, the biological activities of both of our final target products were comparable to previously reported values (Table 3; [12]). Of note, several other TNF-alpha analogues with p*I* values between 7.9 and 8.6 were also efficiently purified by the procedures we have described here.

4. Conclusions

The VRSSSR amino-acid sequences at the N-termini of the trimeric TNF-alpha protein are responsible for the binding of TNF-alpha to immobilized heparin. This was confirmed chromatographically by demonstrating the binding TNF-alpha and various TNF-alpha analogues to heparin–Sepharose. TNF-alpha and its analogue LK-805 (which retains the original N-termini) both bound to the heparin–Sepharose matrix, while their truncated forms that lack the N-terminal VRSSSR sequence, ΔN6TNF and ΔN6LK-805, remained in the flow-through of the heparin–Sepharose. The use of mixed trimers revealed that one RSSSR sequence within a trimer is sufficient for this heparin–Sepharose binding. Based on this finding, an isolation procedure was developed, which used heparin–Sepharose affinity chromatography as the key step in the purification, and which resulted in highly purified TNF-alpha. According to all of the criteria for pharmaceutical proteins, this highly purified TNF-alpha can be applied to *in-vitro* systems as well as to clinical testing. With minor modifications, this purification procedure can also be used for other TNF-alpha analogues that have these conserved N-termini. This purification scheme is also suitable for scale-up for the production of large amounts of TNF-alpha and its analogues.

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