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Rapid purification method for human recombinant tumor necrosis factor alpha

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Abstract

Human recombinant tumor necrosis factor alpha was purified in a single step to about 95% purity from *Escherichia coli* lysate by chromatography on hydroxyapatite. The last traces of contaminants were removed by fast protein liquid chromatography on a Mono Q column. The final product was found to be pure by gel electrophoresis with silver staining. A molecular mass of approximately 17 000 and a specific activity of $4.3 \cdot 10^6$ U/mg after a single purification step were found.

1. Introduction

Tumor necrosis factor (TNF) is a monokine produced by activated macrophages [1,2] and it is believed to be present mainly in two forms, as a monomer of M_r 17 000 and as a trimer [3,4].

This cytokine has an action both as a messenger on the immune system and also in the necrosis of tumor cells [5]. Although the precise role of TNF has not yet been elucidated, it is one of the most important monokines of the immune reaction. This product has been cloned most of the time in *Escherichia coli* [6–8] and it is usually purified by many steps including gel chromatography, ion exchange and absorption chromatography [9–11]. The total yields reported are about 20% [12]. We report here the purification of recombinant human TNF from *E. coli* lysate in a single purification step on hydroxyapatite followed by a rapid Mono Q chromatography to remove the last traces of impurities.

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2. Experimental

2.1. Human recombinant TNF alpha and E. coli

E. coli cells carrying the expression vector for TNF alpha were grown at 37°C in LB medium containing 50 μg/ml of ampicillin. When the absorbance at 600 nm reached about 1.0, the culture was stopped and cells were collected by centrifugation. After washing the suspension, cells were lysed with buffer containing lysozyme at 1 mg/ml in 20 m*M* Tris–HCl (pH 7.4)–25%

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sucrose–10 m*M* EDTA containing 1 m*M* (0.001%) phenylmethylsulfonyl fluoride (PMSF) to block proteolysis. The insoluble fraction was removed by centrifugation at 3000 *g* for 15 min.

2.2. *Residual TNF*

The insoluble fraction was solubilized by boiling for 5 min in a minimum volume of 10 m*M* Tris–HCl (pH 8.0)–1 m*M* EDTA containing 2.5% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol. This fraction was analysed by SDS–polyacrylamide gel electrophoresis (PAGE) as described below.

2.3. *Hydroxyapatite chromatography*

The supernatant was dialyzed against 0.05 *M* phosphate buffer (pH 7.0) and applied on a 50 × 1.6 cm I.D. column filled with HTP hydroxyapatite (Bio-Rad Labs., Mississauga, Ontario, Canada) equilibrated with the same buffer. This sample was applied at 1 ml/min and, after washing with one column volume of starting buffer, a linear phosphate buffer gradient (from 0.05 to 0.30 *M*, pH 7.0) was applied. Proteins were eluted at 2 ml/min and monitored at 280 nm on a fast protein liquid chromatographic (FPLC) system (Pharmacia Canada, Montreal, Canada) using two P-500 pumps connected to a GP-250 gradient programmer and a Pharmacia LK-UV-M2 monitor connected to a Pharmacia–LKB Model 102 recorder.

2.4. *Mono Q chromatography*

Positive fractions were pooled and dialyzed against 20 m*M* Tris–HCl buffer (pH 8.0). The pool was applied to a Mono Q column (Pharmacia Canada) equilibrated with the starting buffer. TNF was eluted from the column with a linear NaCl gradient from 40 to 75 m*M* in starting buffer at 1 ml/min [12]. Positive fractions were screened as described before and analyzed by SDS-PAGE [13].

2.5. *Assay for TNF activity*

The activity of TNF was monitored by a previously described cell lysis assay [12]. Briefly, mouse L-929 fibroblasts obtained from the American Type Culture Collection were grown in a 96-well flat-bottomed plate (Falcon Plastics 3040) at 50 000 cells per well in 0.1 ml of culture medium in the presence of 1 μg/ml of actinomycin D and incubated with a serially diluted test sample of TNF in a humidified atmosphere at 37°C with 5% CO_2. After 18 h, the test sample was removed, the plates were washed and cell lysis was detected by staining the plates with a 0.5% solution of crystal violet in methanol–water (1:4, v/v). The end-point on the microtitration plates was determined with a Thermomax ELISA reader (Molecular Devices, CA, USA) set for absorption at 540 nm. One unit of TNF is defined as the amount required for 50% cell lysis [12].

2.6. *Protein determination*

Protein concentration was determined at 280 nm using a factor of 1.62 for the molar absorptivity of a 0.1% TNF solution as described previously [7].

2.7. *Gel electrophoresis*

SDS-PAGE was performed on the various fractions and on the purified material using an 8–25% polyacrylamide gel gradient on a Phast system (Pharmacia Canada). After electrophoresis, the gels were stained with silver using a silver-staining kit (Pharmacia Canada). Proteins were also stained with 2% Coomassie Brilliant Blue R-250 (Bio-Rad Labs.) in 10% (v/v) acetic acid. Gels were destained in the Phast system using methanol–acetic acid–H_2O (3:1:6). Molecular mass standards (Bio-Rad Labs.) were also run on the same gel.

2.8. *Amino acid analysis*

About 3 mg of the purified TNF were extensively dialysed against HPLC-grade water and

2.5 μg were placed in Corning culture tubes (Cat. No. 9820, 50 × 6 mm I.D.) which were previously heated in a muffle furnace at 450°C overnight. The tubes were placed in Waters reaction vials and samples were dried in a Waters Pico-Tag workstation (Waters, Division of Millipore, Mississauga, Ontario, Canada). Constant-boiling HCl (200 μl) containing 1% of phenol was added to the vial and alternately purged (with dried nitrogen) and evacuated. After three purges, the vial was heated at 150°C for 1, 2, 3 and 4 h under vacuum. The values for ten amino acids stable to acid hydrolysis were not corrected. Values for Ser, Thr and Tyr were extrapolated to zero time and those for Ile and Val were extrapolated to infinity. Tryptophan was determined by alkaline hydrolysis [14] and cysteine was determined as cysteic acid after performic acid oxidation [15]. The analysis was performed on a Beckman System 6300 high-performance analyzer according to the general procedures of Spackman *et al.* [16].

2.9. Capillary electrophoresis

Capillary electrophoresis was performed at 20°C using a BioFocus 3000 (Bio-Rad Labs.). The coated capillary (24 cm × 25 μm I.D.; No. 148-3031, Bio-Rad Labs.) was washed for 1 min with distilled water, followed by a 2-min wash with 0.3 *M* borate buffer (pH 8.5). Electrophoresis was run at 10 kV with a constant current of 50 μA and monitored at 280 nm.

3. Results

3.1. Purification on hydroxyapatite

HTP hydroxyapatite was used in the first step in process chromatography as it eliminates most of the contaminating material and yields about 95% pure TNF at the end of the gradient. Fig. 1 shows the elution pattern in which we observed a large peak of contaminating proteins which is eluted with the void volume whereas TNF under the conditions applied is retained on the hydroxyapatite. The linear gradient used allows the separation of TNF from some of the major constituents of the cell lysate. This material still contains some minor impurities which may be observed by silver staining but not with Coomassie Blue, as shown in Fig. 2.

3.2. Purification on Mono Q

The last traces of impurities could be removed easily in a single step on a Mono Q anion-exchange column, as shown in Fig. 3. We obtained two peaks in this chromatographic step and both contain pure TNF without any difference in specific lytic activity.

As shown in Table 1, this purification pro-

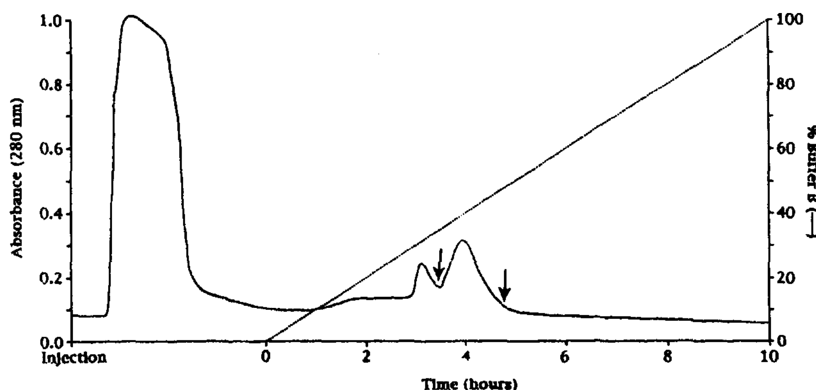

Fig. 1. Elution pattern of TNF on the HTP hydroxyapatite column eluted with a linear gradient. Buffer A = $NaHPO_4$–Na_2PO_4 (0.05 *M*); buffer B = $NaHPO_4$–Na_2PO_4 (0.30 *M*). The arrows indicate the TNF-containing peak.

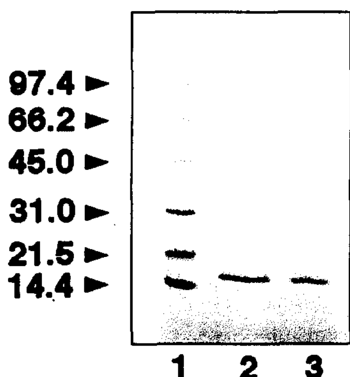

Fig. 2. SDS-PAGE of TNF with a polyacrylamide gel gradient (8–25%, w/v) using 10 m*M* Tris–HCl (pH 8.0)–1 m*M* EDTA–5% β-mercaptoethanol–2.5% SDS. The proteins were detected in the gel by staining with Coomassie Brilliant Blue R-250. Lanes: 1 = molecular mass standards (M_r values × 10^{-3} on the left); 2 = purified TNF; 3 = purified TNF diluted twice.

cedure gives a 3580-fold increase in specific activity while conserving most of the TNF content of the cell lysate. As the number of steps

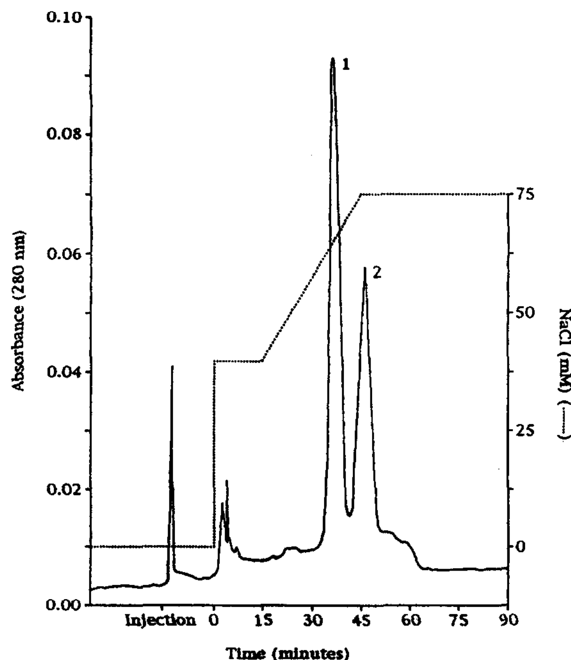

Fig. 3. Elution pattern of TNF using the Mono Q column. Peaks 1 and 2 represent two forms of TNF, as found by identical amino acid content.

was decreased by developing conditions that would eliminate most of the contaminants while TNF was retained on the column, the purification yield was greatly improved. We also found that by using Bio-Gel HTP hydroxyapatite, one could scale up almost linearly the purification procedure from a small Econo HTP column (V_t = 5 ml) to a semi-preparative scale (V_t = 100 ml) without changing the conditions.

3.3. Residual TNF

Integration of three different scans of the electrophoresis of the solubilized residual fraction showed that 15.8% of the total TNF was left in this fraction. This was considered to be negligible compared with the degree of purification obtained with the method described. The specific activity of TNF could not be determined owing to the presence of various proteases and toxic material in the insoluble fraction.

3.4. Analysis of the final product

The final product was found to be pure by SDS-PAGE and silver staining. A single band corresponding to a molecular mass of about 17 000 had the same electrophoretic mobility as reference TNF obtained from Dr. V. Korobko (Shemyakin Institute, Moscow, Russian Federation) (Fig. 4). The results of amino acid analysis of the final product agreed very closely with the theoretical amino acid content reported [11] (Table 2). Capillary electrophoresis gave the expected three peaks corresponding to the monomer, dimer and trimer isoforms of human TNF without any trace of impurities (Fig. 5).

4. Discussion

The method described here for the purification of tumor necrosis factor from *E. coli* is highly reproducible and may be scaled up from an analytical column of Bio-Gel HTP hydroxyapatite to a semi-preparative column that was used for this separation. We found an almost

Table 1
Purification of human TNF from *E. coli*

Step	Total protein (mg)	Specific activity[a] (U/mg)	Total activity (U/mg)	Total protein (%)	Increase in specific activity
E. coli homogenate	217.0	$1.2 \cdot 10^4$	$2.60 \cdot 10^6$	100	–
Hydroxyapatite	13.6	$4.3 \cdot 10^6$	$5.85 \cdot 10^7$	6.26	3580×
Mono Q	8.21	$1.44 \cdot 10^7$	$11.8 \cdot 10^7$	3.78	12 000×

Yields are calculated for 2 l of culture medium.
[a] Specific activity of TNF was calculated as described in under Experimental; this value is an approximation for the *E. coli* homogenate, which contains various toxic factors and possible inhibitors of TNF activity.

linear correlation between the elution volume and the column size under the conditions adopted. Most of the published methods for the purification of recombinant human TNF alpha consist of 5–6 purification steps with yields varying from 5% to 30%. The above procedure using a single step with hydroxyapatite yields 95% pure TNF while a second step with Mono Q anion-exchange chromatography allows the elimination of the last traces of contaminants while conserving the lytic activity. It is believed that this efficient purification method may be used for the large-scale purification of TNF in industrial settings.

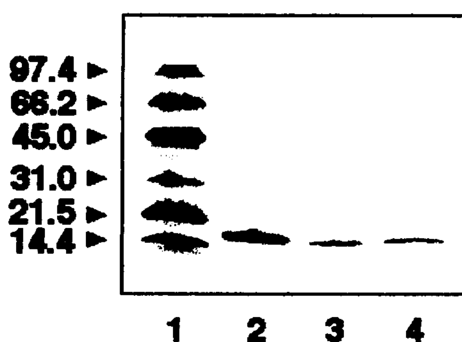

Fig. 4. SDS-PAGE of TNF with a polyacrylamide gel gradient (8–25%, w/v) using 10 m*M* Tris-HCl (pH 8.0)–1 m*M* EDTA-5% β-mercaptoethanol-2.5% SDS. Proteins were detected by silver staining. Lanes: 1 = molecular mass standards (M_r values × 10^{-3} on the left); 2 = reference TNF; 3 = Mono Q-purified TNF (peak at 60 m*M* NaCl); 4 = Mono-Q purified TNF (peak at 75 m*M* NaCl).

5. Acknowledgements

We are grateful to Dr. V. Korobko of the Shemyakin Institute, Moscow, for supplying us

Table 2
Amino acid composition of TNF

Amino acid	Theoretical number of residues	Number of residues found
Ala	13	12.73
Arg	9	8.80
Asx[a]	12	12.00
Cys[b]	2	1.86
Glx	20	20.39
Gly	11	11.07
His	3	3.01
Ile	8	8.02
Leu	18	18.00
Lys	6	6.04
Phe	4	4.01
Pro	10	10.30
Ser	13	12.07
Thr[c]	6	6.10
Trp[d]	2	1.78
Tyr[c]	7	6.89
Val	13	12.22

Proteins were gas-phase hydrolysed in 6 *M* HCl containing 0.1% phenol for 1, 2, 3 and 4 h at 150°C.
[a] As Asx is stable to acid hydrolysis, it was taken as the reference residue.
[b] Determined as cysteic acid by performic acid oxidation prior to acid hydrolysis [15].
[c] Determined by extrapolation to zero time.
[d] Determined by alkaline hydrolysis with 12 *M* NaOH at 110°C for 16 h [14].

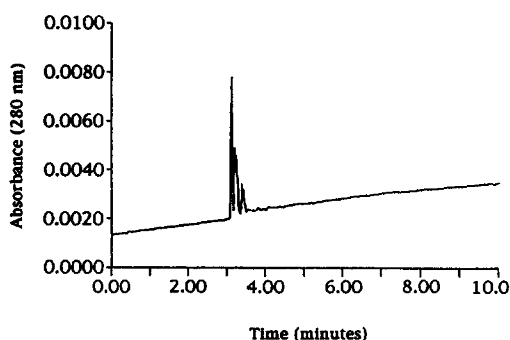

Fig. 5. Capillary electrophoresis of TNF.

with the TNF plasmid and to Dr. B. Gibbs of the Institute of Biotechnology, Montreal, for the amino acid analyses.

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