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Role of polyethyleneimine in the purification of recombinant human tumour necrosis factor beta

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Abstract

The chromatographic behaviour of recombinant human tumour necrosis factor beta (rhTNF-β) (p*I*~9.0) during cation-exchange chromatography at pH 7.5 is investigated. Without prior treatment of the *Escherichia coli* cell extract with polyethyleneimine (PEI), very little rhTNF-β was bound to the column. However, upon addition of 5% PEI (100 μl ml^{-1}) to the cell lysate, rhTNF-β was shown to bind to cation-exchange columns normally. TNF-β was readily precipitated from the clarified cell extract by 20% ammonium sulphate, but only ca. 25% of this precipitate could be re-solubilized for further purification. However, when 5% PEI was included in the solubilization buffer, the balance of the rhTNF-β could be recovered. It is proposed that charge interaction between rhTNF-β and nucleic acids in the cell extract is responsible for both of these anomalous phenomena, and that PEI (a cationic polyelectrolyte) was able to disrupt this interaction by displacing rhTNF-β from the charge complex.

Keywords: Polyethyleneimine; Tumour necrosis factor; Proteins

1. Introduction

Removal of nucleic acids is a common early purification step in downstream protein purification. This step reduces the viscosity of the extract and eliminates the interference of nucleic acids in the subsequent purification steps. Precipitation of nucleic acids can be achieved by numerous methods, but one of the most effective methods is by treatment with the cationic polyelectrolyte, polyethyleneimine (PEI) [1-3]. An additional benefit of using PEI in downstream processing is that it can also precipitate anionic proteins at slightly alkaline pH values. This phenomenon has been exploited in the purification of many proteins from several biological sources [4,5]. In one interesting application, PEI precipitation of β-galactosidase fused to polyaspartic peptide tails was examined as a possible low cost and easily scaled-up separation method for the recovery of proteins from industrial microbial processes [6,7].

In an earlier communication [8], we reported a novel and rapid purification method for recombinant tumour necrosis factor beta (rhTNF-β) using PEI as the precipitating agent at the initial stage of the purification. However, several questions remained unanswered regarding the behaviour of rhTNF-β during purification, especially its solubility problem after ammonium sulphate precipitation and its failure

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to bind to cation-exchangers at neutral pH values prior to PEI treatment. As this phenomenon may be of a general nature in the purification of cationic proteins from cell extracts, it is important to overcome these problems in order to enhance the recovery of this class of proteins. In this paper, we describe the results of our work aimed at resolving these issues by using rhTNF-β as the model cationic protein.

2. Experimental

2.1. Materials

CM-Sepharose CL-6B, CM-Sepharose Fast Flow and Sephadex G-25 were purchased from Pharmacia-LKB Biotechnology, while PEI and phenylmethylsulfonylfluoride (PMSF) were from Sigma. Ammonium sulphate was obtained from Merck and the protein assay kit was supplied by Bio-Rad Labs. Calf thymus deoxyribonucleic acid and ribonucleic acid were purchased from Boehringer Mannheim. All chemicals used were of the highest grades available.

2.2. Methods

2.2.1. Cell line and fed-batch culture

E. coli HB101 cells cloned with an efficient rhTNF-β gene 2/1 in pCG402 was kindly provided by Dr. Alan Porter (Institute of Molecular and Cell Biology, National University of Singapore, Singapore). Cells were obtained from a 10-l fed-batch culture, using growth conditions that were optimised by Mak et al. [9]. Purified rhTNF-β was obtained as described by Loh et al. [8].

2.2.2. Precipitation of rhTNF-β by DNA and RNA

Different amounts of calf thymus DNA (3 to 180 μg) or RNA (6 to 176 μg) in 10 m*M* Tris–HCl buffer, pH 7.5, was added to 1 ml of rhTNF-β (0.64 or 0.55 mg ml^{-1}). After adjusting the solutions to 1.1 ml, they were incubated at 4°C for 15 min. Precipitates that formed were removed by centrifugation at 16 000 *g* for 15 min. The amount of rhTNF-β, DNA and RNA precipitated from the mixture was monitored by determining the amount of protein, DNA and RNA that remained in the supernatant. The content of protein and DNA in the supernatant was determined by the method of Bradford [10] and Burton [11], respectively, while the amount of RNA was estimated using orcinol reagent [12].

2.2.3. Displacement effect of PEI on DNA

First, 1 ml of rhTNF-β (1.45 mg ml^{-1}) was added to 150 μl (0.8 mg ml^{-1}) of calf thymus DNA solution in a 2-ml eppendorf tube. The solution was mixed well by vortex-mixing for 5 min and the resulting precipitate was recovered by centrifugation at 16 000 *g* for 10 min. This precipitate was then resuspended in 1 ml of 10 m*M* Tris–HCl buffer, pH 7.5, and 50 μl of 5% PEI were added. After 5 min of incubation, the mixture was re-centrifuged at 16 000 *g* to pellet any residual precipitate. The amount of rhTNF-β displaced by PEI from the rhTNF-β-DNA complex was calculated from the protein content of the supernatant.

2.2.4. Recovery of rhTNF-β from the ammonium sulphate precipitate

A 20-ml volume of clarified cell extract was precipitated directly with 20% ammonium sulphate and the resulting precipitate was recovered by centrifugation at 39 200 *g* for 30 min. A 20-ml volume of 10 m*M* Tris–HCl buffer, pH 7.5, containing 4 μg ml^{-1} of PMSF (buffer A) was then added to the pellet for resolubilization. After resolubilization, the suspension was centrifuged briefly to pellet any insoluble materials. The clear supernatant was saved while the insoluble precipitate was resuspended in 20 ml of buffer A, but with the inclusion of 1 ml of 5% PEI. The supernatants obtained from the two resolubilization steps were loaded separately onto a CM-Sepharose Fast Flow column (75×16 mm) that had been pre-equilibrated with buffer A. The bound rhTNF-β was eluted with a linear salt gradient and the amount of rhTNF-β recovered was determined.

The experiment was repeated with 20 ml of clarified cell extract that had been pretreated with 1 ml of 5% PEI first. Any precipitate formed after PEI treatment was removed by centrifugation at 39 200 *g* for 30 min at 4°C. The supernatant fraction was similarly treated with 20% ammonium sulphate and the resulting precipitate was re-solubilised with 20 ml of buffer A. The clear supernatant obtained was

loaded onto the same CM-Sepharose Fast Flow column and the amount of rhTNF-β recovered was determined as above.

2.2.5. Cation-exchange chromatography

A 30-ml volume of wet cell slurry (3 g of wet cells; 30 ml) was sonicated three times, each for 5 min at 4°C. The resulting cell lysate was subjected to the following two purification routes:

Method 1

A 10-ml volume of cell lysate was clarified by centrifugation at 39 200 *g* for 30 min at 4°C. The clear supernatant was desalted using a G-25 column (240×16 mm) equilibrated with buffer A and subsequently loaded onto a CM-Sepharose CL-6B column (90×16 mm) that had been pre-equilibrated with the same buffer. The bound rhTNF-β was eluted with a linear salt gradient of 0–1.0 *M* NaCl in buffer A. The fractions collected were checked for the presence of rhTNF-β by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and cytotoxic assay.

Method 2

In this procedure, 5% PEI (100 μl ml^{-1} of cell lysate) was added first to 10 ml of the sonicated cell lysate. Cell debris and any precipitate formed were then removed by centrifugation (39 200 *g* at 4°C for 30 min). The clear supernatant was desalted and purified using a CM-Sepharose column as in Section 2.2.5.1.

2.2.6. Protein assay

Protein concentration was determined by the method of Bradford [10] using the protein assay kit from Bio-Rad Labs. and bovine serum albumin as the standard.

2.2.7. Electrophoretic procedures

SDS–PAGE was performed according to the method of Laemmli [13] using 12% polyacrylamide gels. Following staining with 0.1% (w/v) Commassie blue R-250 and destaining with 30% (v/v) methanol and 10% (v/v) acetic acid, the amount (in %) of rhTNF-β present in each gel lane was quantitated by densitometry at 600 nm (Model 620 video densitometer; Bio-Rad Labs.).

2.2.8. Cytotoxic assay

Mouse fibroblast L929 cells were used to assay for the cytotoxicity of rhTNF-β, based on the method of Seow et al. [14]. Briefly, 96-well microtiter plates were seeded with L929 cells ($2\cdot10^4$ cells $well^{-1}$) in 100 μl of cell culture media and incubated overnight at 37°C. Serial dilution of samples was performed in the presence of cycloheximide (10 μg ml^{-1}). After incubation for 16 h at 37°C, the viable cells were stained with 0.5% crystal violet in 20% methanol for 15 min. Excess stain was washed away with phosphate-buffered saline (PBS) and the cells were solubilized in 33% (v/v) acetic acid. Absorbance was measured at 540 nm with a microplate reader (Bio-Tek, model EL311). The unit of cytotoxicity was defined as the reciprocal of the dilution causing 50% cytotoxicity (cell killing), which was calculated from a graph of percentage cytotoxicity versus dilution of rhTNF-β. The percentage cytotoxicity was calculated from [1−(A_{540} nm test well/A_{540} nm untreated well)]×100.

3. Results

3.1. DNA and RNA precipitation of recombinant tumour necrosis factor beta

The positively charged rhTNF-β (p*I*~9) interacts with the negatively charged phosphate residues of nucleic acids (DNA and RNA) resulting in the formation of a precipitate. Fig. 1 and Fig. 2 show the profile of rhTNF-β precipitated by DNA and RNA, respectively. It was estimated that 1 mg of DNA and RNA would precipitate about 10 and 9 mg of rhTNF-β, respectively, at the optimum dosage.

3.2. Displacement effect of PEI on DNA–protein complex

Table 1 shows that addition of 120 μg of DNA will completely precipitate 1.45 mg of rhTNF-β. However, upon the addition of 50 μl of 5% PEI to the precipitate, rhTNF-β was quantitatively released from the DNA–protein complex. This result showed that PEI has a higher binding affinity for DNA than rhTNF-β, and hence the latter was displaced effectively from the DNA–TNF-β complex by PEI.

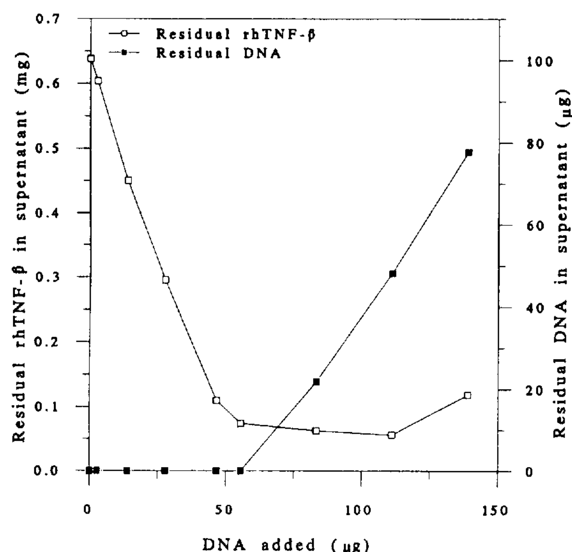

Fig. 1. Precipitation of rhTNF-β (0.64 mg ml^{-1}) with calf thymus DNA (3 to 180 μg) in 1 ml of 10 m*M* Tris–HCl buffer, pH 7.5.

3.3. Recovery of rhTNF-β from the ammonium sulphate precipitate

It was found that only about 25% of rhTNF-β obtained from ammonium sulphate precipitation of the clarified cell extract could be recovered (Fig. 3a). The main problem was that the precipitated rhTNF-β could not be re-solubilized. However, upon addition of PEI to this precipitate, the remaining rhTNF-β could be recovered (Fig. 3b).

By comparison, prior treatment of the clarified *E. coli* extract with PEI before ammonium sulphate precipitation showed that the precipitate was readily soluble and, hence, recovery by cation-exchange chromatography was very much improved (Fig. 3c).

The specific cytotoxicities of rhTNF-β obtained from Fig. 3a,b were $30 \cdot 10^7$ and $29 \cdot 10^7$ U/mg, respectively, suggesting that the rhTNF-β recovered from the insoluble ammonium sulphate fraction by PEI displacement was biologically active.

3.4. Purification by CM-Sepharose chromatography

The elution profiles for Methods 1 and 2 are shown in Fig. 4. It is apparent that the recovery of rhTNF-β has been substantially enhanced by the addition of PEI to the cell extract before chromatography. In addition, since there was no ammonium precipitation step, the purification process was further shortened.

4. Discussion

We had earlier observed that rhTNF-β, although being cationic in nature, showed very weak binding to a cation-exchange column at neutral pH values. It was speculated that some kind of "neutralization or shielding effect" was responsible for this phenomenon, due to the presence of the predominant anionic proteins and nucleic acids in the *E. coli* cell extract [8]. The results presented here are in complete accord with our earlier suggestion, since, after the removal of anionic species from the cell extract by

Table 1
Displacement effect of PEI

	Amount of protein in supernatant (mg)
rhTNF-β	1.450
rhTNF-β+120 μg of DNA	0.004
rhTNF-β+120 μg of DNA+50 μl of 5% PEI	1.429

A 1-ml volume of rhTNF-β (1.45 mg ml^{-1}) was added to 150 μl (0.8 mg ml^{-1}) of calf thymus DNA solution. The solution was vortex-mixed for 5 min and the resulting precipitate was recovered by centrifugation at 16 000 *g* for 10 min. This precipitate was then resupended in 1 ml of 10 m*M* Tris–HCl, pH 7.5, and 50 μl of 5% PEI was added. After 5 min of incubation, the mixture was re-centrifuged at 16 000 *g* to pellet any residual precipitate. The amount of rhTNF-β displaced by PEI was calculated from the protein content of the supernatant.

PEI via charge precipitation, rhTNF-β is now behaving like a typical cationic protein that could bind readily to cation-exchangers at neutral pH values (Figs. 3 and 4).

We have also reported that rhTNF-β, precipitated directly from the clarified cell extract by 20% ammonium sulphate, was not readily soluble [8]. Hence, only about 25% of soluble rhTNF-β was recovered in this step (Fig. 3a). However, when the precipitate (after resuspension in buffer) was treated with PEI, approximately 80% of the precipitated rhTNF-β could be readily resolubilised and recovered by cation-exchange chromatography (Fig. 3b). The poor solubility of the rhTNF-β ammonium sulphate pellet was probably due to the co-precipitation of DNA and rhTNF-β, which resulted in the formation of insoluble protein–DNA complexes. This is supported by our precipitation experiments (Figs. 1 and 2), whereby pure DNA and RNA were shown to precipitate pure rhTNF-β from solution stoichiometrically (1 mg DNA and RNA per 10 and 9 mg rhTNF-β, respectively). However, this process is reversible, since, upon addition of PEI to the insoluble precipitate, rhTNF-β was readily recovered. This is most likely due to the stronger binding of PEI to DNA, leading preferentially to the formation of a more stable PEI–DNA complex, which in turn releases rhTNF-β from the rhTNF-β–DNA complex. This suggestion is confirmed by our displacement experiment using pure DNA and rhTNF-β (see Table 1). In addition, the conformation of rhTNF-β recovered by this process seems to be unaltered, since its specific cytotoxicity ($30 \cdot 10^7$ U/mg) is very similar to that of the corresponding soluble ammonium sulphate fraction of rhTNF-β ($29 \cdot 10^7$ U/mg) obtained from the same experiment (Fig. 3a). This value is also comparable to the published value of $57 \cdot 10^7$ U/mg [8]. Subsequently, we developed a modified and efficient rhTNF-β precipitation procedure in which DNA from the cell extract was first removed by PEI before addition of ammonium sulphate (Fig. 3c). It is noteworthy that the total amount of rhTNF-β recovered in Fig. 3a–b is approximately equal to the quantity of rhTNF-β obtained in Fig. 3c.

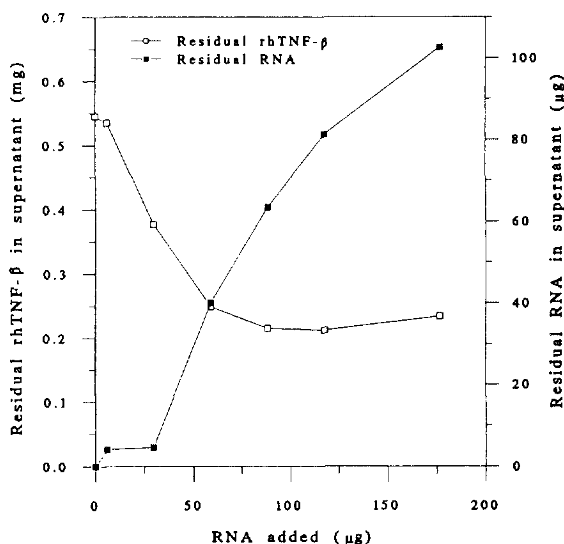

Fig. 2. Precipitation of rhTNF-β (0.55 mg ml^{-1}) with RNA (6 to 176 μg) in 1 ml of 10 mM Tris–HCl buffer, pH 7.5.

This communication has also shown that by addition of PEI directly to the sonicated *E. coli* cell lysate, followed by cation-exchange chromatography, Method 2 (Fig. 4b) has some advantages over our earlier published procedure [8]. Firstly, in the present method, since there is no ammonium sulphate precipitation step, the method is shorter and hence purification will be faster. Secondly, the use of a cation-exchanger for the purification of a cationic protein is more suitable than an anion-exchanger. This is because isocratic elution is usually inferior compared to gradient elution in ion-exchange chromatography, as altering gradient volumes and shapes can improve the separation of proteins in a purification procedure.

In summary, we have shown that PEI is a very useful cationic polyelectrolyte for removing nucleic acids and other anionic proteins during the purification of cationic proteins from bacterial cell extracts. In addition, it is also an effective flocculent [3] and hence by adding it directly to the sonicated/homogenized cell extract, it aids centrifugation and simultaneous removal of DNA and anionic proteins. Moreover, it is relatively cheap compared to protamine sulphate and DNA degrading enzymes (e.g., DNAase) and should be a compound of choice for industrial downstream processing of cationic proteins.

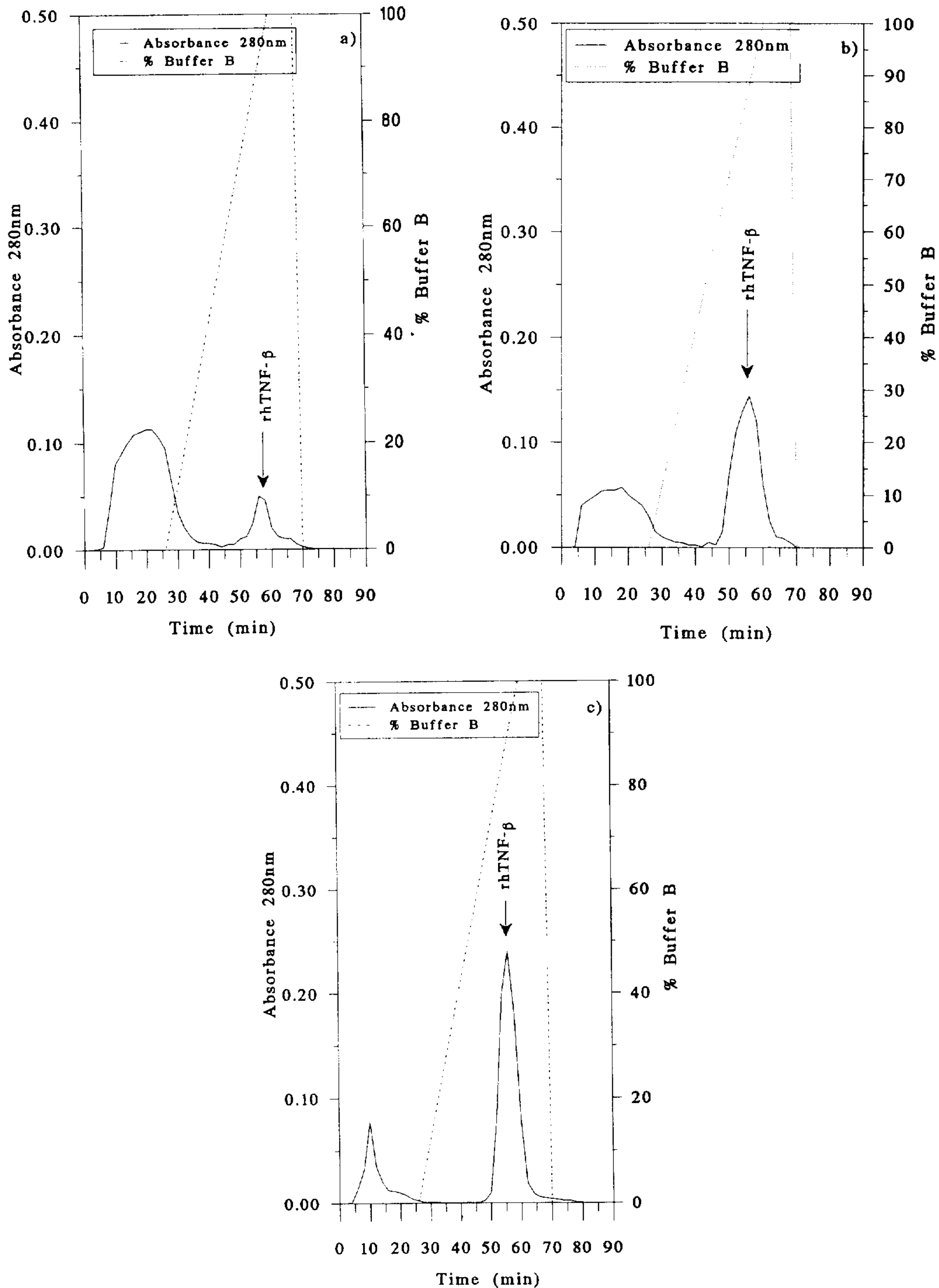

Fig. 3. Elution profiles for CM-Sepharose Fast Flow chromatography of rhTNF-β recovered from ammonium sulphate precipitation. Buffer A: 10 m*M* Tris–HCl, pH 7.5; buffer B: 10 m*M* Tris–HCl, pH 7.5, 1 *M* NaCl. (a) Ammonium sulphate precipitate dissolved in buffer A, (b) redissolved ammonium sulphate precipitate after treatment with PEI and (c) cell extract treated with PEI before ammonium sulphate precipitation.

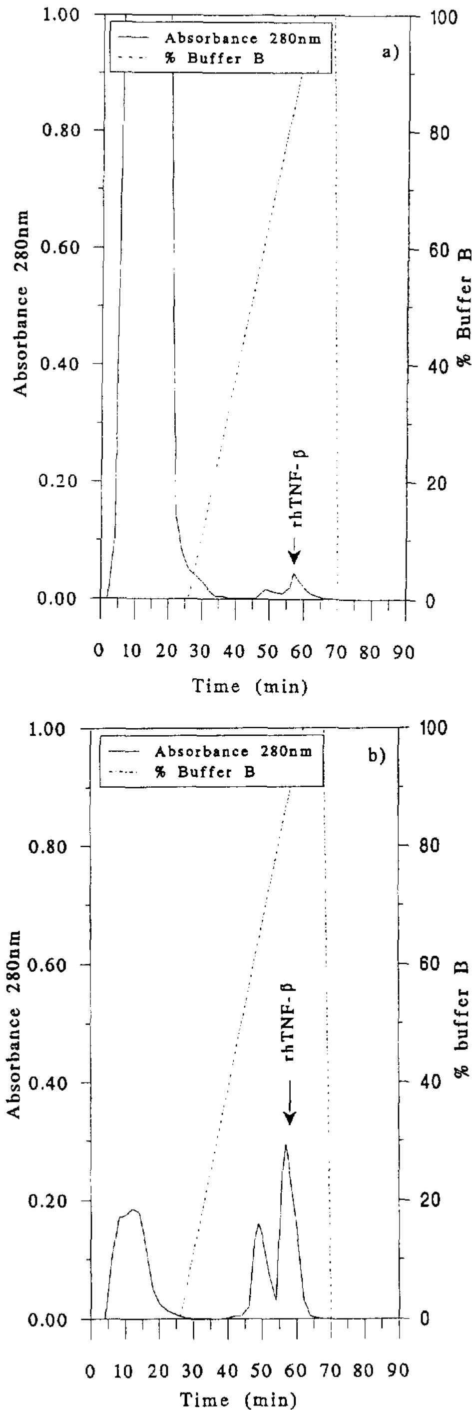

Fig. 4. Elution profiles following CM-Sepharose CL-6B chromatography using Methods 1 (a) and 2 (b), respectively. Buffer A: 10 m*M* Tris–HCl, pH 7.5; buffer B: 10 m*M* Tris–HCl, pH 7.5, 1 *M* NaCl.

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