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Purification of Transforming Growth Factor Type e

Pamela G. Parnell, John Wunderlich, Bobbie Carter, and Jaroslava Halper

Department of Veterinary Pathology, College of Veterinary Medicine (P.G.P., B.C., J.H.), and Molecular Genetics Instrumentation Facility (J.W.), University of Georgia, Athens, Georgia, 30602

Transforming growth factor type e (TGFe) is a heat- and acid-stable polypeptide with an apparent molecular weight of 22,000, which stimulates the proliferation of certain epithelial and mesenchymal cells in monolayer and soft agar. TGFe has been purified to homogeneity. Initial acid-ethanol extraction of bovine kidney was followed by batch ion-exchange chromatography utilizing Bio Rex 70 resin. The activity eluted from the Bio Rex 70 resin was concentrated and diafiltered using an Amicon concentrator equipped with an S1Y10 spiral membrane, then was further purified by Bio-Gel P-60 molecular sieve chromatography. Active fractions from molecular sieve chromatography were pooled and purified by heparin-Sepharose affinity chromatography, followed by reverse-phase high-performance liquid chromatography using a microbore C-8 column. The final purification step involved electroelution of TGFe separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purity of TGFe was assessed to be greater than 90%.

Key words: batch ion-exchange chromatography, protein purification, molecular sieve chromatography, heparin-Sepharose affinity chromatography, reverse-phase high-performance liquid chromatography

Previous investigations have identified a novel transforming growth factor type e (TGFe), which is capable of stimulating the growth of certain epithelial and mesenchymal cells in monolayer and soft agar. TGFe has been detected in extracts from neoplastic and nonneoplastic tissues of mainly epithelial origin, including nonneoplastic lung and kidney [1], as well as in bovine plasma and human platelets [2]. Bovine kidney has been used as a source for purification due to its ready availability. The factor has been characterized as an acid- and heat-stable polypeptide with an apparent molecular weight of 22,000–25,000, requiring disulfide bonds for maximum activity [3]. TGFe stimulates soft agar growth of squamous carcinoma cell lines A431 and D562, adrenal carcinoma cell line SW-13 [3], and bladder carcinoma T24 cells [4] as well as mouse embryo-derived AKR-2B cells [2,3]. TGFe does not bind significantly to TGF-β, epidermal growth factor (EGF) or fibroblast growth factor (FGF) receptors [3]. SW-13 cells serve

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as indicator cells in a soft agar growth assay to detect TGFe as previously described [3]. Colonies of a diameter greater that 50–60 μm in a total of 20 medium-power fields are counted. The ED_{50} is defined as the quantity of protein added to the plate that causes half-maximal stimulation of SW-13 cells.

Initial acid-ethanol extraction of bovine kidney was a modification [5] of the procedure previously reported by Roberts et al. [6]. Approximately 11 mg of protein per gram of tissue was collected. The ED_{50} of the initial extract as measured in the soft agar assay was 40 μg (Table I).

Protein from acid-ethanol extraction, resuspended in 1 M acetic acid, was adsorbed to Bio Rex 70 ion-exchange resin (Bio-Rad, Richmond, CA) preequilibrated with 1 M acetic acid at a volume of 100 ml resin per 1–2 gm of protein [7]. The mixture was stirred overnight at 4°C, then poured into a 2.6 × 40 cm column. The column was washed with 500 ml of 1 mM HCl, followed by 100–150 ml of 0.5 M ammonium acetate, pH 8.0. Both the HCl and the ammonium acetate eluants are discarded. The protein was then eluted with 500 ml of 1 M ammonium acetate. The eluant was concentrated and diafiltered against 4–5 volumes of 1 M acetic acid using an Amicon concentrator equipped with a S1Y10 spiral membrane to a final volume of 100 ml. The ED_{50} of the extract after Amicon concentration and diafiltration was 0.5 μg, with 10–15% protein recovery (Table I).

Protein from Bio Rex 70 ion-exchange chromatography, resuspended in 1 M acetic acid, was further purified by P-60 molecular sieve chromatography (Bio-Rad) as previously described [3]. SW-13 colony-stimulating activity eluted after the majority of the proteins as a broad peak between 520 and 900 ml of eluant (Fig. 1). In the hands of other investigators, TGFe also elutes as a broad peak on P-60 columns [8]. Protein recovery from the P-60 column was approximately 10–15% with an ED_{50} of 0.2 μg (Table I).

The most active fractions (550–700 ml) from molecular sieve chromatography were pooled, lyophilized to dryness, and resuspended in 50 mM Tris HCl, pH 7.4, for further purification by heparin-Sepharose (Pharmacia LKB, Piscataway, NJ) or heparin Affi-Gel (Bio-Rad) affinity chromatography as previously described [9]. TGFe eluted as a major peak from the heparin-Sepharose column at a concentration of 0.5 M NaCl (Fig. 2), with an ED_{50} of 5 ng and a protein recovery of 4.7% (Table I). The purification steps involving Bio-Rex 70, Bio-Gel P-60, and heparin affinity chromatography resulted in marked increase in recovery of activity. We attribute this increase to separation of growth inhibitors (e.g., TGF-β) from TGFe present in the acid-ethanol extraction.

Peak active fractions (27–35) from four or five Heparin-Sepharose runs were pooled, lyophilized to dryness, and further purified by microbore reverse-phase C-8 high-performance liquid chromatography (HPLC) (Fig. 3). Briefly, protein was resuspended in 0.1% TFA/H_2O and 50 μg was applied to a 2.1 × 30 mm column. Protein was eluted from the column with a linear gradient of 10% to 45% solution B (80% acetonitrile/0.085% TFA/20% H_2O) over a 30 min period. TGFe eluted from the C-8 column at approximately 25% solution B and could be separated from the majority of proteins eluting before and after the peak activity. Peak active fractions corresponding to retention times of 16–18 min from 10–12 HPLC runs were pooled for further purification. Protein recovery was estimated to be 5% based on the protein absorbance at 214 nm from the chromatograph and silver stained sodium dodecyl sulfate (SDS)-polyacryla-

TABLE I. Purification of TGFe*

Method	Protein recovery (mg)	ED_{50} (μg)	Units ($\times 10^{-3}$)	Specific activity (U/μg)	Purification degree	Percent recovery of activity
Acid-ethanol	5,500	40	137.5[a]	0.025	1	100
Bio-Rex 70	330	0.5	660	2	80	480
Bio-Gel P-60	55	0.2	275	5	200	200
Heparin-Sepharose	2.6	0.005	520	200	8,000	378

*Bovine kidney (500 gm) was used as a source.
[a]Initial low yields of activity units may be due to coprecipitation of an inhibitory factor removed in subsequent purification steps.

mide gels of each fraction collected (data not shown). ED_{50} was not determined in this and further steps due to the small amounts of protein recovered.

The pooled samples were partially evaporated and rerun on the same column, with an extended acetonitrile gradient of 15–40% solution B over 60 min (Fig. 4). Again, TGFe elutes at approximately 25% solution B, though some tailing of the peak was observed. The most active fractions, corresponding to retention times of 24–26 min, were pooled for final purification steps.

Final purification was achieved by pooling active fractions from six to eight HPLC purification runs, and separating the proteins in a 10% to 20% gradient SDS-polyacrylamide gel (10). Five percent of the total protein was run in a separate lane and silver stained for assessment of purity and identification of active bands eluted from the gel lane containing 95% of the protein. The gel lane containing 95% of the protein was

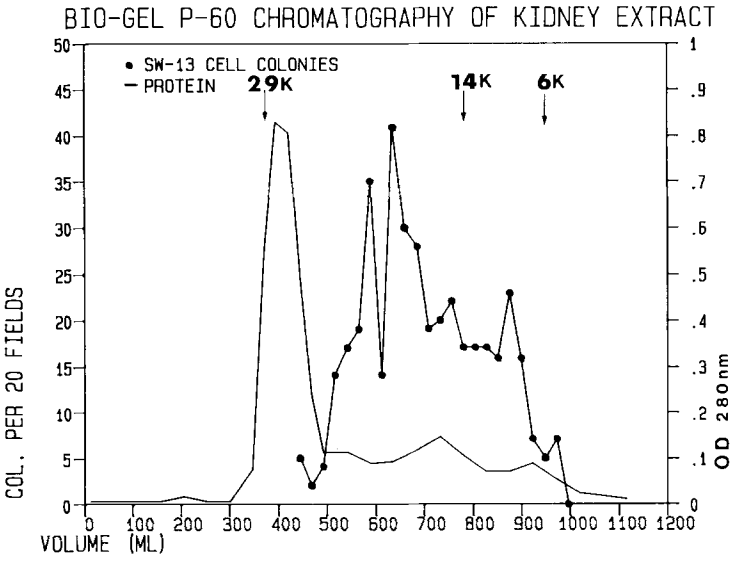

Fig. 1. Molecular sieve chromatography of TGFe; 200 mg of protein eluted from cation-exchange Bio-Rex 70 column and resuspended in 1 M acetic acid was loaded on a 5 ×90 cm molecular sieve Bio-Gel P-60 column equilibrated with 1 M acetic acid. The column was eluted at 22°C, with 1 M acetic acid, at a flow rate of 30 ml/h. Ten milliliter fractions were collected and 1/500 aliquots tested for SW-13 colony-stimulating activity.

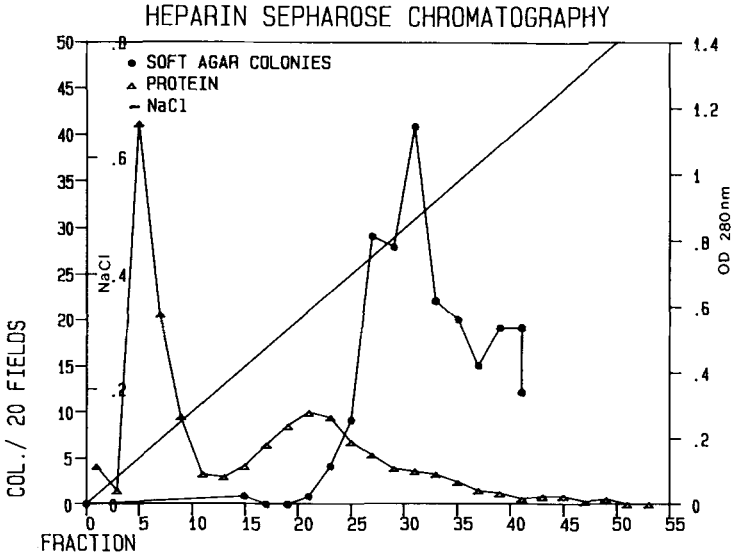

Fig. 2. Heparin-Sepharose affinity chromatography of TGFe; 20 mg of protein containing activity from the P-60 column was resuspended in 50 mM Tris HCl, pH 7.4. The sample was loaded on a 1 ×10 cm heparin-Sepharose column equilibrated with 50 mM Tris HCl, pH 7.4. The column was washed with 5 volumes of equilibrating solution and eluted with a linear gradient of 0–1.5 M NaCl in 50 mM Tris HCl, pH 7.4, at a flow rate of 40 ml/h. Four milliliter fractions were collected, dialyzed against 1% acetic acid, and lyophilized to dryness. The protein was resuspended in 1 ml of 1 M acetic acid and 1/50 aliquots tested for SW-13 colony-stimulating activity.

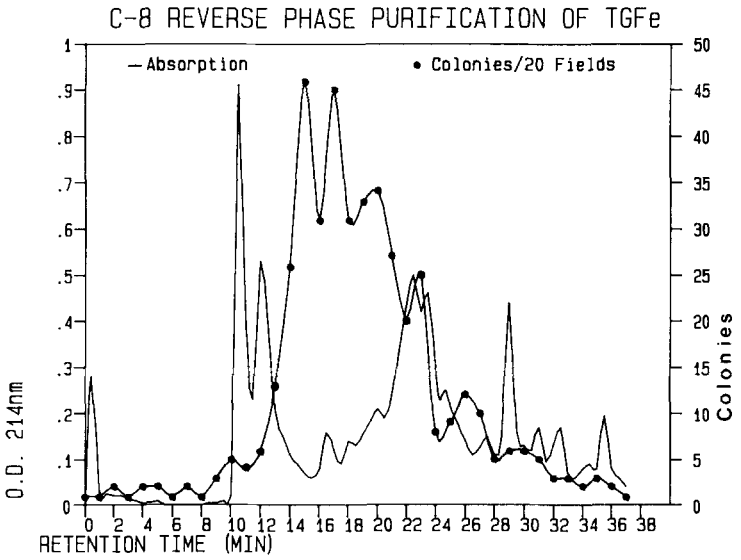

Fig. 3. Reverse-phase high-performance liquid chromatography (HPLC) of TGFe. Active fractions from four or five heparin-Sepharose purification runs were pooled and assayed for protein concentration, and the sample was diluted to a concentration of 1 μg/μl; 50 μg of protein were loaded on a 2.1 ×30 mm RP-300 microbore HPLC column and eluted with a linear gradient of 10% to 45% solution B over 30 min at a flow rate of 200 μl/min. Solution A, 0.1% TFA/H_2O; solution B, 80% acetonitrile/0.085% TFA/20% H_2O. One minute fractions were collected, and a 1/100 aliquot of each was evaporated to dryness and tested for SW-13 colony-stimulating activity.

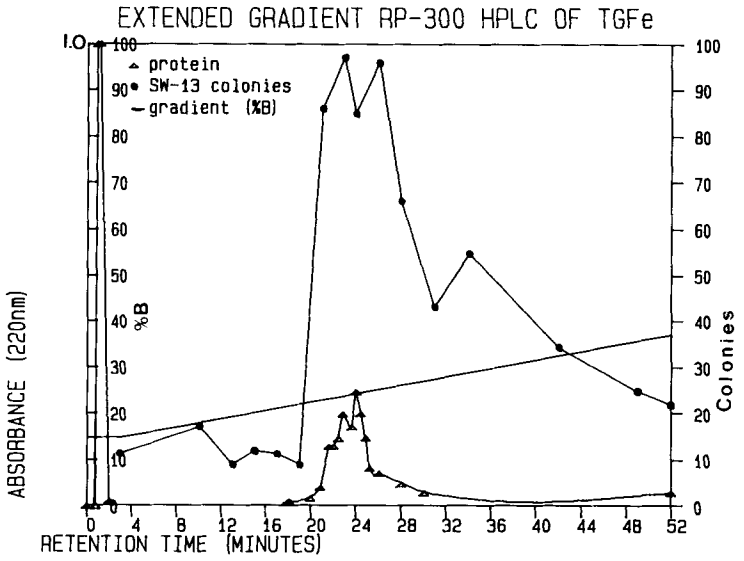

Fig. 4. Extended-gradient reverse-phase HPLC of TGFe. Active fractions from 10–15 RP-300 separation runs were pooled, partially evaporated to a volume of 75 μl, and loaded on the same column. The column was eluted with a 15% to 40% gradient of solution B over 60 min at a flow rate of 200 μl/min. One minute fractions were collected, and 1/20 aliquots were evaporated and tested for SW-13 colony-stimulating activity.

cut into 3 mm strips, and the protein was eluted from each strip utilizing Elutrap electroelution chambers (Schleicher & Schuell, Keene, NH) and 15 mM ammonium carbonate, with 0.1% SDS as the elution buffer [11]. Gel strips were eluted at 70 V for 1 h. Following electroelution, samples were purified by HPLC using the microbore RP-300 column, with a rapid (20 min) linear gradient of 0 to 80% acetonitrile in 0.085% TFA in water. Samples were applied directly to the column. Many spurious peaks were present, which corresponded to absorbance of ammonium carbonate and SDS in the

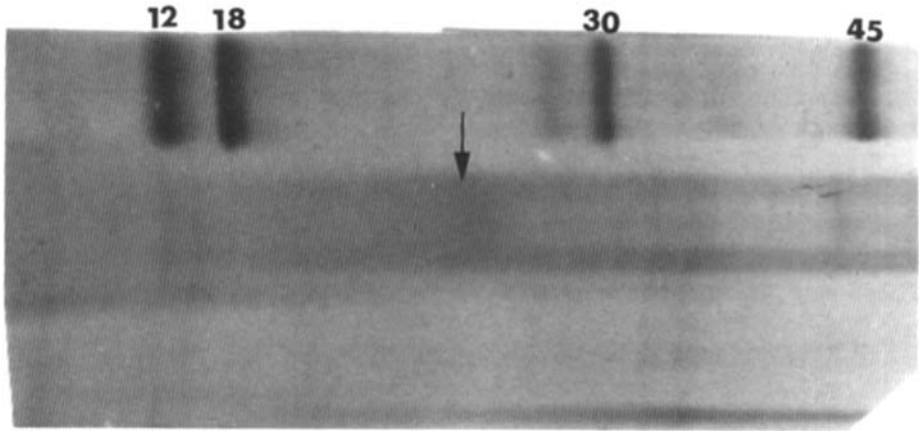

Fig. 5. SDS-PAGE of protein eluted from initial acrylamide gel. A major band is visible migrating at approximately 22,000 daltons (arrow), with a minor band visible at approximately 40,000.

elution buffer. The retention of TGFe by the column was not significantly affected by the ammonium carbonate buffer. Individual peaks were collected, and 1/100 aliquots of all fractions eluting from the column were tested for colony-stimulating activity. The peak activity eluted from a strip corresponding to an apparent molecular weight of 25,000. The entire sample containing the colony-stimulating activity was rerun on a SDS-polyacrylamide gel and silver stained to assess recovery and purity of the sample. Figure 5 is a photograph of the gel indicating a major biologically active band migrating at 22,000–25,000, with a minor biologically inactive band migrating at approximately 40,000. The remaining bands are due to background staining and are not attributed to proteins; they appear in the blank lanes as well. Based on intensity of staining, protein recovery is estimated to be in the range of 10–15 ng, and purity is assessed at greater than 90% for this particular run (Fig. 5). Characteristically, TGFe appears as a fuzzy band on all silver-stained gels; we therefore assume that TGFe is a glycoprotein.

Although the above-described procedure is useful for purification of analytical quantities of TGFe, and will be used to obtain TGFe for sequence analysis, it is insufficient for large-scale purification of TGFe for biological studies. Future purification schemes will include semipreparative RP-300 C-8 columns, which will accommodate larger quantities of protein.

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