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RAPID PURIFICATION OF TOPOISOMERASE I FROM HUMAN BREAST CANCER CELLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The DNA regulatory enzyme topoisomerase I (TpI) from human breast cancer cells has been analyzed by high-performance liquid chromatography (HPLC) for the first time. Cells were homogenized in Tris buffer and TpI activity was extracted with 0.5 *M* sodium chloride. Negatively supercoiled plasmid pBR322 was used as the substrate to monitor TpI activity, as judged by relaxed products, analyzed on 1% agarose gels. HPLC in the anion-exchange mode (HPIEC) provided an approximately 6-fold purification of the enzyme. Enhanced purification was subsequently obtained by chromatography of a HPIEC eluate on size-exclusion columns (30- to 60-fold). Recovery of TpI from size-exclusion columns, whether used in multistep analysis or as the first step, was dependent on inclusion of organic solvent, 1-propanol (0.5%, v/v), in the mobile phase. Marked resolution of TpI activity was observed with HPIEC on a SynChrom CM-300 column. Enzyme activity was noted in the void volume, at 150–200 m*M* phosphate and at 250–350 m*M* phosphate. TpI purification was 10- and 120-fold in the latter two peaks, respectively. Silver-stained polyacrylamide gels of TpI-containing activity, eluted from a CM-300 column, showed considerable purification of all but the void volume fraction. A distinct protein band at approximately 88–90 kD was seen in the peak eluted from the CM-300 column with 250–350 m*M* phosphate. These results indicate that HPLC is useful for rapid purification of the labile enzyme, TpI, in the analysis of its structure–function relationship.

INTRODUCTION

Topoisomerase enzymes are involved in the alteration of topological properties of DNA molecules by concerted breaking and rejoining of phosphodiester bonds[1]. In eukaryotes, two types of topoisomerases have been described. Topoisomerase I (TpI) transiently introduces a single-strand break, which leads to changes in the linking number of DNA by one[2]. Topoisomerase II (TpII) causes a double-strand break in DNA and changes the linking number of DNA by steps of two[3]. Both enzymes are now thought to be involved in several cellular processes, such as relax-

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ation of supercoiled DNA[4], knotting/unknotting[5], and catenation/decatenation[6] via a covalent enzyme–DNA intermediate, which has been well characterized[7].

This report concerns the purification of TpI. Several attempts have already been made to purify this enzyme, with varying degrees of success[8–11], in order to obtain further insight into its function *in vivo*. However, a wide range of molecular weights (60–200 kD) have been reported for the purified enzyme[8–15]. It has been suggested by some[15,16] that the lower-molecular-weight components represent the enzymatically active, proteolyzed form of the parent enzyme, but others argue against this proposal[10]. It may be noted that all purification processes reported so far require considerable time for completion. This increases the possibility of introducing artifacts *in vitro*.

Recent developments in high-performance liquid chromatography (HPLC) and column technology have provided a fast and efficient procedure for the purification and analysis of certain proteins[17–19]. Alternatively, other proteins have been shown to undergo pressure induced conformational changes[20]. Still other proteins show denaturation due to incompatibility with either a particular stationary or bonded phase in the HPLC column. This is particularly true in the case of reversed-phase chromatography.

In this report we describe our experience with TpI purification by HPLC in several modes. Our objective was to ascertain whether this methodology is useful for the rapid isolation of this labile protein. Whereas partial purification of TpI was obtained with every HPLC mode employed, a combination of anion-exchange and size-exclusion chromatography (HPSEC) provided enhanced purification. However, when a single HPLC step was performed in the cation-exchange mode, a purification greater than that of the multistep procedure was obtained. This study provides the first demonstration that HPLC may be used as a rapid, efficient method of purifying TpI, a labile regulatory enzyme, so that kinetic and molecular analyses may be conducted.

EXPERIMENTAL

Materials

All reagents were of analytical grade. Agarose for gel electrophoresis was obtained from Bethesda Research Labs. (Gaithersburg, MD, U.S.A.), supercoiled DNA plasmid pBR322 was from NEN/DuPont (Boston, MA, U.S.A.). Disodium ethylenediaminetetraacetic acid (EDTA), glycerol and 1-propanol were obtained from Fisher Scientific (Cincinnati, OH, U.S.A.). Dithiothreitol (DTT) was from Sigma (St. Louis, MO, U.S.A.). The materials for polyacrylamide gel were from the following: sodium dodecyl sulfate (SDS), β-mercaptoethanol and tetramethylethylenediamine (TEMED) were from the Bio-Rad Labs. (Richmond, CA, U.S.A.), thrice recrystallized acrylamide was from PolySciences (Worthington, PA, U.S.A.) and bisacrylamide was from Eastman Kodak (Rochester, NY, U.S.A.). The reagents for silver staining of polyacrylamide gels were from Pierce (Rockford, IL, U.S.A.). Dulbecco modified Eagle Medium and fetal calf serum were from GIBCO Labs. (Grand Island, NY, U.S.A.).

Cell culture

The procedure for cell culture of T47D and MCF-7 human breast cancer cells was as described elsewhere[21,22]. The cells were harvested by the addition of EDTA saline solution and the cellular pellets were collected by centrifugation at 600 *g* for 5 min.

Enzyme extract preparation

Cellular extracts for TpI activity measurements were prepared as described elsewhere[22]. Briefly, cellular pellets were resuspended in 1 μl per 10^3 cells in TKM buffer [10 m*M* Tris–HCl (pH 7.5), 25 m*M* potassium chloride, 5 m*M* magnesium chloride, 1 m*M* DTT, 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]. Lysis was achieved by pipeting the suspension 40 times with a commercial 50-μl Eppendorf pipette (Brinkmann Instruments, Westbury, NY, U.S.A.). This technique provides gentle cell lysis, solubilizing topoisomerases into the cytosol. Cell lysates were adjusted to 0.5 *M* sodium chloride by addition of an equal volume of TEM buffer [50 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 1 *M* sodium chloride, 1 m*M* DTT, 1 m*M* PMSF] and homogenized. Nuclear topoisomerases were extracted for 2–3 h at 0°C. PEG-8000 from Fisher Scientific was added, to give 5% (w/v) suspensions which were centrifuged for 1 h at 50 000 *g* to precipitate endogenous DNA. Glycerol (20%, v/v) was added to the extract, which was kept at −20°C until assayed. Extract were assayed either immediately or stored for less than one week.

Topoisomerase assay

TpI activity was determined by the decreased mobility of relaxed pBR322 DNA in 1% agarose gel after treatment of the supercoiled form with either cellular extracts[22] or with various fractions obtained by HPLC, as described in the figure legends. Assay mixtures contained 50 m*M* Tris–HCl (pH 7.5), 0.2 *M* sodium chloride, 1 m*M* DTT, 30 μg/ml bovine serum albumin (BSA), 1 m*M* EDTA, and 0.1 μg of supercoiled pBR322. These reactions were allowed to proceed for 15 min at 37°C and then terminated by the addition of a solution composed of 5% SDS, 40% glycerol, and 0.005% bromophenol blue (all w/v). Samples were analyzed by electrophoresis on 1% agarose gels in TEA buffer [40 m*M* Tris–acetate (pH 8.0), 1 m*M* EDTA]. Gel electrophoresis was performed at 40 V for 12–15 h or at 80 V for 3–4 h. The TpI activity was then visualized by UV irradiation of the products on a Spectroline Transilluminator (Model TR-302, Fisher Scientific). To calculate the activity of TpI, the plasmid bands were scanned on a Beckman Du 8B spectrophotometer. One unit is defined as the amount of protein which reduces the plasmid concentration by half.

High-performance liquid chromatography

Chromatography was performed in a Puffer-Hubbard (Asheville, NC, U.S.A.) cold box (2–6°C) except HPSEC, which was performed in the cold room at 4–6°C. An Altex (San Ramon, CA, U.S.A.) Model 344 liquid chromatography system was used with Model 114 solvent delivery pumps (operated in either isocratic or gradient mode) and a Model 210 injection valve. The system was controlled with a Model 421 system controller. All buffers were filtered through Millipore (Bedford, MA, U.S.A.) 0.45-μm HA-type filters.

High-performance anion-exchange chromatography

The flow-rate for high-performance ion-exchange chromatography (HPIEC) separations was 1 ml/min. Cellular extracts from either T47D or MCF-7 cells (0.5–1.0 ml) containing TpI activity and dialyzed against 10 m*M* phosphate, 1.5 m*M* EDTA, 1 m*M* DTT and 10% (v/v) glycerol (P_{10}EDG), were injected into a polyamine-coated SynChropak AX-1000 anion-exchange column (250 × 4.1 mm I.D.), obtained from SynChrom (Linden, IN, U.S.A.). The column was previously equilibrated with buffer A [P_{10}EDG or P_{10}EDG, containing 0.5% (v/v) 1-propanol]. Buffer B was the same as buffer A, except that the phosphate concentration was 500 m*M*.

The gradient elution program was as follows: The sample was injected at t = 0.5 min with 100% buffer A. The column was washed for 9.5 min with 100% buffer A, then the salt gradient was initiated. It approached 50% of B in the next 25 min. At t = 35 min the gradient was changed to give 100% of B in the next 10 min, and at t = 45 min B was held at 100% for 2 min. At t = 47 min, 100% A was delivered for the next 8 min to return to the original low salt conditions. Thus the total elution time was 55 min. Fractions (1 ml) were collected with an ISCO (Lincoln, NE, U.S.A.) fraction collector.

During the programmed elution time, the salt gradient was monitored with an in-line Bio-Rad conductivity monitor, which recorded the signal generated by the monitor on a Kipp & Zonen BD-41 chart recorder. The flow cell was directly calibrated with standard phosphate solutions, ranging from 10 m*M* to 500 m*M* phosphate concentration so that the approximate salt concentration of the eluted protein could be directly read[23].

Following HPIEC, the TpI activity was monitored as described earlier. Either aliquots (100 μl) from three successive tubes were mixed, dialyzed, and assayed for TpI activity, or every second fraction was analyzed. Later in the study, we determined that after HPIEC, dialysis leads to considerable loss of activity. Therefore, we avoided the dialysis step and assayed for TpI activity directly following HPIEC.

High-performance cation-exchange chromatography

This mode of HPIEC was performed in a manner similar to that described in the previous section, except that the silica based CM-300 column was used (SynChrom) and the mobile phase consisted of P_{10}EDG (buffer A) and P_{10}EDG plus 0.5 *M* sodium chloride (buffer B). Fractions (1 ml) were collected, and every second fraction was assayed for TpI activity. Conductivity was monitored in-line with the Bio-Rad conductivity monitor.

High-performance hydrophobic-interaction chromatography (HPHIC)

HPHIC was performed as previously described[23]. Briefly the active fractions obtained by HPIEC were directly injected (1 ml) into a SynChropak Propyl 500 column (SynChrom), previously equilibrated with mobile phase P_{500}EDG (buffer A). A reverse salt gradient was developed to reach P_{10}EDG in 30 min, and that salt concentration was maintained for an additional 30 min. Fractions (1 ml) were collected and assayed for TpI activity.

High-performance size-exclusion chromatography (HPSEC)

HPSEC was performed on a TSK 3000SW (60 × 0.75 cm I.D.) column (Toyo-Soda, Japan) previously equilibrated with P_{50}EDG containing 0.5% 1-propanol (like the P_{10} buffer previously described, except with 50 m*M* phosphate). Up to 1 ml of crude extract of T47D or MCF-7 cells or of the HPIEC fractionated TpI activity was loaded onto the column. HPSEC was performed at a flow-rate of 0.7 ml/min. The calibration of the TSK column with standard proteins provided a regression line with $r = 0.998$. The standard proteins utilized and their retention times were as follows: thyroglobulin, 12.5 min; ferritin, 15.5 min; catalase, 16.5 min; BSA, 19.0 min; cytochrome *c*, 24 min. The void volume of the column (V_0) was 8.4 ml, which corresponded to 12 min. Following HPSEC, 0.5 to 1.0 ml fractions were collected with a Buchler LC-100 fraction collector (Saddle Brook, NJ, U.S.A.). The first 7 ml of the effluent were discarded. Aliquots (5–10 μl) were then taken from every second fraction for TpI activity tests. The first fraction taken, therefore, represented tube 8 (1-ml fractions) or tube 16 (0.5-ml fractions).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[24] in 7.5% slab gels, and proteins were visualized by a highly sensitive silver-staining procedure, using a Pierce Gelcode staining kit according to the manufacturer's instruction.

Protein determination

Protein determinations were performed according to Bradford[25], using a Bio-Rad reagent and BSA as standard.

RESULTS AND DISCUSSION

Although several studies have aimed at understanding the physiological role of TpI, the function(s) of TpI remain(s) poorly understood. Recently it was demonstrated[26] that TpI in HeLa cells is bound in active nucleosomes, presumably in association with high-mobility-group proteins. This has renewed interest in the purification of this interesting enzyme to investigate its molecular and functional properties. Furthermore, reports that increased TpI activity was associated with cellular transformation[21,27] of normal cells prompted our efforts to understand the properties of topoisomerases. Because of its lability, a rapid and efficient purification of TpI will aid significantly in characterization of this important, regulatory protein.

The various reports of TpI purification involve conventional purification techniques such as open column chromatography in conjunction with salting out processes. For example, Ishii *et al.*[9] reported a procedure for the purification of TpI activity from mouse mammary carcinoma cells FM3A which required 16–48 h. To our knowledge, no attempt has been made to purify TpI by HPLC, which is capable of reducing the purification time substantially. This preliminary report describes our experience with the HPLC purification of TpI capitalizing upon its properties of size, shape, surface charge and hydrophobicity.

Our initial studies were directed toward analytical separation and detection of TpI activity by HPLC. It is our goal to scale-up to a preparative procedure so that

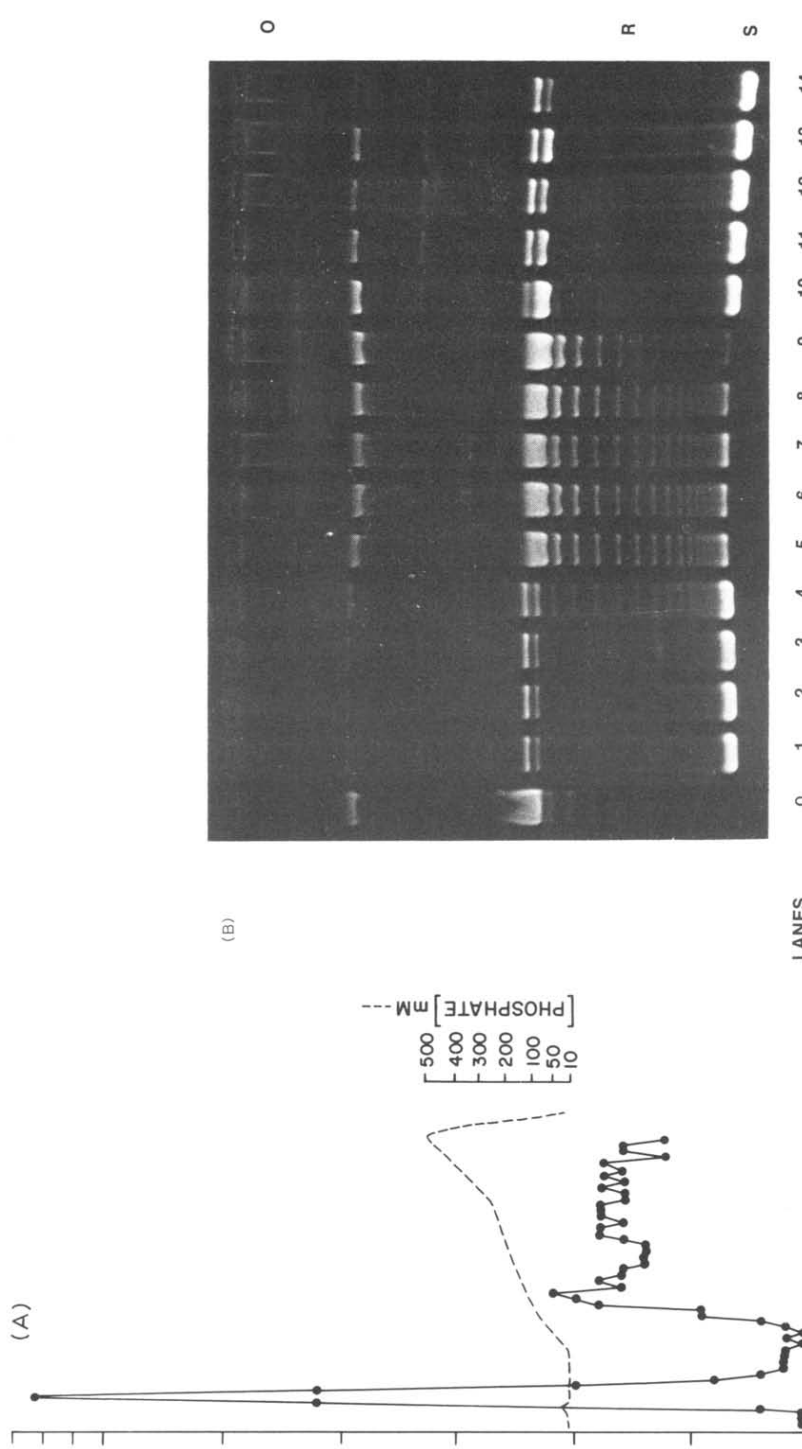

Fig. 1. HPIEC of TpI activity from T47D cells on a SynChrom AX-1000 anion-exchange column. Nuclear extracts from T47D cells in TKM were dialyzed against P_{10}EDG (pH 7.4). A dialyzed sample (1 ml) containing 1.34 mg protein, was injected onto an AX-1000 column and fractionated with a salt gradient from P_{10}EDG to P_{500}EDG, as described in Experimental. (A) Protein concentration and ionic gradient profile of a representative TpI extract on an AX-1000 column. Recovery of protein in this experiment was 98%. (B) Visualization of TpI activity, which had been eluted from an anion-exchange column and then analyzed on a 1% agarose gel. Lane 0 represents activity in the original extract. Lanes 1–14 show TpI activity in successive fractions, as indicated in the HPIEC separation. S = Supercoiled pBR322; R = relaxed DNA plasmid pBR322; O = origin.

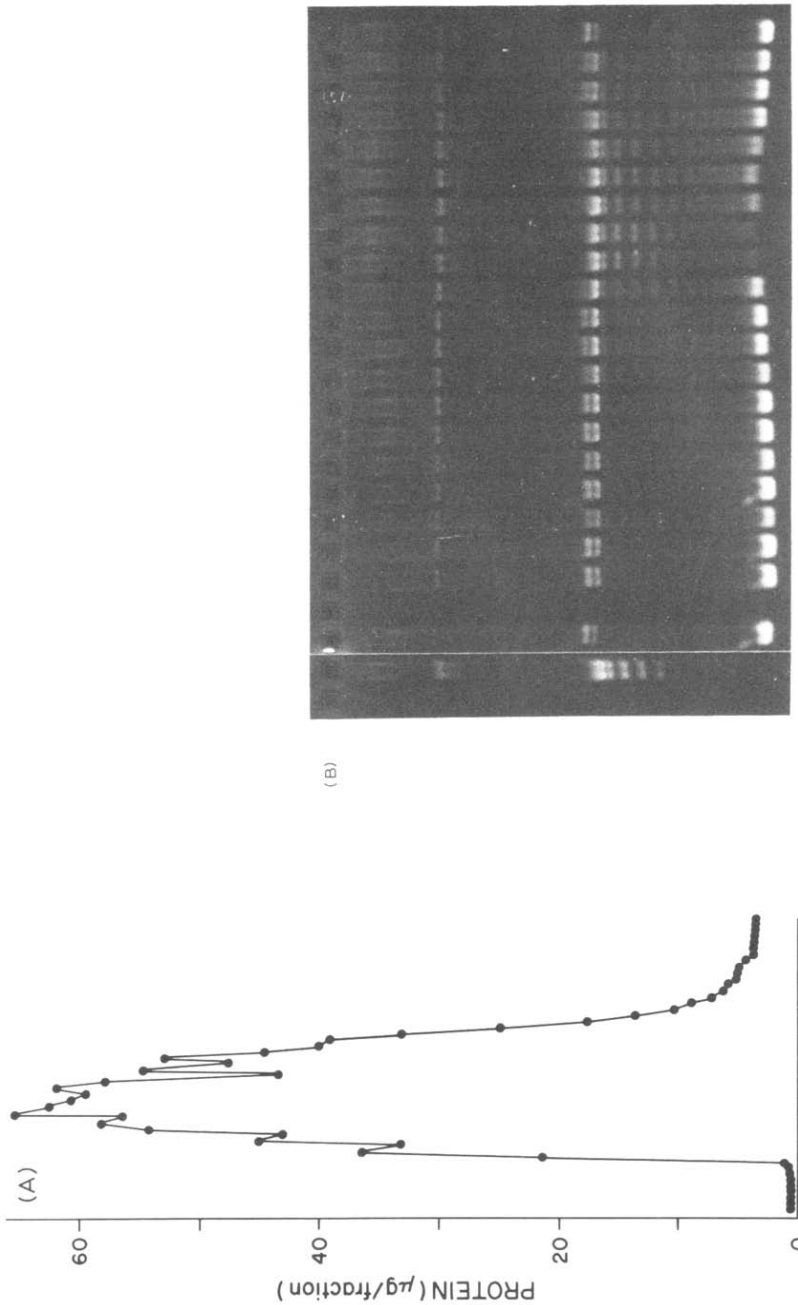

Fig. 2. HPSEC of Tpl activity from T47D cells. Extracts from T47D cells (0.5 ml containing 1.2 mg protein) were injected into a TSK 3000-SW column, equilibrated with P_{50}EDG containing 0.5% 1-propanol. Chromatography was performed at a flow-rate of 0.7 ml/min. Fractions (1 ml) were collected and tested for Tpl activity and protein content. (A) Protein concentration and ionic gradient profile of a representative Tpl extract on a TSK 3000SW column. Recovery of protein in this experiment was *ca.* 100%. (B) Visualization of Tpl activity which had been eluted from a TSK 3000SW column. The first seven 1-ml fractions were discarded, then starting with fraction 8 (void volume) every second fraction was analyzed. Lanes 1–20 represent the fractions from the HPSEC separation. Lane C_1 represents a 1:100 dilution of the original extract (10 μl), C_2 is the plasmid control.

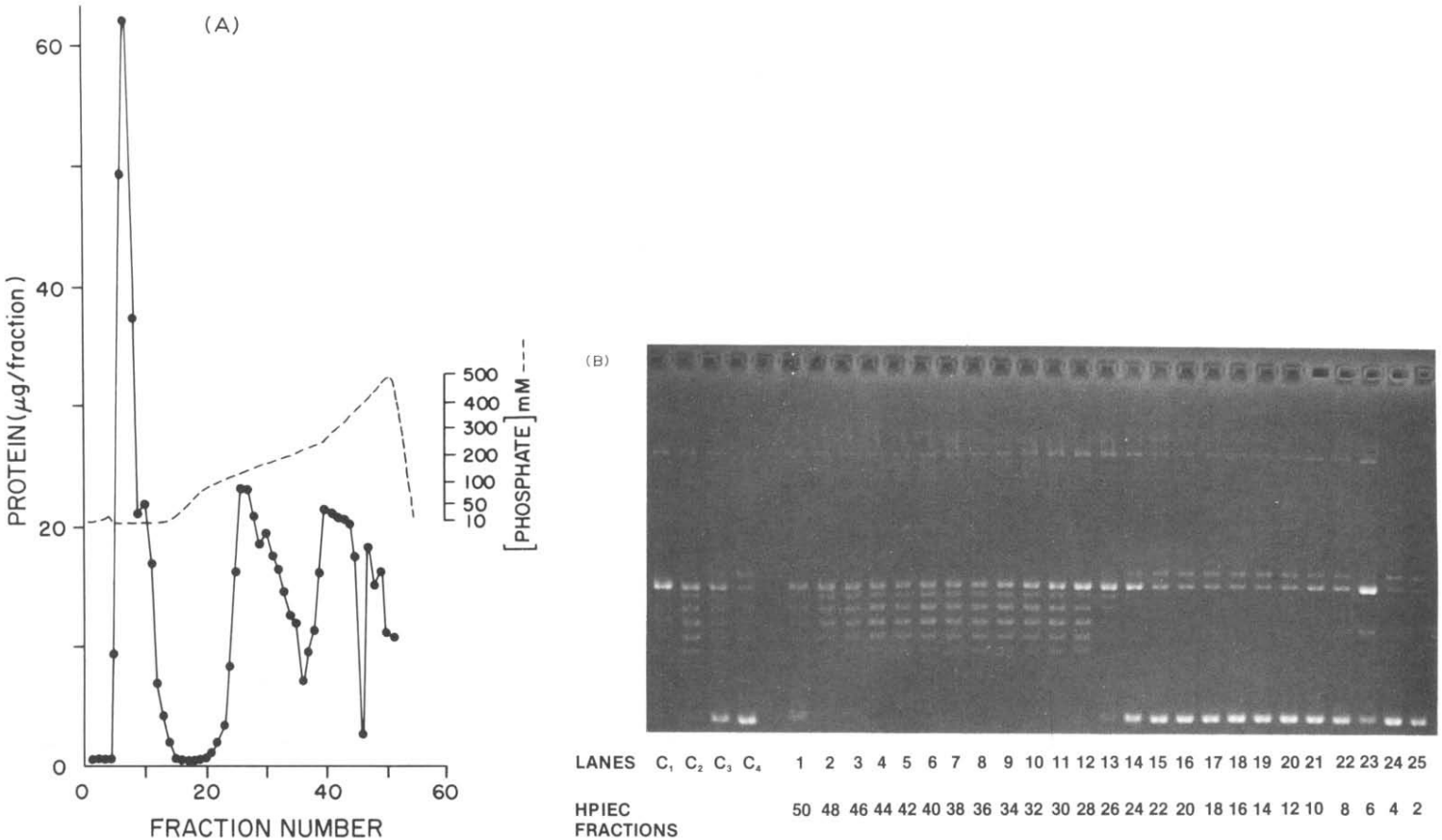

Fig. 3. HPIEC of TpI from T47D cells on an AX-1000 anion-exchange column. Extracts from T47D cells in TKM were dialyzed against P_{10}EDG, (pH 7.4). A dialyzed sample (1 ml, containing 0.78 mg protein) was injected into an AX-1000 column. Fractionation was performed with a salt gradient from P_{10}EDG containing 0.5% 1-propanol to P_{500}EDG containing 0.5% 1-propanol. (A) Protein and ionic gradient profiles of TpI extract on an AX-1000 column in the presence of 0.5% 1-propanol. Recovery of protein in this experiment was 89%. (B) Visualization of TpI activity in the effluent on 1% agarose gel (lanes 1–25). Indicated also are the HPIEC fractions to which each lane corresponds. Lane C_1 is the TpI activity from a 1:10 dilution of the original extract, C_2 is the activity from a 1:100 dilution and C_3 is the activity from a 1:200 dilution of the original extract. C_4 is the plasmid control.

large quantities of the enzyme can be purified. We report that, in contrast to the previously described procedure[9] requiring 16–48 h, HPLC technology provided similar results within 4–6 h.

Partial purification of TpI activity on an anion-exchange column

Purification of TpI activity from human breast cancer cells was investigated on a silica based, polyamine coated anion-exchange column (SynChrom AX-1000). Fig. 1A shows the separation of total protein under the programmed elution conditions. Recovery of protein from the column was 98%. The eluted fractions were tested for TpI activity. Fig. 1B shows the electrophoretic analysis of TpI activity eluted from the anion-exchange column. TpI activity was observed in lanes 5–9 with a maximum in lane 9, corresponding to fractions 28–30 of HPIEC (Fig. 1A). The activity profile (lanes 5–9) in Fig. 1B corresponded to salt concentrations of 150–250 m*M* phosphate. The total protein concentration in fractions 28–30 (Fig. 1A) was 6% of the total applied.

To enhance purification, we combined HPIEC fractions 28–30 (Fig. 1A) and injected 1 ml into a SynChrom Propyl-500 column. The separation on that column is based on the hydrophobicity of the protein molecule. An advantage of this form of chromatography is that the intermediate dialysis procedure, which in our hands had a deleterious effect on TpI activity, can be avoided. Hydrophobic interactions are promoted by high salt concentrations. Following injection of the HPIEC eluted sample into the column, a reverse salt gradient was developed, as previously described[23]. However, fractions eluted from the hydrophobic column did not exhibit any TpI activity (data not shown). Although we have not studied in detail the influence of hydrophobic matrices on TpI, we do not use HPHIC as a first step in TpI purification currently. Several explanations for the loss of TpI activity include (1) denaturation of TpI on the column, (2) irreversible binding of the protein to the stationary phase and (3) considerable dilution during chromatography resulting in loss of a co-factor.

Further attempts to purify the sample first separated by HPIEC with HPSEC on a TSK 3000SW column also failed (data not presented). This is surprising, since this mild type of separation usually preserves receptor activity[18].

Partial purification of TpI by HPSEC

Although HPSEC of most proteins is performed with a mobile phase of moderate ionic strength[18], some proteins may interact strongly with the stationary phase. Also moderate concentrations of organic solvents may be added to negate the hydrophobic effects. Such interactions often result in separations of proteins which are not based upon their molecular weight alone. It appears that the failure of TpI to elute from the TSK 3000SW column may have been the result of strong interactions between the protein and the stationary phase. However, loss of biological activity as a result of TpI interaction with the column cannot be ruled out since a time-dependent loss of TpI activity was noted in extracts left in contact with a phosphocellulose column[10].

Since HPSEC is performed under mild conditions and gave effective fractionation of various proteins such as the estrogen receptor[28], we examined conditions for optimal separation. Our earlier attempts to recover biological activity of TpI from

TABLE I

PURIFICATION OF DNA TpI FROM HUMAN BREAST CANCER CELLS

Fraction	*Total protein (μg)*	*Total activity (units)*	*Specific activity (units/μg)*	*Purification*
Original sample used in first purification step	780	50 000	64	–
Original sample stored for 24 h in 20% glycerol at −70°C	780	26 000	33	–
Fraction No. 46 from HPIEC (for 2nd purification step)	2.6	1000	385	6
Fraction No. 90 from HPSEC (Fig. 4)	0.5*	1000	2000	31 (61)**

* This is the assumed protein concentration, since the sensitivity of Bio-Rad is 1.0 μg/ml.

** Since the HPIEC fractions were stored for 24 h at −70°C in 20% glycerol prior to separation by HPSEC, this could result in loss of *ca.* 50% activity, as shown above. The purification value is therefore corrected and is shown in parenthesis, relative to the stored sample.

a HPSEC column with buffers such as P_{50}EDG or TKM followed by TKM containing 1% 1-propanol resulted in poor recoveries of enzyme activity. 1-Propanol was included in the mobile phase to decrease the hydrophobic effect of the column. Interestingly although 1% 1-propanol destroyed TpI activity, inclusion of 0.5% propanol in the mobile phase resulted in the elution of TpI activity (Fig. 2) with a retention time of 32 min. Cytochrome *c* (12.5 kD) under the same conditions eluted with a retention time, t_R, of 24 min, suggesting that TpI interacted with the column matrix to a large extent even in the presence of 0.5% 1-propanol. Analysis of HPSEC fractions by PAGE followed by silver staining (data not shown) indicated that high-molecular-weight proteins were still present in the fractions with retention times (t_R) ranging from 32 to 42 min. This indicates that many proteins were not fractionated according to their molecular weight (MW) but may have been eluted late due to interaction with the column.

Partial purification of TpI by HPIEC in the presence of 1-propanol

Since inclusion of 1-propanol in the mobile phase proved effective in the isolation of TpI activity by HPSEC, we analyzed its effect on the separation of TpI activity on the anion-exchange column (Fig. 3). Fig. 3B shows the agarose gel analysis of TpI activity eluted from an AX-1000 column. Although a narrow range of phosphate concentration was required for elution of TpI activity with the propanol-free mobile phase (Fig. 1), inclusion of the organic solvent lead to a broad separation of TpI activity, from 150 to 500 m*M* phosphate. Maxima in enzyme activity were ob-

served at 150–250 m*M* and 300–400 m*M* phosphate concentration suggesting molecular heterogeneity. The activity in fraction 46 (*ca.* 375 m*M* phosphate) was selected for sequential analysis by HPSEC. Representative purification of this fraction was approximately 6-fold compared to the non-fractionated sample (Table I).

Sequential purification of TpI activity by HPIEC–HPSEC

Following HPIEC (Fig. 3) the partially purified TpI activity (2–3 μg protein) was injected into a TSK 3000SW column and eluted with P_{50}EDG containing 0.5% 1-propanol. No protein was observed in the eluted fractions by the Bio-Rad protein method indicating the samples were below the limit of detection, *i.e.* 1 μg/ml. However, small amounts of TpI activity were present in several fractions, including 22, 30, 46, 58, but most of the activity was located in fraction 90, t_R = 75 min (Fig. 4). The main TpI activity (t_R = 75 min) eluted with a retention time more than twice that obtained when crude cytosol was analyzed under similar conditions (Fig. 2B). This may be due either to difference in the protein concentration or to the ionic strength of the sample injected into the HPSEC column.

When the HPSEC fractions eluting after the total column volume containing

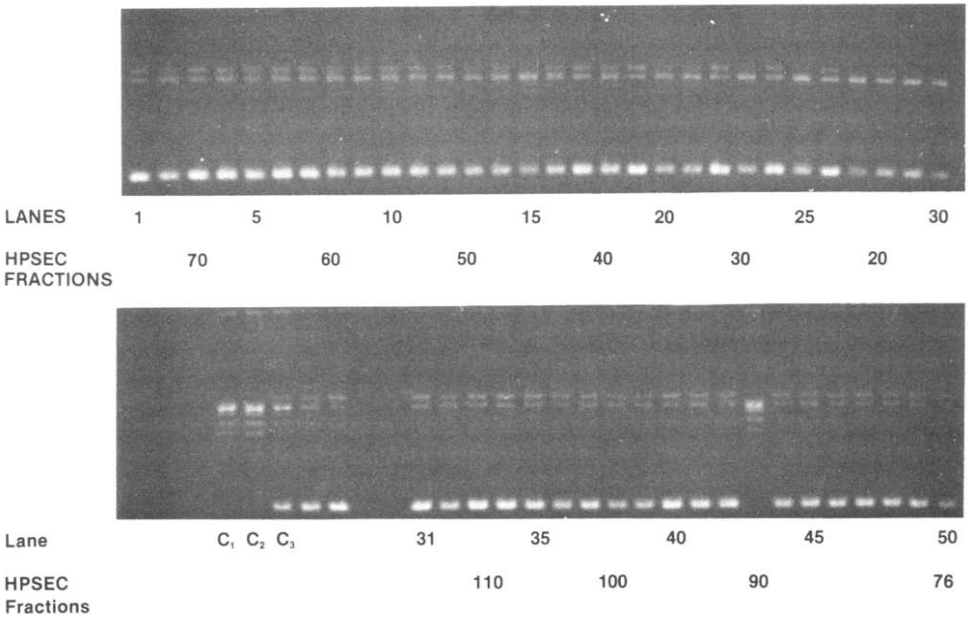

Fig. 4. HPSEC of a HPIEC fractionated (fraction 46, Fig. 3) containing TpI activity. Immediately following HPIEC and TpI activity analysis (within 3–4 h, Fig. 3B), the TpI active fraction (fraction 46; 1.0 ml containing 2.6 μg protein) was injected into a HPSEC column (TSK 3000SW), previously equilibrated with P_{50}EDG containing 0.5% 1-propanol. Fractionation was performed with the equilibration buffer at a flow-rate of 0.7 ml/min. Fractions (0.5 ml) were collected and tested for TpI activity and for protein content. No protein was detectable by the Bio-Rad assay in the HPSEC fractions. The TpI activity profile, visualized on 1% agarose gel by the plasmid relaxation assay, was conducted as described in Experimental. Lanes 1–50 represent TpI activity analysis following HPSEC. The corresponding HPSEC fraction numbers (0.5 ml each) are also indicated. TpI activity in C_1, C_2 and C_3 represent 1:10, 1:100 and 1:200 dilutions of the original extract, respectively.

TpI were analyzed by SDS-PAGE, with silver staining of the gels, no predominant protein bands were detected (data not shown). Again, this appears to be due to the protein concentration which is below the limit of detection of this method. An artifact in the silver-stained gels in the 60 kD range was observed (*e.g.* see Fig. 5C) as reported by others[19]. As shown in Table I, the protein was purified 30- to 60-fold, depending on whether the purity calculations was based on the activity of the original cytosol or on the stored sample. The latter showed a 50% loss in activity during overnight storage. The HPIEC fraction used for purification by HPSEC had been stored in 20% glycerol overnight. Although TpI activity was isolated by HPSEC and HPIEC, storage stability requires immediate processing. The reasons for its instability are under study.

Purification of TpI by HPLC in the cation-exchange mode

Whereas multistep HPLC provided a 30- to 60-fold purification of TpI activity, better separation and resolution of TpI activity was observed when HPLC was performed as a single step in the cation-exchange mode on a CM-300 column. As shown in Fig. 5A, when the crude extract containing TpI activity was fractionated on a

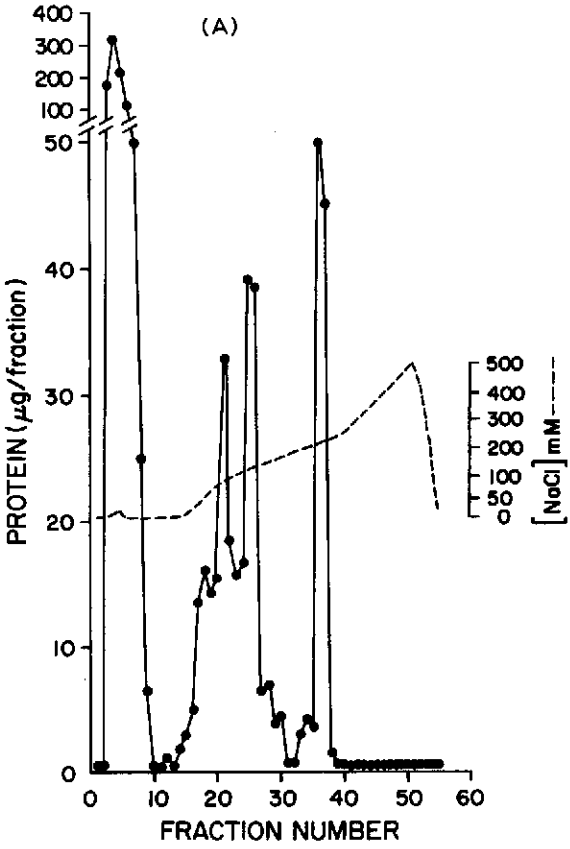

Fig. 5.

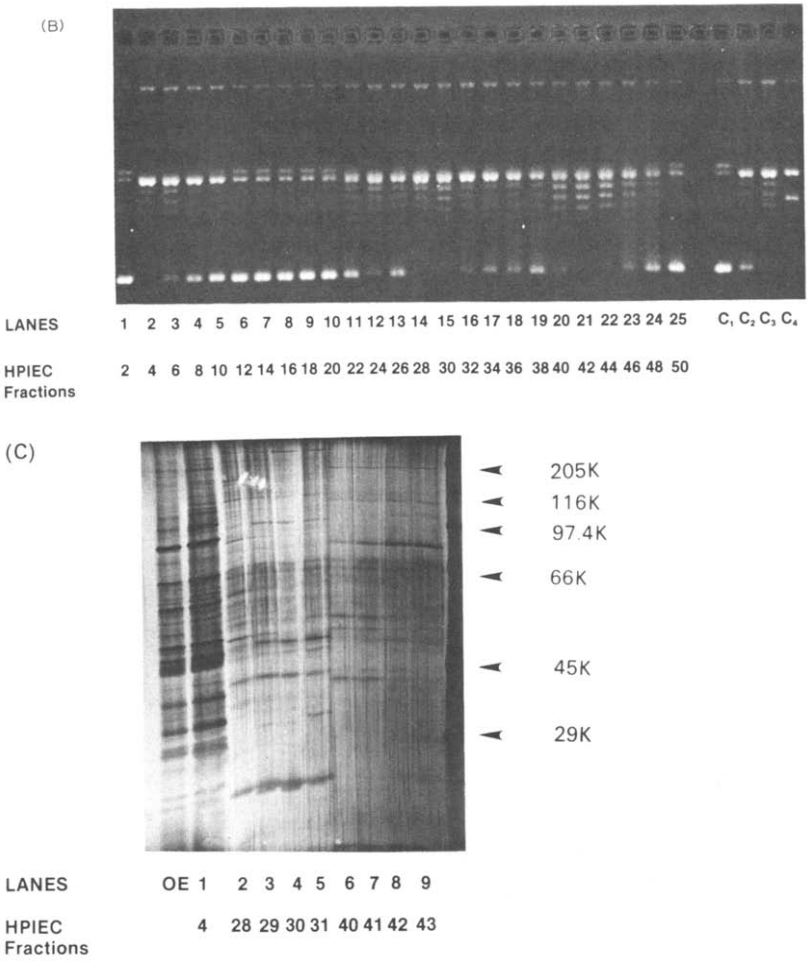

Fig. 5. HPIEC of TpI from MCF-7 cells on a CM-300 cation-exchange column. A nuclear extract from MCF-7 cells in TKM was dialyzed against P_{10}EDG, (pH 7.4). A dialyzed sample (1.0 ml containing 1.27 mg protein) was injected into a CM-300 column and fractionated as described in Experimental. (A) Protein concentration and the ionic gradient profiles of a representative extract chromatographed on a CM-300 column. Recovery of protein in this experiment was ca. 100%. (B) Visualization of TpI activity which had been eluted from an cation-exchange column and then analyzed on a 1% agarose gel. Lane C_1 shows a plasmid control, *i.e.* no sample was added. Lanes C_2–C_4 represent 1:200, 1:100 and 1:10 dilution of the original extract respectively. Lanes 1–25 represent TpI activity analysis of every second fraction eluted from the CM-300 column. (C) Silver stained polyacrylamide gel of HPIEC fractionated samples. Lane OE represents the original cytosol. Lane 1 represents fraction 4 (5 μg), lanes 2–5 represent HPIEC fractions 28–31 (*ca.* 0.2 μg) and lanes 6–9 represent fractions 40–43. In fractions 40–43 protein was not detectable by the Bio-Rad detection procedure.

CM-300 column, more than 75% of the applied protein was detected in the void volume.

TpI activity (Fig. 5B) was eluted in the void volume (fraction 4), at 150–200 m*M* phosphate, and fractions corresponding to 250–350 m*M* phosphate concentration. In the latter, no protein was detected with the Bio-Rad method which has a

TABLE II

PURIFICATION OF DNA TpI FROM HUMAN BREAST CANCER CELLS (MCF-7 ON CM-300 COLUMN)

Fraction	*Total protein (μg)*	*Total activity (units)*	*Specific activity (units/μg)*	*Purification*
Sample injected into a CM-300 column	1020.00	34 000	33.0	–
Void fraction (lane No. 2 on agarose gel)	312.00	3000	9.6	–
Fraction 28 (lane 14 on agarose gel)	6.75	2250	333.0	10
Fraction 42 (lane No. 21)	0.50*	2000	4000.0	121

* This value is assumed since the sensitivity of the Bio-Rad protein assay is 1.0 μg/ml. Therefore, the result may be subject to error resulting in an underestimate of purification.

sensitivity of 1 μg/ml. Table II shows that, following fractionation by CM-300, peak II (150–200 m*M* phosphate) was purified at least ten times relative to the original cytosol injected. However, peak III (250–350 m*M* phosphate) was purified at least 120-fold relative to the original cytosol on the assumption that there was a minimum of 500 ng protein in these fractions. This is an underestimation of the purity of the protein exhibiting TpI activity and a scale-up of the purification (in progress) should give a more accurate value. PAGE analysis of fractions containing TpI (Fig. 5C) showed a predominant protein band of 88–90 kD in the most highly purified TpI peak (250–350 m*M* phosphate, purity *ca.* 120-fold). TpI activity eluted at 150–200 m*M* phosphate showed several protein bands while the TpI peak eluted in the void volume showed heavy contamination with other proteins. Use of a CM-300 column as the first step in TpI purification provided a rapid means of processing this labile enzyme for further characterization.

CONCLUSION

We conclude that TpI from human breast cancer cells is a highly charged molecule, exhibiting binding to both anionic and cationic matrices. Further, TpI appears to display strong hydrophobic characteristics as judged by its separation on both size exclusion (TSK 3000SW column) and hydrophobic interaction columns (SynChropak Propyl-500). These properties may be expected of a chromatin-binding protein.

Sequential analyses using a combination of HPIEC (Synchrom AX-1000) and HPSEC (TSK 3000SW) gave 30- to 60-fold purification of TpI. However the greatest resolution and purification (at least 120-fold) was obtained with a single step using

the SynChrom CM-300 column. Analysis of the TpI activity isolated with the CM-300 column, provided a rapid method (4–6 h) of processing the labile enzyme for further characterization.

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