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Activation of topoisomerase II during partial purification by heparin–Sepharose chromatography

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ABSTRACT

Partial purification of topoisomerase II from small samples (10^7–10^8 cells) of human leukaemic cells was achieved by isolation of cell nuclei, hyper-osmotic extraction of nuclear proteins, sorption of nuclear proteins by heparin–Sepharose and elution with potassium phosphate. Similar results were obtained by gradient and batchwise elution. The calatylic activity of topoisomerase increased *ca.* eightfold after removal of *ca.* 95% of the contaminating nuclear proteins. The conserved enzymatic activity after partial purification indicates that the enzyme was not damaged. The half-life of enzymatic activity is increased by the chromatographic procedure. Owing to its high yield and technical simplicity, this could be a candidate procedure for the study of topoisomerase II in patient-derived blood samples.

INTRODUCTION

Recently, the analysis of DNA topoisomerase (topo) II has come into the focus of oncological interest because many potent anti-cancer drugs formerly regarded as DNA-intercalating or chromosome-fragmenting agents are now known to act as topo II inhibitors [1]. The function of this enzyme system appears to be to disentangle chromosomal DNA during replication and transcription by cleaving both strands of the DNA helix [2]. The enzyme forms a protein bridge across the ends of the divided DNA molecule until continuity is restored, but topo II inhibitors stabilize the DNA–protein complex so that the normally rapid process of strand division, disentangling and rejoining is arrested at mid-stage [3]. Much, if not all, of the cytotoxic acitivity of podophyllotoxins, anthracyclins, mitoxantrone, *m*-amsacrine and a number of other cytostatics is attributable to this specific interaction with topo II [1]. Two forms (α and β) have been recognized in mammalian cells, which are encoded by separate genes and differ biochemically but also with respect to drug sensitivity [4]. Resistance to topo II inhibitors is known to develop in both forms by point mutations in the 5′-triphosphate (ATP)-binding domain of the enzyme [5,6]. In addition, the drug sensitivity can be modulated by post-translational modifications, such as phosphorylation [7,8]. Recently, we observed that both forms of topo II, purified from a human leukaemic cell line, can be further fractionated by anion-exchange chromatography [9] and that the fractions differ considerably with respect to drug sensitivity and optimum reaction [10]. In the treatment of human leukaemia the pre-/peri-therapeutic study of drug sensitivity therefore necessarily includes the preparative fractionation of topo II and the separate analysis of the fractions. For this purpose, we have developed an extraction and partial purification procedure that might be suitable for preparing nuclear topo II from patient-derived samples of leukaemic cells in a routine clinical setting. The emphasis in this study is on the miniaturization and the optimization of a single step of heparin–Sepharose chromatography sufficient for preparing partially purified topo II

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from very small samples of leukaemic cells. The structural and functional characterization of this partially purified enzyme preparation is described.

EXPERIMENTAL

Preparations

The culture of human leukaemic HL-60 cells, isolation of nuclei and extraction of nuclear proteins by 400 m*M* NaCl was carried out as described previously [9]. All buffers for chromatography contained 10% glycerol, 100 m*M* NaCl, 12 m*M* β-mercaptoethanol, 0.5 m*M* phenylmethylsulphonyl fluoride, 1 μg/ml diisopropyl fluorophosphate, 0.1 m*M* benzamidine, 1 mg/ml soybean trypsin inhibitor and 0.5 m*M* EGTA. For heparin–Sepharose chromatography, prefilled 2 × 1 cm I.D. columns (HiTrap-Heparin, 1 ml; Pharmacia, Uppsala, Sweden) were used. Loading, washing and elution were performed at 4°C, using either an FPLC-System (Pharmacia) and a flow-rate of 1 ml/min or a Luer-lock syringe and a flow-rate of 3 drops/s. Usually, 5–7 ml of nuclear extracts were diluted 1:10 with ice-cold 5 m*M* potassium phosphate (pH 7.5) and immediately loaded on to the columns, followed by washing with 6 ml of 5 m*M* potassium phosphate. Bound proteins were either eluted with a 15-ml linear gradient from 5 to 600 m*M* potassium phosphate, as specified later. Eluted proteins were stored at −20°C after addition of concentrated glycerol to a final concentration of 60%. Further purification by gel chromatography was carried out using a 60 × 1.6 cm I.D. Superose 200 column (Pharmacia), which was equilibrated and developed with 50 m*M* Tris (pH 7.5) at a flow-rate of 1.7 ml/min. Fractions of 2 ml were collected and analysed.

Assays

Topo II catalytic activity was detected by ATP-dependent relaxation of 500 ng of pBR322 plasmid DNA as described previously [9,10]. It was determined by serial dilution of the enzyme and expressed as units. One unit was defined according to ref. 11 as the amount of topoisomerase that will give a 90% relaxation of 500 ng of pBR322 plasmid DNA under the described conditions [9]. As a control for topo II-specific activity, phage P4 DNA unknotting or decatenation of kinetoplast DNA was also measured [12,13].

Immunoblot analysis of topoisomerases was carried out as described previously [9]. Protein concentrations were determined according to Peterson [14], and the protein composition was studied by sodium dodecyl sulphate–polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE) [15], followed by silver staining [16].

RESULTS

Topo II from human leukaemic cells strongly binds to heparin-Sepharose at low salt concentrations. After loading the crude extract from *ca.* 10^9 nuclei on to a 1-ml HiTrap-heparin column, only traces of the enzymatic activity were found in the effluent and wash fractions, which contained *ca.* 95% of the nuclear proteins (Fig. 1). Topo II was eluted from the heparin column with 300–400 m*M* potassium phosphate as a single, sharp peak. Interestingly, the total recovery of activity usually significantly exceeded 100% (Table I), indicating that the enzyme is activated during chromatography. A similar result was also obtained when elution was carried out discontinuously, using 200 m*M* potassium phosphate for washing and 400 m*M* potassium phosphate for elution. As Fig. 2b shows (one of

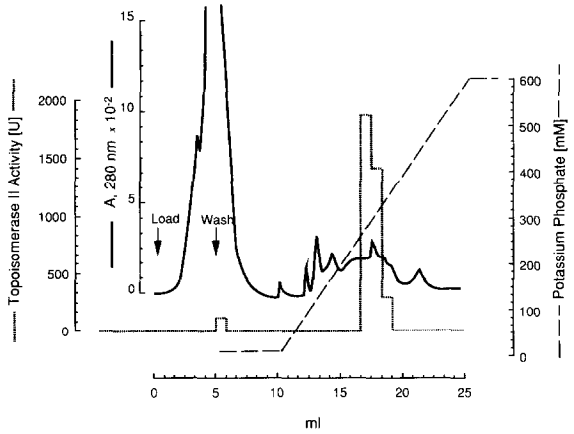

Fig. 1. Chromatography of crude nuclear extracts on heparin-Sepharose. A 5-ml portion of crude nuclear extract was loaded on to a 1-ml HiTrap-heparin column at a flow-rate of 1 ml/min. The column was washed with 5 m*M* potassium phosphate buffer (pH 7.5) and eluted with a 15-ml linear gradient from 5 to 600 m*M* potassium phosphate (pH 7.5). Fractions (1 ml) were assayed for pBR322 plasmid DNA relaxation activity in the presence of 2 m*M* ATP.

TABLE I

PARTIAL PURIFICATION OF TOPO II BY HEPARIN–SEPHAROSE CHROMATOGRAPHY

Mean results of four independent experiments ± standard error.

Fraction	Protein concentration (mg per 10^8 nuclei)	Specific activity of topo II (U/mg)	Recovery of topo II activity (%)
Crude nuclear extract	2.5 ± 1.6	96 ± 33	100
Heparin–Sepharose eluate	0.125 ± 0.078	15 667 ± 1385	816 ± 273

four similar experiments), large proteins (M_r 60 000–20 000) were preferentially recovered in the fraction eluted with 400 mM potassium phosphate.

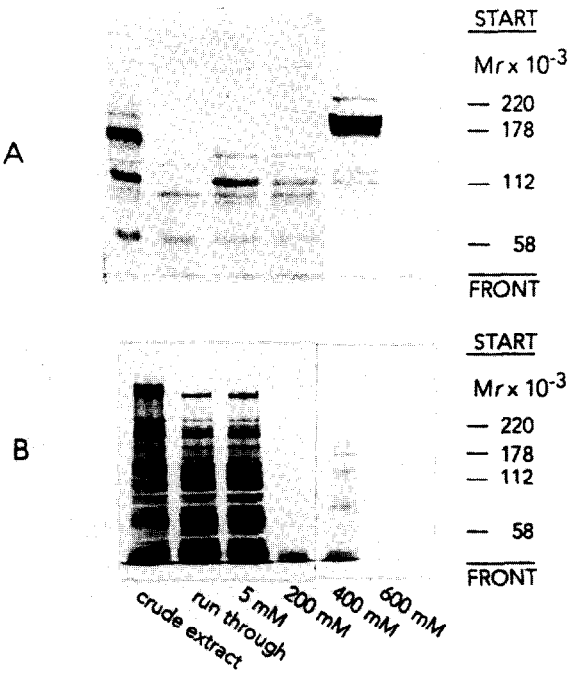

Fig. 2. Enrichment of topoisomerase II by a single-step batch chromatography on heparin–Sepharose. A 50-ml portion of crude nuclear extract, diluted tenfold with 5 mM potassium phosphate buffer (pH 7.5), was loaded on to a 1-ml HiTrap-heparin column with a syringe (3 drops/s). After washing with a 6-ml portion of the above buffer, bound proteins were eluted with 3-ml portions of potassium phosphate (pH 7.5, 200, 400 and 600 mM). Equal proportions of starting material, effluent, wash and eluted fractions were precipitated with 7.5% trichloroacetic acid and subjected to SDS-PAGE 7.5% gels. (A) Immunoblot analysis with rabbit–anti-human topoisomerase II antiserum and (B) silver staining of the precipitated proteins are shown.

By immunoblot analysis of the fractions (Fig. 2a) with a rabbit–anti-human topoisomerase II antiserum, a double band of M_r 170 000–180 000, equivalent to the known α- and β-forms of the enzyme [4], was recognized in crude nuclear extracts and in the fraction eluted by 400 mM potassium phosphate, but not in any of the other fractions. Crude nuclear extracts additionally contained various proteolytic fragments of topo II, which were also recognized by the antibody. These fragments did not bind to the heparin column or exhibited a weaker affinity than the intact enzyme, and they could be removed by washing with 200 mM potassium phosphate.

Titration of topo II pBR322 DNA relaxation activity is shown in Fig. 3 (one of four similar experiments). ATP-dependent DNA relaxation could be observed in crude extracts and in eluate fractions (400 mM potassium phosphate), but not in the effluent or in the wash fractions. The electrophoretic mobility of both relaxed and supercoiled pBR322 DNA was markedly reduced not only in the presence of high concentrations of crude extract or elution fraction but also in the 200 mM wash fractions (Fig. 3). The band-shift phenomenon indicates that some of the DNA-binding proteins present in crude extracts are removed during the chromatography. This might explain why the enzyme is at least eight times more active after removal of *ca.* 95% of the contaminant proteins. By the additive effect of activation and purification, a 160–200-fold increase in the specific activity is obtained in a single chromatographic step (Table I). The half-life of topo II activity in crude extracts was less than 3 days when stored at −20°C (in the presence of 50% glycerol), but it increased to 2 weeks after partial purification (not shown).

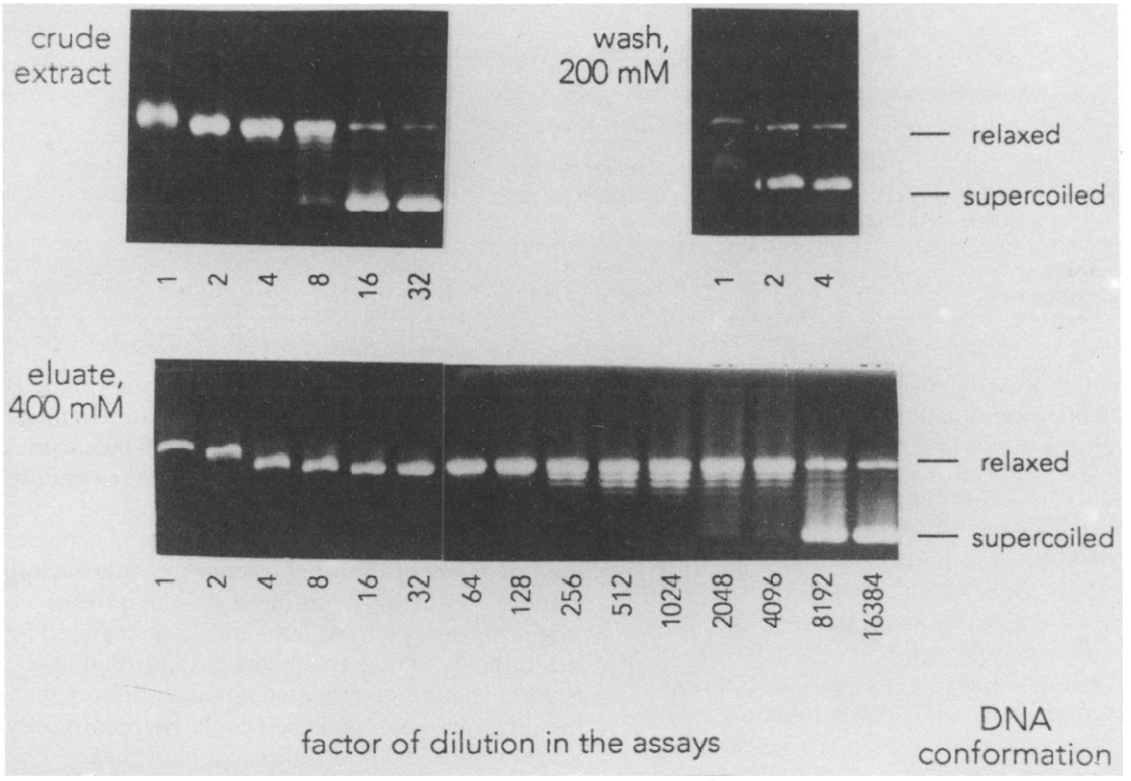

Fig. 3. pBR322 DNA relaxation activity of crude and partial purified topoisomerase II. Batch chromatography on heparin–Sepharose was caried out as described in the caption of Fig. 2. DNA relaxation activity was titrated by serially diluting the fractions prior to assessing the relaxation of 500 ng of pBR322 DBA. DNA conformers were separated by agarose gel electrophoresis and revealed by ethidium bromide staining. The results obtained with the crude extract and with the fractions eluted by 200 and 400 m*M* of potassium phosphate are shown.

DISCUSSION

A variety of purification methods have been described for topo II, all of which start from gram amounts of primary tissue [17,18] or litres of cultured cells [19,20] to obtain milligram amounts of homogeneous enzyme. The yield of these methods (from the cells to the homogeneous enzyme) is usually less than 5%. Whereas these procedures have been suitable for obtaining material for enzymological studies and for raising antibodies, they are of little use when the amount of starting material and/or analysis time are limited, as with patient-derived blood samples.

Studies of the effectiveness of topo II inhibitors in primary leukaemic cells require a certain degree of purification of the target protein for a number of reasons:

(i) As we have reported previously [10] and also shown here, DNA fragments and a variety of DNA-binding proteins present in crude nuclear extracts are potent inhibitors or modulators of topo II activity. The composition and concentration of these interfering substances varies from extract to extract. The determination of topo II activity in crude nuclear extracts is therefore very unreliable and prone to yield artifacts resulting from these variations in the simultaneously extracted nuclear protein matrix.

(ii) Hyperosmotic disintegration of the nucleus releases a high proteolytic potential, which can hardly be inhibited, even by elaborate preparations

of protease inhibitor. We have shown here that topo II quickly becomes degraded in crude nuclear extracts. Partial purification helps to remove proteolytic fragments and to increase the half-life of the enzyme activity.

(iii) We have previously shown [21] that nuclear topo II activity from leukaemic cells is highly dependent on the pH and salt concentration of the reaction medium. Moreover, these cells contain relatively low levels of topo II, so that the enzyme assay is not sensitive enough to allow dilution of the crude extract with the appropriate buffer sufficiently to optimize the assay conditions. Therefore, it is necessary to concentrate the sample prior to the enzyme assay.

The procedure described fulfils the above needs with a single-step heparin–Sepharose batch chromatography. By this procedure the enzyme activity is enhanced eight-fold and the protein concentration is decreased twenty-fold. The increase in specific enzyme activity is virtually 160-fold. Judging from immunoblot analysis, the partially purified enzyme is free from proteolytic degradation products.

This purification procedure is within the capability of a routine clinical application because it is fast (less than 3 h), relatively simple, highly reproducible (prefilled ready-to-use chromatographic columns are commercially available) and does not require complex laboratory equipment. It should be equally suitable for research applications, because it can easily be scaled up by using a larger size of the same brand of prefilled columns. A further purification of topo II to more than 50% homogeneity can be obtained by a single step of gel filtration or anion-exchange chromatography [9].

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