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Partial Purification of Topoisomerase I from *Mycobacterium phlei**

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Aim: DNA topoisomerases control several cellular activities by catalyzing the relaxation of superhelices, which are produced during the replication of DNA. Investigation of the inhibitory action of the candidate substances on topoisomerase activity is widely used in drug development. The purpose of this study was to purify topoisomerase I from *Mycobacterium phlei*, which is closely related to *Mycobacterium tuberculosis*, and investigate some of its properties.

Materials and Methods: Topoisomerase I from *Mycobacterium phlei* was partially purified with the method including homogenization followed by DNase treatment, Sephadex G50 and Heparin Sepharose column chromatography steps.

Results: The enzyme was purified 31.8-fold with a yield of 16.7%. Molecular weight of the enzyme was estimated to be 125 kDa. Enzyme activity was found to be stable in the pH range of 6.0 - 8.5. ATP and spermidine were not required for the activity of the enzyme. Camptothecin, which is an inhibitor of eukaryotic topoisomerase I, did not inhibit the enzyme at the concentrations studied.

Conclusions: The investigated properties of partially purified topoisomerase I from nonpathogenic strain *Mycobacterium phlei* were found closely related to those of *Mycobacterium tuberculosis*. Characterization of the enzyme that is completely purified with additional purification steps will hopefully lead to the development of selective antimycobacterial drugs.

Key Words: *Mycobacterium phlei*, DNA topoisomerase type I, purification, molecular properties

Mycobacterium phlei'den Topoizomeraz I'in Kısmen Saflaştırılması

Amaç: DNA topoizomerazlar DNA'nın replikasyonu sırasında ortaya çıkan süpersarmalın açılmasına aracı olarak birçok hücresel aktiviteyi kontrol ederler. Topoizomeraz inhibitör etkisinin araştırılması, yeni ilaç geliştirilmesinde yaygın olarak kullanılan bir yöntem haline gelmiştir. Bu çalışmanın amacı *Mycobacterium tuberculosis* ile yakın benzerliği olan *Mycobacterium phlei* topoizomeraz I'inin saflaştırılarak bazı özelliklerinin incelenmesidir.

Yöntem ve Gereç: Topoizomeraz I *Mycobacterium phlei*'den sonikasyonu takiben DNaz muamelesi, Sephadex G50 kromatografisi ve Heparin Sepharose kromatografisi yöntemleri kullanılarak kısmen saflaştırılmıştır.

Bulgular: Enzim % 16.7 verimle, 31.8 kez saflaştırılmıştır. Enzimin molekül ağırlığı 125 kDa olarak tesbit edilmiştir. Enzim aktivitesinin pH 6.0 - 8.5 arasında stabil olduğu, aktivite için ATP ve spermidine gerek duymadığı gözlenmiştir. Ökaryotik topoizomeraz I'in inhibitörü olan kamptotesin tarafından, çalışılan konsantrasyonlarda enzim inhibe edilmemiştir.

Sonuç: Patojen olmayan *Mycobacterium phlei*'den kısmen saflaştırılan enzimin incelenen özelliklerinin *Mycobacterium tuberculosis*'e benzer olduğu bulunmuştur. Daha ileri saflaştırma basamaklarının ardından elde edilecek enzimin incelenmesi antimikobakteriyel ilaçların gelişimine yarar sağlayabilecektir.

Anahtar Sözcükler: *Mycobacterium phlei*, DNA topoizomeraz tip I, saflaştırma, moleküler özellikler

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Introduction

DNA topoisomerases control and modify the topological states of DNA. They are ubiquitous enzymes catalyzing the relaxation of superhelices produced during the replication of DNA. Topoisomerases have mainly two forms (type I and type II). Topoisomerase I breaks a single DNA strand, while topoisomerase II breaks both strands and requires ATP for full activity (1-3). In recent years, topoisomerases gained importance as the target of several antibacterial and anticancer drugs. Investigation of the inhibitory action of the candidate substances on topoisomerase activity is widely used in drug development (4-7).

Topoisomerase I was purified and studied extensively from several prokaryotic sources including *Escherichia coli,* which is the prototype prokaryotic enzyme (8-9), *Micrococcus luteus* (10), *Diplococcus pneumoniae* (11), thermophilic archeabacterium *Desulfurococcus amylolyticus* (12), *Fervidobacterium islandicum* (13), *Rhodobacter capsulatus* (14), *Mycobacterium smegmatis* (15,16), and *Mycobacterium tuberculosis* (17).

According to the World Health Organization (WHO) global report of 2006, 9 million people are newly diagnosed with tuberculosis every year (18). In Turkey 18,000 to 20,000 tuberculosis patients have been reported annually (19). Furthermore, the spread of multidrug resistant (MDR) strains continuing for decades worldwide and emergence of a new form termed as extensively drug resistant (XDR) tuberculosis will worsen the situation (20). This can only be stopped by discovery of new drugs. Since those bacteria easily gain resistance to known antibiotics, the development of new drugs is urgently needed (20).

This study aimed to purify topoisomerase I from a nonpathogenic strain, *Mycobacterium phlei,* and to investigate some of its properties. Characterization of the topoisomerase I from *Mycobacterium phlei*, which has not been published elsewhere, and comparison of these properties with the previously published properties of *M. smegmatis* and *M. tuberculosis* enzymes and eukaryotic topoisomerases may hopefully lead to the development of selective drugs against tuberculosis, which would contribute to the solution of an important health problem.

Materials and Methods

Material: Middlebrook 7H9 broth was obtained from Difco (USA), Heparin Sepharose 6 Fast Flow from Amersham Biosciences (Uppsala, Sweden), and Sephadex G50 from Sigma Chemical Company (USA). pBluescript plasmid was isolated from *E. coli* XL1-blue strain culture by alkaline lysis technique and plasmid concentration was determined spectrophotometrically (21). All other chemicals were of analytical grade.

Bacterial strain and growth conditions: *M. phlei* ATCC 11758 strain was grown in Middlebrook 7H9 broth, supplemented with 0.3% Tween-80, 0.5% glycerol, 0.005% oleic acid, 0.5% bovine serum albumin (BSA), 0.2% dextrose, 0.0004% bovine liver catalase, and 0.085 NaCl for three days at 37°C, with shaking. Bacteria in the initial log phase (4 days of culture at 37°C in a shaking incubator) were harvested by centrifugation at 20,000 x g for 20 min.

DNA topoisomerase activity measurement: Relaxation activity of DNA topoisomerase I was measured as described by Stewart and Champoux (22) with some modifications. The reaction was performed in 20 µl of reaction mixture containing 40 mM Tris-HCl buffer (pH 8.0), 20 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 50 µg/ml BSA, 40 µg plasmid DNA and 10 µl of enzyme sample. Mixtures were incubated at 37°C for 30 min. The reaction was stopped by the addition of 5 µl electrophoretic loading solution consisting of 100 mM EDTA, 60% sucrose, 6% sodium dodecyl sulfate (SDS), and 0.05% bromophenol blue (pH 8.0).

Agarose gel electrophoresis was used to follow the enzyme activity (23). The reaction products were subjected to 1.0% agarose gel electrophoresis in a buffer consisting of 40 mM Tris, 20 mM glacial acetic acid, and 1 mM EDTA (pH 8.0) at 110 V for 2 h. Gels were stained with ethidium bromide (1 µg/ml) and photographed under UV light. Band distribution was analyzed with a GDS 8000 Complete Gel Documentation and Analysis System (Gel Works 1D Intermediate, version 2.5; Ultra Violet Products). The change in the density of the fastest moving band during the incubation period was used as a measure of the enzyme activity. At each enzyme measurement, parallel assays were run with eukaryotic topoisomerase I of known activity.

Protein concentration at different stages of purification was estimated by the Bradford method (24), using BSA as standard. Polyacrylamide gel electrophoresis (PAGE) with or without SDS was carried out according to Laemmli (25).

Unit definition: Known units of eukaryotic topoisomerase I (*Calf thymus,* Takara Bio Inc) were used as a standard for the calculation of the mycobacterial enzyme activity units. Under the assay conditions, eukaryotic topoisomerase I activity was linear up to 0.1 units. One unit of eukaryotic topoisomerase I activity was defined by the producer as the enzyme that completely relaxes 0.5 µg of supercoiled pBR322 DNA in 50 µl of the reaction mixture (35 mM Tris-HCl, pH 8.0; 72 mM KCl; 5 mM $MgCl_2$; 5 mM DTT; 5 mM spermidine; 0.01% BSA) for 30 min at 37°C (http://bio.takara.co.jp).

Purification of topoisomerase I: All steps were performed at + 4°C and the samples were stored on ice. The cells were suspended in Buffer A [50 mM Tris-HCl, pH 8.0, 10% glycerol (w/v)], 50 mM NaCl, 1 mM EDTA, 0.5 mM phenyl methyl sulfonyl fluoride (PMSF), and 10 mM β-mercaptoethanol (ME), and vortexed for 4 x 1 min with 0.5 mm glass beads (0.5 g/ml). The cells were kept on ice for 1 min at each interval.

Deoxyribonuclease (DNase) I was added to the homogenate (0.11 mg of DNase I / ml homogenate) and the mixture was incubated at + 4°C for 30 min followed by centrifugation at 20,000 x g for 40 min.

The supernatant was loaded onto a Sephadex G50 column (90 cm x 2.6 cm) equilibrated with Buffer A at a flow rate of 20 ml/hour. Active fractions were loaded onto a Heparin Sepharose column (3.0 cm x 2.6 cm) equilibrated with the same buffer at a flow rate of 10 ml/hour. The column was washed with 130, 50 and 25 ml Buffer A containing 150 mM, 300 mM and 600 mM NaCl, respectively. Fractions eluted after each step were combined separately, concentrated and desalted before measurement of topoisomerase I activity.

Results

M. phlei topoisomerase I was purified 31.8-fold with a yield of 16.7%, with a method employing homogenization followed by DNase I treatment, Sephadex G50 and Heparin Sepharose column chromatography steps. Specific activity of the partially purified enzyme was calculated as 126.32 U/mg (Table).

Elution profile of Sephadex G50 column is shown in Figure 1. The enzyme was eluted in the void volume. The inset shows the relaxation activity of the fractions. Figure 2 shows the elution profile of the Heparin Sepharose column. Topoisomerase I was eluted from the column at 600 mM NaCl. The activity was assayed after concentration and desalting of the combined fractions. The electrophoregram showing the topoisomerase activities at each step is shown in Figure 3.

Molecular weight of the partially purified enzyme from *M. phlei* was estimated to be 125 kDa by SDS PAGE (Figure 4).

The effect of pH on the enzyme activity was investigated between pH 7.0 and pH 9.0. The enzyme activity was quite stable between pH 7.0 and 8.2 (Figure 5).

One mM ATP did not change the activity of the partially purified enzyme, and the DNA relaxation activity of the enzyme was not inhibited by 2 mM spermidine and 4.2-42.0 µg camptothecin per unit of enzyme.

Discussion

In this study, partially purified topoisomerase I was obtained from *M. phlei*. In the early purification steps, nuclease activity interfered with the topoisomerase activity (Figure 3, lanes 2 and 3) and the specific activity of the homogenate could not be calculated. This has also been reported by other authors (12,14,26). Specific activity of the enzyme after Sephadex G50 and Heparin

Table . Purification of *M. phlei* topoisomerase I.

Purification Step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Sephadex G50 Chromatography	35	0.696	24.360	2.76	96.60	3.97	1.0	100.0
Heparin Sepharose Chromatography	6.7	0.019	0.127	2.40	16.08	126.32	31.8	16.7

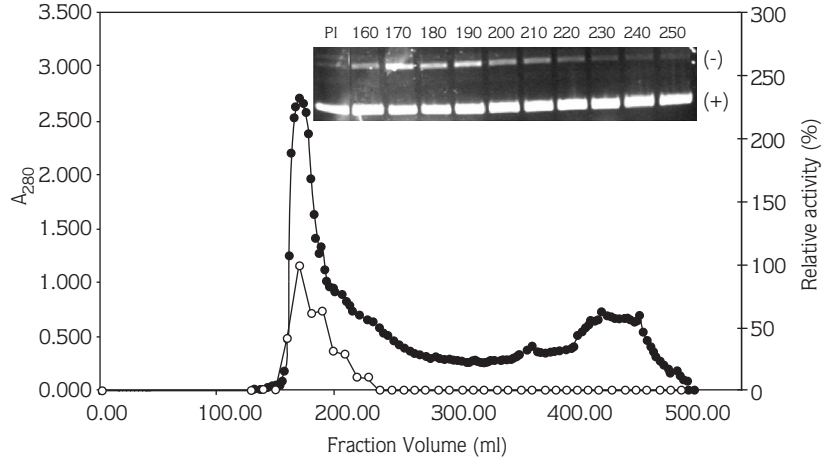

Figure 1. Elution profile of Sephadex G50. Column size, 90 cm x 2.6 cm; flow rate, 20 ml/h. (●): A_{280} measurements, (❍): % relative activity. Inset: Relaxation activity of the fractions. Percentage relative activity was calculated by assigning 100% to the peak activity.

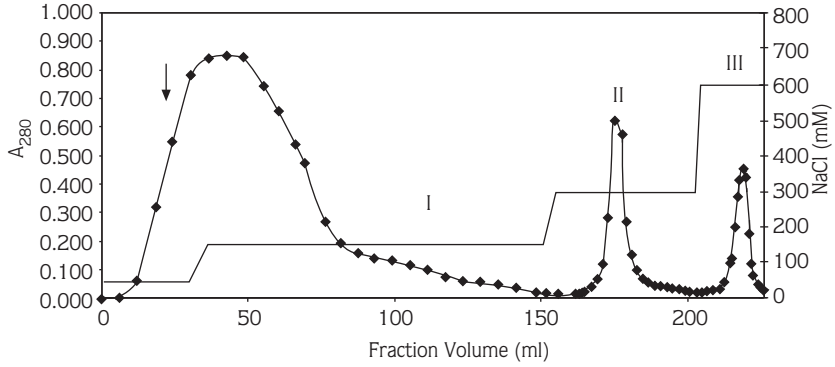

Figure 2. Heparin Sepharose column stepwise NaCl elution profile. Column size, 3.0 cm x 2.6 cm; flow rate, 10 ml/hour. ◆: A_{280} measurements. Peak III shows topoisomerase I activity after concentration and desalting. The arrow indicates the starting point of stepwise NaCl elution.

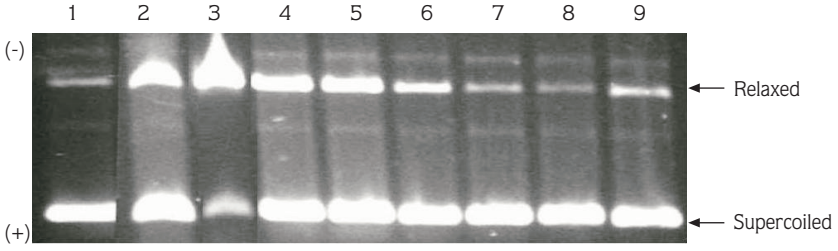

Figure 3. Agarose gel electrophoresis of purification steps. Lane 1: plasmid; lane 2: homogenate; lane 3: homogenate after DNase I incubation; lanes 4,5: peak of Sephadex G50 void; lane 6: sample loaded onto the Heparin Sepharose column. Lanes 7-9: Heparin Sepharose column elution with 150 mM, 300 mM and 600 mM NaCl, respectively, after concentration and desalting. Relaxation assay and electrophoresis were performed as described in Materials and Methods.

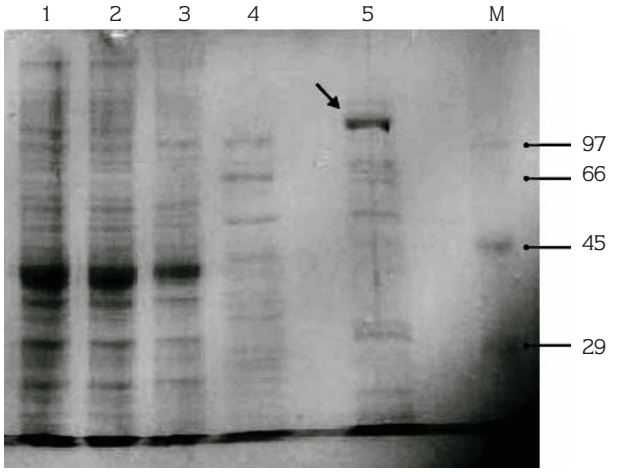

Figure 4. SDS PAGE analysis of different purification steps. M: Molecular weight markers: phosphorylase B 97 kDa (1.0 µg), BSA 66 kDa (1.5 µg), egg albumin 45 kDa (1.5 µg), 29 kDa carbonic anhydrase (1.0 µg). Lane 1 (5 µg total protein): homogenate, lane 2 (5 mg total protein): homogenate after DNase I treatment, lane 3 (5 µg total protein): sample that is loaded onto Heparin Sepharose column. Lanes 4 (0.8 µg total protein) and 5 (0.8 µg total protein) indicate Heparin Sepharose column elutions with 300 mM and 600 mM NaCl, respectively. The arrow indicates probable topoisomerase I.

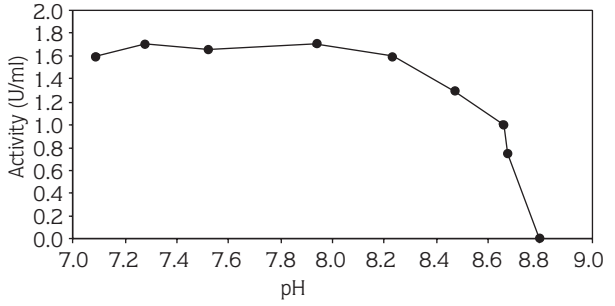

Figure 5. The effect of pH on topoisomerase relaxation activity. The relaxation activity was assayed in 40 mM Tris-HCl buffer between pH 7.0 and pH 9.0.

Sepharose chromatography steps was 3.97 and 126.32 U/mg protein, respectively, which makes up a 31.8-fold purification. Thus, excluding the initial homogenization step, the overall yield is calculated as 31.8% (Table). Total activity of the enzyme after Sephadex G50 and Heparin Sepharose chromatography steps was 96.60 and 16.08 U, respectively, which gives an overall yield of 16.7% (Table). The low yield is most likely due to the presence of bacterial proteases, which gradually degrade the enzyme.

Prokaryotic type I topoisomerases have molecular weights ranging from 75 to 112 kDa (8,12,27,28). The gene encoding *M. tuberculosis* topoisomerase I has been cloned and the molecular weight of the expressed protein was found to be 99.353 kDa (17). The molecular weight of the topoisomerase I purified from *M. smegmatis* was 110 kDa (16), which is similar to our result. On the other hand, several low molecular weight bands in addition to the 125 kDa protein were observed in SDS-PAGE (Figure 4, lane: 5). These bands are thought to be the proteolytic products of the enzyme.

The activity of the partially purified enzyme was stable over a wide range of pH. It has been reported that *M. smegmatis* topoisomerase I activity is also stable in a wide pH range from 7.0 to 8.5 (15), and our finding is in agreement.

Type I topoisomerases can relax supercoiled DNA in the absence of ATP, but type II topoisomerases require ATP. In our study, partially purified enzyme activity did not change with the addition of 1 mM ATP to the reaction mixture. This result is in accordance with the previously published reports (13-16,28). Prokaryotic type I topoisomerases do not require ATP for relaxation activity (22). With this result, we also concluded that the enzyme was not contaminated with type II topoisomerase.

Spermidine stimulates the relaxation activity of eukaryotic type I topoisomerases and inhibits *E. coli* topoisomerase I relaxation activity (29), but does not affect the activity of *M. smegmatis* topoisomerase I (16). We observed that 2 mM spermidine in the assay mixture was ineffective on the DNA relaxation activity of partially purified topoisomerase I from *M. phlei*, which is closely related to *M. smegmatis*.

Camptothecin is an inhibitor of eukaryotic topoisomerase I, but does not affect the activity of prokaryotic topoisomerase I. In our study, camptothecin did not inhibit the partially purified enzyme over a wide range of concentrations. These results are in agreement with the published data (16).

This paper describes the preliminary data for purification of type I topoisomerase from *M. phlei.* Further purification and characterization of mycobacterium topoisomerase I may hopefully lead to the development of selective antimycobacterial drugs, which would contribute to the treatment of tuberculosis and to the solution for this important health problem.

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