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A Simplified Protocol for High-Yield Expression and Purification of Bacterial Topoisomerase I

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Abstract

Type IA topoisomerases represent promising antibacterial drug targets. Data exists suggesting that the two bacterial type IA topoisomerase enzymes—topoisomerase I and topoisomerase III—share an overlapping biological role. Furthermore, topoisomerase I has been shown to be essential for the survival of certain organisms lacking topoisomerase III. With this in mind, it is plausible that topoisomerase I may represent a potential target for selective antibacterial drug development. As many reported bacterial topoisomerase I purification protocols have either suffered from relatively low yield, numerous steps, or a simple failure to report target protein yield altogether, a high-yield and high-purity bacterial topoisomerase I expression and purification protocol is highly desirable. The goal of this study was therefore to optimize the expression and purification of topoisomerase I from *Streptococcus mutans*, a clinically relevant organism that plays a significant role in oral and extra-oral infection, in order to quickly and easily attain the requisite quantities of pure target enzyme suitable for use in assay development, compound library screening, and carrying out further structural and biochemical characterization analyses. Herein we report the systematic implementation and analysis of various expression and purification techniques leading to the development and optimization of a rapid and straightforward protocol for the auto-induced expression and two-step, affinity tag purification of *Streptococcus mutans* topoisomerase I yielding >20 mg/liter of enzyme at over 95% purity.

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Supplementary Material: Data supporting the preliminary characterization of *SmtopI16b* is included as supplementary material. This includes a mini expression SDS PAGE analysis comparing IPTG to auto-induction method for expression (Figure S1), *E. coli* catalase contaminant identification on SDS PAGE analysis (Figure S2), peptide fingerprinting data used to properly identify the target enzyme (Figure S3), freeze trials conducted to analyze enzyme activity after different methods of storage including freezing and basic refrigeration (Figure S4), analysis of enzyme activity after cleavage of the His-tag (Figure S5), DNase contaminant analysis (Figure S6), and thermal shift data from the Hampton Research Solubility and Stability Screen 2™ kit and protocol (Figure S7).

Authors' Contributions: K.E.H. and J.A.J. designed all experiments and analyzed the data. K.E.H. provided laboratory space and resources. J.A.J., E.P., and D.M. conducted the experimental studies. K.E.H. performed the protein comparative homology modeling. J.A.J. wrote the manuscript.

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Keywords

Type IA topoisomerase; topoisomerase I; *Streptococcus mutans*; purification; characterization; autoinduction

Introduction

Viridans Group Streptococci (VGS) are represented by a plurality of heterogeneous streptococcal organisms that constitute a common component of the human microbiome found primarily within the oral cavity and upper respiratory tract, as well as in the gastrointestinal tract and female genitourinary tract.^{1,2} With historically low consideration for pathogenicity, VGS have been increasingly implicated in a wide array of problematic diseases such as endocarditis, pneumonia, cellulitis, bacteremia, and viridans streptococcal shock syndrome (VGSS), among others.^{1,3} Moreover, VGS stand as the predominant group of microorganisms within the oropharynx, with a member of the group, *Streptococcus mutans*, representing the main culprit in dental cariogenesis.⁴ Considering the vast clinical and economic burden now known to be caused by VGS, the necessity for effective antibacterials targeting these pathogens has escalated. While the majority of VGS infections are still susceptible to penicillin, the proliferation of drug-resistant VGS organisms, including those resistant to penicillins, macrolides, and lincosamide antibiotics, is becoming more of a concern.^{1,2} Additionally, as the beneficial bacteria that cohabitate the usual sites of VGS residence are concomitantly eradicated by the traditional use of these broad-spectrum antibacterials, significant collateral consequences such as candidiasis and *Clostridium difficile* infections have increased over time.⁵⁻⁷ Therefore, novel targets for new VGS-focused antibacterials are in dire need.

One such potential target lies within the topoisomerase family of enzymes. Topoisomerases are ubiquitous enzymes that manage the topological complications DNA undergoes during biological processes and, accordingly, are indispensable in their duties and represent highly attractive and efficacious chemotherapeutic targets. Up to four topoisomerases can be present in the prokaryotic genome, but only two (the type IIA topoisomerases, gyrase and topoisomerase IV) have inhibitors that have been developed and marketed as antibacterial drugs. While these type IIA topoisomerases stand as well-established and well-validated antibacterial targets via the quinolone class of topoisomerase poisons (which form bactericidal topoisomerase-DNA-quinolone ternary complexes), the type IA topoisomerases (topoisomerase I and III) remain relatively unexplored and without respective approved inhibitors.⁸ Current efforts are being undertaken, however, to discover novel bacterial topoisomerase IA inhibitors.⁹ Unlike the ATP-dependent type IIA topoisomerases, which cleave both strands of DNA to introduce strand passage and relieve or generate supercoiling, the type IA topoisomerases are ATP-independent and cleave only one strand of DNA to produce strand passage and relieve hyper-negative supercoiling or decatenate interlinked DNA.⁸ Also unlike type IIA topoisomerases, which are heterotetramers encoded by multiple genes (*gyrA/B* and *parC/E*), type IA topoisomerases are monomers encoded only by single genes—*topA* for topoisomerase I and *topB* for topoisomerase III (Figure 1).^{8,10}

As our understanding of the human microbiome evolves, the deficiencies in traditional, broad-spectrum antibacterial therapy are becoming increasingly apparent. Both the advent of multi-drug resistant bacterial infections and the devastating disturbance of our normal bacterial ecology testify of the alarming need for novel antibacterial agents capable of selectively targeting pathogens, drug-resistant or otherwise, while mitigating the impact on beneficial and commensal organisms. With evidence supporting the bacterial essentiality of at least one type IA topoisomerase (topoisomerase I or topoisomerase III), an overlapping role between the two enzymes, and the fact that a select few bacterial species solely possess topoisomerase I without topoisomerase III, the catalytic inhibition of bacterial topoisomerase I represents an attractive prospect for novel, selective antibacterial development.^{8,11-14} The streptococci are one of the few bacterial species that possess topoisomerase I as their sole type IA topoisomerase and a representative of the VGS, *Streptococcus mutans*, exists as a particularly promising candidate for the exploration and validation of topoisomerase I as a novel, selective antibacterial target.¹⁵⁻²¹

As expected, inherent difficulties are involved with the purification of DNA-binding proteins such as bacterial topoisomerase I. The initial need to separate cellular DNA from the target enzyme introduces an early obstacle in the protocol. To overcome such an obstacle, previous methods including treating extracts to batch anion exchange, DNase treatments, and DNA precipitations using different compounds like streptomycin sulfate and polyethyleneimine have been employed.²² While each DNA-separation method has its own advantages and disadvantages, they all share a certain degree of costliness with respect to time and resources. Previous studies regarding the expression and purification of bacterial topoisomerase I have yielded varied results. While truncated N-terminal fragments of *E. coli* topoisomerase I have been well studied and characterized, the full-length enzyme is desirable for thorough characterization and inhibitor development.^{23,24} In one study, the purification and analysis of full-length topoisomerase I from the extremophile *Thermotoga maritima* was carried out (expressed in *E. coli* with IPTG induction; heated to precipitate non-thermophile proteins and DNA; purified using a heparin column followed by size exclusion chromatography) and yielded 2-3 mg/Liter.^{25,26} However, information gleaned from an extremophile would not necessarily be expected to provide data that could be reliably applied to clinically relevant bacteria. Another study detailed the purification of full-length topoisomerase I from *Streptococcus pneumoniae* (expressed in *E. coli* with IPTG induction followed by dialysis, IMAC purification, and a second dialysis step), but did not report yield.²⁷ Despite their noteworthy contributions to our understanding of bacterial topoisomerase I, these studies either failed to report the amount of protein yielded from their respective protocols, or the yield was relatively low, making biophysical and biochemical assay studies logistically difficult. A study developing the purification of topoisomerase I from *E. coli* yielded a significant amount of the enzyme, but would not be appropriate for expressing and purifying topoisomerase I from non-*E. coli* bacteria as the protocol lacked a method for separating non-native enzyme from native.²⁸ More recent studies discussed the purification of full-length *E. coli* and *Mycobacterium tuberculosis* topoisomerase I used for the subsequent crystallization and characterization of the respective enzymes, but did not report the yields.^{29,30}

Herein we report the expression, rapid two-step purification, and preliminary characterization of topoisomerase I from *Streptococcus mutans* with yields significantly greater than previously reported purifications of bacterial topoisomerase I. Notably, the following expression protocol requires significantly less effort than classical induction methods and the purification protocol bypasses traditionally cumbersome DNA-removal methods and can be carried out in a single day with an estimated purity of 95% or higher. We also report our work to date regarding the development of biophysical and biochemical topoisomerase I assays.

Material and Methods

Reagents, chemicals, biologicals, and equipment

Biological materials, equipment, and reagent-grade chemicals used were from the following sources: *Streptococcus mutans* genomic DNA, ATCC[®] 700610D-5[™] from ATCC[®] (Manassas, VA); ArcticExpress(DE3) Competent Cells, pUC18 plasmid, XL1-Blue Supercompetent cells, and BL21(DE3)-Gold Competent cells from Agilent Technologies (Santa Clara, CA); Champion[™] pET SUMO Protein Expression System, including One Shot[®] Mach1[™]-T1[®] and BL21(DE3) One Shot[®] Chemically Competent *E. coli* cells from invitrogen[™] by Life Technologies[™] (Carlsbad, CA); HyperLadder[™] 1kb Plus from Bioline USA Inc (Taunton, MA); pET-15b vector, pET-16b vector, and pET-21d vector from Novagen[®] EMD Millipore[®] (Billerica, MA); Quick Ligation[™] Kit, BamHI, NdeI, and NcoI restriction enzymes from New England Biolabs, Inc. (Ipswich, MA); Difco[™] LB, Miller and Terrific Broth from BD[™] (Franklin Lakes, NJ); Thermo Scientific[™] GelCode[™] Blue Safe Protein Stain and Spectra[™] Multicolor High Range Protein Ladder from Thermo Fisher Scientific (Waltham, MA); HiMark[™] Pre-stained Protein Standard, Novex[™] InVision[™] His-Tag In-Gel Stain, Platinum[®] PCR SuperMix High Fidelity, NuPAGE[®] Novex[®] 3-8% Tris-Acetate Protein Gels, Novex[™] NuPAGE[™] LDS Sample Buffer (4×), and Novex[™] NuPAGE[™] Tris-Acetate SDS Running Buffer and Molecular Probes[™] SYPRO[™] Orange Protein Gel Stain from Invitrogen[™] by Life Technologies[™] (Carlsbad, CA); QIAGEN Plasmid Maxi Kit, QIAquick PCR Purification Kit, and QIAprep Spin Miniprep Kit from Qiagen (Valencia, CA); DTT, glycerol, IPTG, lysozyme, NaCl, MgCl₂, protease inhibitor tablets (EDTA-free), Tris, Tris base, and Triton X-100 from Fisher Scientific (Pittsburgh, PA); DNase I from Worthington Biochemical Corporation (Lakewood, NJ); ampicillin, kanamycin, and KH₂PO₄ from Acros Organics, part of Thermo Fisher Scientific (Waltham, MA); 30,000 MWCO Amicon[™] Ultra-15 Centrifugal Filter Units and Imidazole from EMD Millipore[®] (Billerica, MA); K₂HPO₄ from Sigma Aldrich (St. Louis, MO); GelRed[™] nucleic acid gel stain from Biotium, Inc. (Hayward, CA); primers synthesized by Integrated DNA Technologies (Coralville, IA); Hampton Solubility & Stability Screen 2[™] from Hampton Research (Aliso Viejo, CA).

ÄKTA Purifier FPLC, HisTrap-HP 5 mL column, and HiLoad 26/600 Superdex 200 PG size exclusion column from GE Healthcare Life Sciences (Pittsburgh, PA).

Buffers

The mini-expression lysis buffer is composed of 75 μ L of 1% n-octyl beta-D-thioglucofuranoside (OTG) in 20 mM Tris HCl (pH 7.5), 0.1 mg Lysozyme, 0.1 mg DNase I, and 0.5 μ L of 0.5 M $MgCl_2$ for one cell pellet. The 2 $\times$ LDS dye is composed of 5 mL Novex™ NuPAGE™ LDS Sample Buffer (4 $\times$), 500 mM DTT, and brought to 10 mL with ddH₂O. The standard lysis buffer is composed of 20 mM sodium phosphate (pH 7.4), one Pierce® EDTA-free protease inhibitor tablet per 50 mL, 1 mM DTT, 0.5% Triton X, 0.5 M NaCl, 5% glycerol, 0.5 mg/mL lysozyme, 5 μ g/mL DNase, and 5 mM imidazole. Lysis buffer 2 is composed of standard lysis buffer without DNase I. Buffer A is composed of 20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 5 mM imidazole, and 5% glycerol. Buffer B is composed of 20 mM sodium phosphate (pH 7.4), 0.15 M NaCl, 250 mM imidazole, and 5% glycerol. The size exclusion buffer is composed of 50 mM sodium phosphate (pH 7.4) and 150 mM NaCl. Buffer C is composed of buffer A with 1M NaCl. Buffer D is composed of buffer B with 0.5M NaCl and 350 mM imidazole. Size exclusion buffer 2 is composed of 20mM Tris (pH 8.0), 1 mM $MgCl_2$, 0.5M NaCl, and 1 mM DTT. The gel assay buffer and assay stop buffer were prepared as previously reported.³¹ The gel assay buffer is composed of 10mM Tris pH 8.0, 50mM NaCl, and 6 mM $MgCl_2$. The assay stop buffer is composed of 50mM EDTA, 50% glycerol, and 0.5% w/v bromophenol blue.

Cloning and Plasmid Construction

Initially, the *topA* gene was cloned from *Streptococcus mutans* UA159 genomic DNA for insertion into the pET-15b vector based for the NdeI and BamHI restriction sites using the following primers:

TopA_Foward: 5'-TGT AGA CAT ATG ACA AGT AAA ACA ACG ACA ACA-3'

TopA_Reverse: 5'-TCA TCA GGA TCC TTA TTT AAC AGC TTT TTC CTT-3'

PCR was carried out via Platinum® PCR SuperMix High Fidelity protocol and denatured at 94 °C for 30 seconds, annealed at 58 °C for 40 seconds, and extended at 68 °C for two minutes for 30 cycles and then held at 4 °C. Success was analyzed via 1% agarose gel electrophoresis based upon the confirmed presence of the ~2Kbp SmTopA_pET PCR product. SmTopA_pET was then purified using QIAQuick® PCR Purification Kit per protocol. The pET-15b vector was then digested with NdeI and BamHI restriction enzymes per New England Biolabs (NEB) protocol, purified, then mixed and ligated with the SmTopA_pET PCR product via NEB Quick Ligation™ Kit protocol. The ligation product (SmTopA15b) was then transformed into XL1-Blue Supercompetent cells per protocol. Upon successful transformation, the plasmid was isolated with the QIAprep Spin Miniprep Kit via protocol and the sequence was confirmed via Retrogen, Inc. (San Diego, CA). The plasmid was also transformed into BL21(DE3)-Gold Competent cells via protocol.

The *topA* gene was sub-cloned from the pET-15b vector in XL-1 Blue into the pET-16b vector using NdeI and BamHI restriction enzymes and NEB Quick Ligation™ Kit per manufacturer protocols.

For the pET 21d vector, the SmTopA gene was amplified via PCR around NcoI and BamHI restriction sites using the following primers:

NcoI_Site_For: TAT ACG ATG GGC AGC AGC CAT CAT C

NdeI_Site_For: GGC AGC CCC ATG GCA AGT AAA AC

BamHI_Site_Rev: TTC GGA TCC TTT TTA ACA GCT TTT TCC TTA TAG
TCA CAC

PCR was carried out via Platinum® PCR SuperMix High Fidelity protocol and denatured at 94°C for 30 seconds, annealed at 58°C for 45 seconds, and extended at 68°C for two minutes and thirty seconds for 35 cycles and then held at 4 °C.

For the pET-SUMO vector, the *topA* gene was cloned from PCR with “hanging T” ends per Champion™ pET SUMO Protein Expression System protocol using the following primers:

TopA_SUMO_For: AGC ACA AGT AAA ACA ACG ACA ACA GTA AAA
AAG ACG

TopA_SUMO_Rev: TTA TTT AAC AGC TTT TTC CTT ATA GTC ACA
CTC CTT ATT GC

PCR was carried out via Platinum® PCR SuperMix High Fidelity protocol and denatured at 94°C for 30 seconds, annealed at 60°C for 45 seconds, and extended at 68°C for two minutes and thirty seconds for 35 cycles and then held at 4 °C.

All gene and respective plasmids were ligated and transformed per respective protocols per respective protocols into XL1-Blue Supercompetent cells and BL21(DE3)-Gold Competent cells except the SmTopA_SUMO plasmid used with the Champion™ pET SUMO Protein Expression System, which utilized included One Shot® Mach1™-T1^R and BL21(DE3) One Shot® Chemically Competent *E. coli* cells. Sequences were confirmed by sequencing via Retrogen, Inc. (San Diego, CA).

Mini-expression of SmTopI5b

A mini-expression was initially conducted in order to determine preliminary expression conditions, starting with a 5-mL LB and 100 ug/mL starter culture of *SmTopI5b* in BL21(DE3)-Gold grown at 32°C overnight. Upon the next day, the sample was diluted 100-fold into three 5-mL sterile TB and 100 ug/mL ampicillin samples for each of three different temperatures: 37°C, 25°C, and 18°C. Cultures were then grown for two hours, then one sample from each temperature was induced with 1 mM IPTG, one with 0.5 mM IPTG, and one remained un-induced. Samples were then grown an additional twenty-four hours, then centrifuged at 10,000 rpm for five minutes. The supernatant was then removed and each pellet was dissolved in Mini_Lysis_Buffer for five minutes. Cells were re-suspended and vortexed for 30 seconds to completely dissolve and the samples were centrifuged at 13,000 rpm for five minutes. The supernatants were then separated into clean 1.5 mL microcentrifuge tubes as the soluble fractions. Pellets were washed by re-suspending in 30 uL of 1% OTG in 20 mM Tris (pH 7.5) and centrifuging at 13,000 rpm for five minutes. The supernatant was then discarded. Pellets were once again re-suspended in 30 uL of 1% OTG

in 20 mM Tris (pH 7.5) and mixed with 30 μ L of LDS_Sample_Buffer, then centrifuged again at 13,000 rpm for five minutes with the resulting supernatant containing the insoluble fraction. 10 μ L samples were then heated at 95°C for five minutes and loaded on SDS-PAGE gels for analysis. Mini-expression was repeated for 48-hour growths as well. (Data not shown.) Mini-expression was again repeated with *SmTopI16b* at 18°C.

Expression of *SmTopI* constructs and cell lysis

Expression and purification experiments were conducted in duplicate. *SmTopI15b* initial starter cultures were grown in BL21 cell lines at 32°C overnight in 5-mL LB with 100 μ g/mL ampicillin (pET constructs) or 50 μ g/mL kanamycin (SUMO construct) and used to inoculate 500mL TB with respective antibiotics. Cultures were grown to OD600 ~0.6, induced with 0.5 mM IPTG, and expressed at 18°C for 24 hours. Auto-induction was carried out per Studier protocol with *SmTopI16b* and grown for 24 hours at 37°C.³² All samples were then centrifuged at 15,000 g for 30 minutes at 4°C and the supernatants were discarded. Pellets were stored at -80°C.

Cell pellets for all constructs were thawed at room temperature and immediately lysed in the standard lysis buffer by stirring for 1 hour at 4°C. Samples were then sonicated for 8 minutes, centrifuged at 18K rpm at 4°C for 15 minutes, filtered at 0.22 μ m, and collected. Auto-induction samples were lysed in the same fashion, except lysis buffer 2 was used, and then samples were sonicated and filtered as above.

Purification of *SmTopI* constructs

Each initial construct sample was purified using a HisTrap-HP 5 mL column on an ÄKTA Purifier FPLC. Samples were loaded at 1 mL/min, washed with 20 column volumes (CVs) Buffer A at 2.5 mL/min, and eluted via linear gradient from 100% Buffer A to 100% Buffer B over 20 CVs at 2.5 mL/min. Samples were then collected and concentrated using a 30,000 MWCO Amicon™ Ultra-15 Centrifugal Filter Unit. The *SmTopI16b* sample was collected and loaded onto a GE HiLoad 26/600 Superdex 200 PG size exclusion column and eluted with 1.5 CV of SE Buffer. The sample expressed via auto-induction (*SmTopI16b_AI*) was purified similarly, except it was loaded and washed with Buffer C for 40 CVs and eluted with a step gradient of Buffer D using 7CV steps at 15%, 20%, and 100% with the sample eluting in a sharp peak in the 100% step. The sample was then loaded onto a pre-equilibrated GE HiLoad 26/600 Superdex 200 PG size exclusion column and eluted with SE Buffer 2. All sample concentrations were attained via Bradford assay with BSA as the standard.

Biochemical Assays

Gel assays were used to confirm target protein activity using published methods.³¹ The pUC18 plasmid was isolated via QIAGEN Plasmid Maxi Kit per protocol. Enzyme assays were conducted in standard volumes of 20 μ L comprised of 25, 50, 75, 100, 150, 200, or 400 ng *SmTopI*; 500 ng negatively supercoiled pUC18 plasmid; and a sufficient quantity of gel assay buffer to bring the reaction volume to 20 μ L. One unit of commercially obtained *E. coli* topoisomerase I was substituted for *SmTopI* as a positive control. Assays were incubated at 37°C for 30 minutes and then terminated using 5 μ L of assay stop buffer per reaction. DNA relaxation activity was monitored on 0.7% agarose gels in Tris-Acetate-

EDTA TAE buffer at 20V for 10 hours and then stained with GelRed™ nucleic acid gel stain per protocol. Freeze trials were conducted similarly using 1 ug (high concentration) or 400 ng (low concentration) of enzyme in 50% glycerol or 0% glycerol and either snap-frozen in liquid nitrogen, frozen drop-wise in liquid nitrogen and stored at -80°C overnight, or simply refrigerated. Non-specific DNase activity assay was conducted using published methods and incubated for 6 hours.²⁸

Thermal Stability Assay

Protein stability and solubility conditions were analyzed via the use of Hampton Research Solubility and Stability Screen 2™ per protocol.

Peptide fingerprinting

Target protein identity was confirmed by standard in-gel tryptic digest and LC-MS analysis.³³⁻³⁵ Peptide spectra matching and protein identification was achieved by database search using Sequest HT algorithm in Proteome Discoverer 1.4 (Thermo Scientific).

Protein Homology Modeling

A comparative homology model of the *SmTopI* protein was built using the *E. coli* (3PX7) and *T. maritima* (2GAI) topoisomerase I crystal structures as templates, the *Streptococcus mutans* (UA159 strain) protein sequence, and the program MODELLER.^{36,37} The model was refined by MD simulation using the program Amber.³⁸

Results

Cloning and Expression of recombinant *SmTopI*

Considering the need to rapidly isolate *SmTopI* from host *E. coli* topoisomerase I, cloning into a poly-histidine affinity tag system was chosen and implemented. The *S. mutans topA* gene was first cloned and inserted into the pET15b vector (*SmTopA15b*), introducing a hexa-Histidine (hexa-His) tag at the N-terminus of the target enzyme.³⁹ The target gene was also cloned or sub-cloned into several alternate vectors in order to analyze the effects of different constructs, including affinity tag location and size, as well as the usefulness of a protein fusion tag. BL21(DE3)-Gold Competent cells were used for the over-expression of target protein. The target gene was also transformed into the ArcticExpress Competent Cell system to evaluate the utility of expressing at lower temperatures (12°C) in order to increase solubility, but was not found to significantly improve results in this instance (data not shown).^{40,41}

Preliminary mini-expression results from the *SmTopI15b* construct led to a traditional IPTG induction and expression that was implemented across all constructs in duplicate. Expression products were then carried into initial immobilized metal-ion affinity chromatography (IMAC) nickel column purification trials using a standard linear imidazole elution protocol. While *SmTopI16b* from the N-term deca-His construct and *SmTopI-SUMO both* significantly outperformed the others, *SmTopI16b* was ultimately selected and carried forward (Table 1). Of note, as the *SmTopI-SUMO* enzyme approached the customary 100-kDa *E. coli* expression cutoff, the additional 11 kDa in size added by the SUMO fusion tag

showed no apparent deleterious effect on the expression in this respective system, demonstrated by the average calculated amounts of expressed recombinant protein from the construct trials resulting in 88.9 pmol for the ~83 kDa *SmTopI16b* and 100 pmol for the ~93 kDa *SmTopI-SUMO*, respectively.

As further loss of target protein was anticipated with additional purification steps, different conditions were also employed in order to further increase target protein expression and convenience, including the implementation of auto-induction for protein expression.³² Upon transitioning from a classical IPTG-induction method to a standard auto-induction method, the yield of *SmTopI16b* increased dramatically, as did the convenience of the protocol (Table 1).³² A subsequent IPTG mini-expression profile of *SmTopI16b* was also conducted in order to determine favorable IPTG expression conditions, which was also compared to auto-induced expression and illustrated no apparent loss of solubility from adopting an auto-induction method (Figure S1).

Purification of recombinant *SmTopI*

Early exploratory nickel-nitrilotriacetic acid (Ni^{2+} -NTA) column IMAC purification with the pET15b N-term hexa-His tag system showed the affinity of the tagged target protein for the Ni^{2+} -NTA column to be markedly weak, leading to both an unacceptable loss of target protein during wash steps and very early dissociation upon elution. Furthermore, it was discovered upon peptide fingerprinting analysis that what was initially presumed to be the ~83 kDa *SmTopI15b* target protein upon SDS-PAGE analysis was actually the ~84 kDa native *E. coli* catalase HP11 protein co-eluting with the target protein (Figure S2).

Comparative analysis of chromatographic linear elution profiles from the *SmTopI* IMAC purification trials showed a slight increase in the retention time for the deca-His tagged target protein relative to the hexa-His tags (Figure 2). Therefore, the precise point of elution of *SmTopI16b* from the linear gradient was observed to be at 25% elution buffer (about 90 mM imidazole), and this aspect was used to develop a step elution protocol. Upon implementation of a 15%, 20%, 100% step elution protocol, *SmTopI16b* eluted in a sharp peak at the 100% elution step and was successfully separated from contaminating proteins, including *E. coli* catalase HP11, as determined by SDS-PAGE analysis and His-staining (Figure 3). As such, the pET16b N-term deca-His tag system was deemed the most successful and carried forward for further experimentation.

While analyzing mini-expression data, it was determined that target protein solubility could likely be improved. A simple transition from 50 mM Tris-HCl pH 8.0 to 20 mM phosphate buffer pH 7.4 in the lysis and nickel column buffers showed an apparent increase in yield and increasing the ionic strength of the nickel column binding and wash buffers (from 150 mM to 1 M NaCl) and the nickel column elution buffer (from 150 mM to 500 mM NaCl) greatly increased the overall purification yield of *SmTopI16b* (Table 1 and Figure 3). A long wash step over 40 CVs with the higher ionic strength binding buffer also resulted in satisfactory removal of DNA from the target protein. Absorbance at 260 nm and 280 nm was monitored, ensuring the maximal removal of DNA. As stated above, transitioning to an auto-induction expression protocol also significantly improved yield and convenience.

Following successful IMAC purification, *SmTopI16b* was identified upon SDS-PAGE analysis as an overexpressed band (Figure 3a). Target protein identity was confirmed initially using mass spectrometry peptide fingerprinting (Figure S3), secondarily upon His-epitope tag antibody directed western blot (data not shown), and again using His-tag staining (Figure 3b). Protein concentrations and yield were calculated using the standard Bradford protein assay with bovine serum albumin (BSA) as the protein standard, which is compatible with the IMAC elution buffer.⁴²

Following optimization of the IMAC purification step, analyses with several alternative secondary purification methods, including the use of a heparin column, cation and anion exchange columns, a Cibacron Blue column, and a size exclusion (SE) column were conducted. Ultimately, transitioning straight from IMAC to a second, polishing size exclusion step was chosen due to simplicity and the fact that it showed the overall greatest yield. The target protein size of ~83 kDa was judged to be distinct enough from any of the remaining ~97 kDa native *E. coli* topoisomerase I to separate them using size exclusion chromatography. After increasing the ionic strength of the size exclusion buffer (from 150 mM NaCl to 500 mM NaCl), the target protein solubility was found to be sufficient for purification without the need for an intermediate buffer exchange or dialysis step of the Ni²⁺-NTA purification product, which again afforded an additional gain in speed and convenience. Following the size exclusion chromatography polishing step, the target protein was collected and again analyzed via SDS-PAGE, indicating purity deemed greater than 95%. Yield was again determined from duplicate trials by Bradford protein assay with BSA as a standard, which indicated over 23 mg/L of target protein (Tables 1 and 2).

Characterization of *SmTopI*

In order to track protein activity, a gel-based DNA-relaxation assay was employed using published methods (Figure 4).³¹ The activity of *SmTopI16b* was maintained over three months in SE Buffer 2 refrigerated at 4°C, as well as after overnight freeze trials at -80°C including enzyme with and without 50% glycerol using both snap-freezing and drop-wise freezing methods in liquid nitrogen (Figure S4). Protein activity after cleavage of the decahis tag was also analyzed, showing no apparent increase in activity (Figure S5). Furthermore, the absence of DNase activity was determined via the implementation of a non-specific DNase activity assay using published methods (Figure S6).²⁸

To characterize the stability of *SmTopI16b*, a number of methods were employed. Hampton Research Solubility & Stability Screen 2™ was used to assess conditions that mitigate target protein precipitation and increase its overall stability.⁴³ Optimal buffer conditions as determined via Differential Scanning Fluorimetry (DSF) from the screen were found to be 0.05 M sodium citrate tribasic dihydrate (pH 5.0) with 4.0 M sodium chloride as showcased by an optimal melting temperature (T_m) of 48°C (Figure S7). An apparent trend was seen showing optimal buffer conditions included relatively low pH and high salt content. Protein solubility was also analyzed via overnight dialysis trials into lower-salt-containing buffers across a wide range of pH values. Results showed considerable visual precipitation regardless of buffer and pH when ionic strength was decreased below 200 mM NaCl with

protein concentration at 1 mg/mL, indicating the necessity of a higher ionic strength buffer for the solubility of this particular enzyme and construct (data not shown).

Discussion

An active bacterial topoisomerase I enzyme has been purified to a relatively high yield and purity from the clinically relevant bacteria *S. mutans*. A systematic analysis of different protein constructs was necessary as an unacceptable amount of the prototype hexa-his tagged protein showed weak affinity for the nickel column and was therefore lost to flow-through and overly contaminated with early co-eluting proteins. In order to analyze the possibility of the obstruction of the N-term His tag in the protein's folded state, the target gene was cloned into a pET21d vector (*SmTopA21d*), placing a hexa-His tag at the C-terminus. As increasing the length of histidine tags has been shown to increase affinity to Ni^{2+} -NTA, allowing for the use of more rigorous wash steps, and generally resulting in improved removal of non-specific binding contaminant proteins, this concept was also explored by sub-cloning the target gene from the pET15b vector into a pET16b vector, placing an extended deca-His tag at the N-terminus.^{44,45} Finally, as the small ubiquitin-related modifier (SUMO) fusion system has been reported to increase both target protein solubility and target protein expression levels, the target was cloned into the pET SUMO vector system (*SmTopA-SUMO*) in order to analyze the usefulness of an N-term SUMO fusion protein along with an N-term hexa-His tag.^{46,47}

Of the various constructs examined, the product of the deca-his-tagged pET16b construct (*SmTopI16b*) resulted in enough of an apparent increase in affinity for the nickel column to be successfully exploited via a step-wise elution to satisfactorily isolate the tagged target protein from the co-eluting contaminant proteins that plagued the hexa-his-tagged pET15b construct. Furthermore, enzyme activity was maintained with the deca-his-tagged construct, bypassing the immediate need for cleaving the affinity tag for downstream applications—a luxury not necessarily expected with the larger SUMO fusion tag construct (which also showed promising results in the purification trials).

Because the separation of DNA from DNA-binding proteins like topoisomerase I is essential, yet particularly onerous, a protocol implementing a rapid and efficient means of addressing this obstacle was highly desired. As an alternative to more traditional DNA removal methods, the ionic strength of the buffers used in the IMAC purification step were increased and the Ni^{2+} -NTA-bound target protein was exposed to a high salt wash over an extended period of time in order to successfully disrupt the largely electrostatic nature of DNA-protein binding.⁴⁸ Therefore, in order to further optimize *SmTopI16b* yield, higher ionic strength buffers were employed to both increase the solubility of the target protein as well as to aid in the expedited removal of DNA during purification steps. Additionally, auto-induction was successfully adopted in order to increase yield and greatly decrease the effort required during the expression and purification protocol. As mini-expression analysis showed no apparent gain or loss in target solubility by adopting auto-induction, it was deduced that the overall increase in target protein yield seen between auto-induction and IPTG-induction likely resulted from greater overall growth.

Conclusion

Using auto-induction for expression and IMAC and size exclusion for purification, a simple and rapid protocol has been developed for acquiring a substantial amount of high-purity bacterial topoisomerase I in just over two days. Via the systematic analysis and implementation of different methods, the yield of *SmTopII16b* was increased dramatically to a final and consistent yield of over 20 mg/L after two steps of purification resulting in an estimated 95% purity or greater—a consistent yield that is both higher than those previously reported for bacterial topoisomerase I and one that results from a substantially faster and less arduous purification protocol than those reported to date.

With the ease and speed of this protocol, a substantial amount of bacterial topoisomerase I can be easily obtained and used in assay development and screening large libraries for further bacterial topoisomerase I characterization and drug development. With the addition of an intermediate purification step, i.e., ion exchange or hydrophobic interaction chromatography, it is presumed that even greater purity could be attained. The substantial yield of this protocol affords the ability to sacrifice target protein yield for overall purity if necessary, for example, for protein crystallography trials. Overall, the optimized expression and purification protocol for topoisomerase I from *Streptococcus mutans* here represents a rapid and convenient tool in the continued study of bacterial type 1A topoisomerases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>SmTopI</i>	<i>Streptococcus mutans</i> topoisomerase I
VGS	Viridans Group Streptococci
VGSS	Viridans Group Streptococcal Shock Syndrome
<i>SmTopA15b</i>	<i>Streptococcus mutans</i> <i>topA</i> in pET15b vector
<i>SmTopA16b</i>	<i>Streptococcus mutans</i> <i>topA</i> in pET16b vector
<i>SmTopA21d</i>	<i>Streptococcus mutans</i> <i>topA</i> in pET21d vector
<i>SmTopA-SUMO</i>	<i>Streptococcus mutans</i> <i>topA</i> in pET SUMO vector
<i>SmTopI15b</i>	N-terminus hexa-Histidine-tagged <i>Streptococcus mutans</i> topoisomerase I enzyme
<i>SmTopI16b</i>	N-terminus deca-Histidine-tagged <i>Streptococcus mutans</i> topoisomerase I enzyme
<i>SmTopI21d</i>	C-terminus hexa-Histidine-tagged <i>Streptococcus mutans</i> topoisomerase I enzyme
<i>SmTopI-SUMO</i>	N-terminus hexa-Histidine-tagged and SUMO-tagged <i>Streptococcus mutans</i> topoisomerase I enzyme
IMAC	immobilized metal-ion affinity chromatography
Ni²⁺-NTA	nickel-nitrilotriacetic acid
IPTG	isopropyl beta-D-1-thiogalactopyranoside
OTG	n-octyl beta-D-thioglucopyranoside
TAE	Tris-Acetate-EDTA

Impact Statement

Type II bacterial topoisomerase enzymes are well-validated antibacterial targets of the fluoroquinolone drug class. There are, however, no marketed drugs targeting the type I bacterial topoisomerases and, though known to be essential, these enzymes are less characterized. The work reported here supports drug discovery efforts targeting this latter class of enzymes by detailing an optimized and highly efficient expression and purification protocol for the topoisomerase I enzyme from *Streptococcus mutans*.

Highlights

- Topoisomerase I is a potentially novel and selective antibacterial target.
- A method of purifying *S. mutans* topoisomerase I to high yield is presented.
- The use of an auto-induction method for protein expression improved yield.
- >95% purity is achieved in just over 48 hours with a simple two-step protocol.

Figure 1.
S. mutans topoisomerase I structural model with single-stranded DNA bound. Full structure of the enzyme (blue ribbons), and single strand DNA bound to active site (yellow ribbons).

Figure 2.

Chromatogram of nickel column linear elution from preliminary construct trials. (A) N-term hexa-His-tagged SmTopI_15b construct. (B) N-term deca-His-tagged SmTopI_16b construct. (C) C-term hexa-His-tagged SmTopI_21d construct. (D) N-term SUMO and hexa-his-tagged SUMO construct.

Figure 3.

SDS-PAGE gels of *SmTopI_16b* purification results. L, ladder; CE, clarified extract from auto-induction growth; FT, nickel column flow-through; P1, nickel column first peak from first step elution using high salt buffers; Ni^{2+} , target fraction from nickel column; SE, target fraction from size exclusion; Ec, commercially available *E. coli* topoisomerase I; TB, target fraction from nickel column from TB growth with high salt buffers. (A) SDS-PAGE after blue-staining. (B) SDS-PAGE after His-staining in order to verify target protein bands.

Figure 4. Gel-based DNA relaxation assay. Lane 1, ladder; lane 2, supercoiled pUC18 plasmid (DNA substrate as negative control); lane 3, commercially available *E. coli* topoisomerase I (positive control); lanes 4-10, SmTopI16b enzyme in increasing concentration (nM). N, nicked; FR, fully relaxed; PR, partially relaxed; SC, supercoiled.

Table 1
Comparative summary of purification yields from *SmTopI* constructs

Vector system	System characteristics	Growth/induction method	Overall yield (mg/500 mL) ^a
pET-15b	N-term 6× His tag	500 mL TB; 0.5 mM IPTG; 18°C × 24 h	4.94 (IMAC)
pET-16b	N-term 10× His tag	500 mL TB; 0.5 mM IPTG; 18°C × 24 h	7.11 (IMAC) 6.24 (SE)
pET-21d	C-term 6× His tag	500 mL TB; 0.5 mM IPTG; 18°C × 24 h	4.11 (IMAC)
SUMO	N-term SUMO fusion tag and N-term 6× His tag	500 mL TB; 0.5 mM IPTG; 18°C × 24 h	9.12 (IMAC)
pET-16b	N-term 10× His tag	500 mL TB; 0.5 mM IPTG; 18°C × 24 h; high salt	8.30 (IMAC)
pET-16b	N-term 10× His tag	500 mL auto-induction media; 37°C × 24 h; high salt	14.21 (IMAC) 11.66 (SE)

^aOverall yields are an average of two 500 mL growths in respective media.

Table 2**Summary of *S. mutans* topoisomerase I purification**

Step	Total protein (mg) ^a	Target protein (mg) ^b	Total activity (units) ^c	Specific activity (units/mg)	Yield (%)	Purity (%) ^d
Clarified Extract	1445	107.3	N/A	N/A	100	7.4
Nickel Column	28.4	25.3	N/A	N/A	24	89
Size Exclusion Column	23.3	22.6	58,250	2,575	21	97

^aProtein concentration determined by Bradford assay using BSA as the standard protein.

^bAmount of target protein determined based off of SDS-PAGE gel analysis.

^cActivity measured as described under Materials and Methods section.

^dPurity determined based off of SDS-PAGE gel analysis.