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Purification of Recombinant E. Coli Topoisomerase III for Structure-Based Drug Design Using Protein Crystallization

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Cover Page Footnote

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Purification of Recombinant E. Coli Topoisomerase III for Structure-Based Drug Design Using Protein Crystallization

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Type IA Topoisomerases are ubiquitous enzymes found throughout all life forms and species. These topoisomerases relieve the topographical constrains formed by DNA during processes like replication and transcription via a cleavage-religation mechanism performed through a catalytically active tyrosine residue in the primary structure of the enzyme. E. coli Topoisomerase III (EtopIII) is a type of Type IA topoisomerase, and its main function in the cell is as a decatenase, which means that it unlinks circular or intertwined pieces of genetic material and creates two unlinked segments of DNA from a singular linked chain. Structure-based determination of the enzyme's three-dimensional structure via crystallization could be beneficial in finding a novel antibiotic drug that is able to inhibit this enzyme in vivo, a solution that is sorely needed in a world where antibiotic-resistant bacteria are an ever-growing problem. The crystallization of the enzyme can be undertaken by truncating a part of the C-terminal of the enzyme that makes it harder for it to crystallize and introduce a DNA/RNA ligand to observe its binding interactions with said substrate. For this to happen, the truncated recombinant mutant must be purified and concentrated to desirable levels for crystallization. The results of the experiment show that out of 4L of bacterial starting culture, a total of 34.57 mg of protein was able to be purified. This protein was then sent for crystallization experiments, of which results are still pending, but preliminary results indicate that the protein quickly aggregates in one of the RNA ligand conditions, which elicits a change in protocol for future purifications. Furthermore, results from a relaxation assay indicate that the protein exhibits some relaxation activity even after the truncation of the C-terminal chain. These results could expand the knowledge of this enzyme for future structure-based drug development.

Keywords: *Topoisomerase III, Type IA Topoisomerase, inhibitors, recombinant protein, protein purification*

Introduction

Importance

In the last few decades, the problems caused by antibiotic-resistant bacteria have grown exponentially. Bacteria are adapting in novel ways to combat what once were very successful antibiotic therapies, costing millions of lives in the process (Pulingam et al., 2021). Given this, millions of dollars are being invested in research to find new types of drugs or antibiotics that the bacteria have not adapted to. One of these are classified as topoisomerase inhibitors. Topoisomerase inhibitors are compounds that stop the function of any type of topoisomerase enzyme; common types are already available for the general public including ciprofloxacin, topotecan, and etoposide (LiverTox, 2020). Although antibiotics such as ciprofloxacin are effective therapies for bacterial infection treatment, these market drugs are inhibitors of type TopIIA and TopIB enzymes. Much less is known, however, about inhibitors of the Type IA TopIII enzyme. Currently there are no inhibitors for this enzyme reported in the literature and limited research in possible inhibitors of the human TopIIIβ is being done (Pommier, 2009; Pommier, 2013; Wang et al., 2023). Discovery of a new type of inhibitor for this enzyme could prove to be extremely beneficial in expanding the options of therapeutics available.

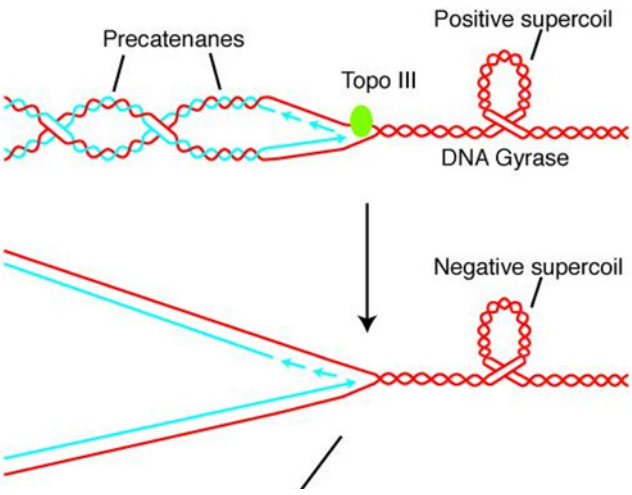
Literature Review

One subtype of topoisomerase Type IA enzyme is of special importance for replication: Topoisomerase III. In prokarya it exists only as TopIII, and in eukarya as TopIIIα or TopIIIβ. In prokaryotes, specifically *Escherichia coli*, there are two topoisomerase IA enzymes: ETopI and ETopIII. ETopI is one of the main enzymes behind relaxing negative DNA supercoils. On the other hand, ETopIII's main role is unlinking two circular pieces of DNA from each other, also known as catenates. Because of this, ETopIII's role is mainly as a decatenase. One of the roles where decatenase activity is required is the segregation of the sister chromosomes into daughter cells after replication (Pereze-Cheeks et. al, 2012). The role of ETopIII in vivo is further corroborated by its ability to only bind single-stranded DNA sequences, which allows it to mainly handle and unwind precatenanes. Precatenanes are intertwining structures between the parent DNA strand and the newly synthesized DNA strand that form behind the replication fork because of the severe positive overwinding of the DNA helix ahead of the replication fork (Champoux et al., 1980). ETopIII is one of the main enzymes that resolve precatenanes

Figure 1

Model of EtopIII resolving precatenane formation behind the replication fork.

Note: Adapted from Perez-Cheeks B.A., et al., 2012.

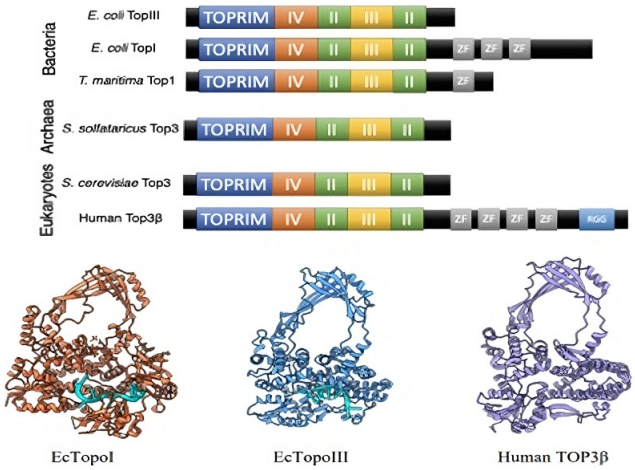


in-vivo, preventing incorrect chromosomal segregation due to precatenane presence before chromosomal separation.

This enzyme's RNA-binding capabilities have been studied and confirmed in the past, as the protein was able to unknot a cyclic RNA structure, a feat that can only be done with an enzyme that can perform a strand passage mechanism (Wang et al., 1996). Thus, the RNA-binding capabilities of ETopIII could be further studied.

Figure 2

Structure comparison of different Type 1A Topoisomerase proteins



Note: Structure is mostly conserved except for unique C-terminus domains Adapted from Ahmad, et al., 2017.

A possible factor that could be targeted to expand the scope of the RNA binding capabilities of ETopIII would be by visualizing a complex between the ETopIII enzyme and an RNA substrate. This could be done by performing crystallization experiments on the protein. Crystallization is a widely used tool to visualize the three-dimensional structure of the protein-ligand complex, relying on the concept of supersaturation to occur reliably (McPherson et al., 2014). Supersaturation is a non-equilibrium state of a system in which solute molecules are present in an amount exceeding their solubility limit concentration. Different compounds and macromolecules have unique complications when forming stable crystal structures. For example, an ionic salt compound such as NaCl forms stable lattices by the strong ionic interactions between the ions in the structure. On the other hand, it is known that large macromolecules like proteins do not share the same strong ionic interactions between themselves, rather limited to hydrogen bonding, salt-bridges, and hydrophobic interactions to form the lattice.

It is due to this difference in bonding interactions that forming a stable protein lattice is a difficult process, with varying degrees of lattice formation depending on the amount of these bonds formed in each type of protein (Chernov et al., 2003). The process of forming these bonds in crystallization happens in 2 different steps; the first one, nucleation, is the initial formation of the pseudo-crystal structure. For a stable crystal to

form, the energy created during crystal formation must exceed the energy created by the interaction of the water molecules with the individual protein molecules (Chernov et al., 2003). The second step, the growth of the crystal, happens after a semi-stable structure (nucleus) is formed in the first step, other protein molecules will randomly aggregate and detach from the growing nucleus. After a critical mass is reached, it will add protein molecules at certain points in the lattice structure until a stable crystal forms. This is known as growth by two and three-dimensional nucleation.

The nucleation and growth steps can only occur, as previously mentioned, under supersaturating conditions. Supersaturation of a solution is done by changing the environment of the solvent. Some ways that this is done in the laboratory are: changing the pH of the solvent, changing salt concentration of the solvent, or adding molecules which will change the interactions of the protein with the solvent, such as certain polymers (McPherson et al., 2014). However, these are only a narrow range of ways the researcher could change conditions for crystallization.

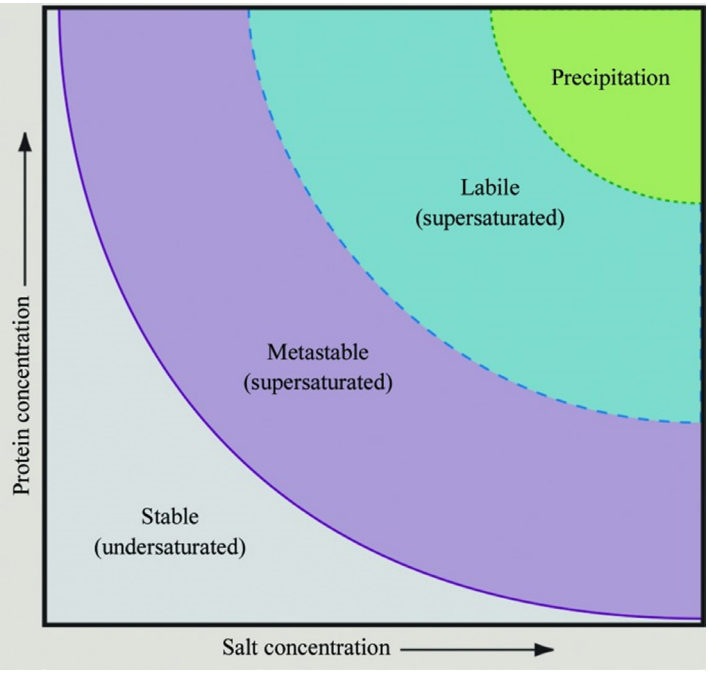
With this mechanism for protein crystallization, it is easy to picture how larger proteins are harder to crystallize since larger proteins have more entropic energy when in contact with the solvent due to their greater surface area (Abdallah et al., 2013). Furthermore, an area that is not static in the protein structure would not create proper areas of nucleation; this phenomenon is visualized in Intrinsically Disordered Proteins (IDPs), which lack tertiary and even secondary structure, leading to high disorder and thus yield lower-to-null protein crystallization (Musselman et al., 2021). However, if these sections were truncated, the intrinsically disordered region (IDR) would no longer be inhibiting the formation of the crystal. Given that the majority of these IDRs are present in the N and C- terminus of the proteins, sometimes truncating these loose amino acid chains is beneficial for the overall crystal formation (Cooper et al., 2017).

Once a stable crystal is formed and correctly visualized in high-imaging pictures, the three-dimensional structure of the enzyme can be analyzed. Doing so will allow for visualization of areas that are involved in the DNA/RNA processing. After this, it could be possible to create a compound that could selectively bind to these areas and inhibit them. Previous research has shown compounds that have been isolated using Structure Based Drug Design (SBDD) techniques, with more

Figure 3

Phase diagram of the crystallization of macromolecules

Note: Phase diagram showing the stages of supersaturation by the addition of salt. Adapted from McPherson A., et al., 2014.

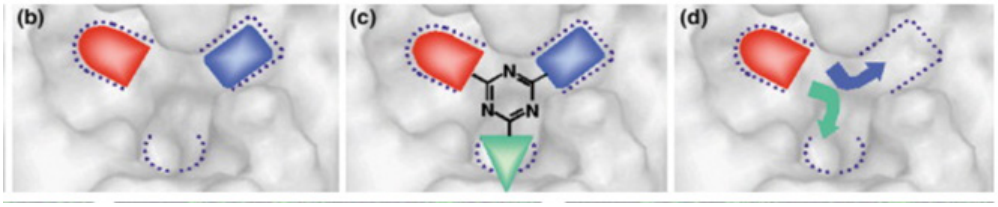


than 40 compounds passing FDA clearance for testing in the general public being found using this method (Hardy et al., 2003).

Even if the whole druggable pocket is not able to be fully occupied by the inhibitor molecule, some partial binding of the molecule would give enough information to then build up on (Blundell et al., 2004). Much like a puzzle, connecting one of the relevant pieces of the inhibitor to the pocket helps visualize what the next piece could be. A schematic of this is shown in **Figure 4.**

Figure 4

Model showing different structure-based drug design techniques.



Note: Adapted from Blundell, T. et al., 2004.

Despite this, the C-terminus tail of the ETopIII protein has already been identified to have some role in the binding of the DNA and RNA substrates. Literature indicates that extensive cleaving of the C-terminus region in the ETopIII enzyme leads to proportional loss in the ability of the enzyme to bind DNA or RNA substrates (Li et al., 2000; Zhang et al., 1994; Zhang et al., 1996). So, to determine the amount of activity, if any, present in the truncated ETopIII mutant compared to wild type, an activity assay must be run.

Methods

Transformation of competent cells

First, the competent Rosetta 2DE3 (Novagen®) cells were thawed on ice. After, 1 µl of pET15tbEtop621 plasmid was added to the cells. These cells and plasmid were placed in an ice bath for 5 minutes. After, they were heated for 30 seconds in a 42-degree Celsius water bath, followed by placing them in ice for 2 minutes. After, 250 µl of SOC medium was added. The solution was mixed and serially diluted into agar plates which contained 50 µg/ml carbenicillin (carb), 34 µg/ml chloramphenicol (chl), and 1% glucose. Cells were then allowed to grow overnight. 12 colonies were re-streaked into fresh carb/chl/glucose plates. Plates were incubated at 37° C until colonies grew.

Checking for expression of recombinant protein

Four colonies were picked and grown to check their expression. The day after, 60 µl of this media was transferred to 6 mls of a new batch of media. These new cells were then incubated at 37° C until they reached an optical density (OD_{600}) between 0.4-0.5. After, Isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to each of the 4 batches to a final concentration of 1mM. After the addition of IPTG, the OD_{600} was allowed

to reach ~2.0.

To check for protein presence a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was run; the gel was made with 12% Protogel Quick-Cast solution from National Diagnostics, as well as tetramethylethylenediamine (TEMED) from GoldBio and ammonium persulfate (APS) from GoldBio.

Large scale preparation of protein culture

Once expression was confirmed in a colony, the transformant was used to scale up the protein purification to 1L, followed by 3L cultures. A loopful of bacteria was taken from the master plate's expressing colonies and swirled in 15 mL of 50 µg/ml carb, 34 µg/ml chl, and 1% glucose. Cells were allowed to grow overnight. After, 7.5 ml of this culture was transferred into two different 1L flasks, each containing 750 mL of autoclaved media (17.25 g Luria Broth, 7.45 g NaCl). These cultures were allowed to reach OD_{600} of 0.4-0.5. After this, IPTG 1mM was then added to the flasks until OD_{600} of ~2. Cells were then centrifuged at 6000 G's for 15 mins at 4°C, the supernatant was discarded and the pellets frozen at -80°C.

Protein purification process

After the pellet was collected, the cells were resuspended in lysozyme (1 mg/ml final concentration) + 1X LEW buffer (50mM NaH_2PO_4 + 300mM NaCl at pH 8.0). This lysate sample was then placed in ice for 30 mins. Afterwards, cells were sonicated in 40% power in 15 second intervals for 3 minutes. Samples were then centrifugated at 10,000 Gs for 2 hours in the Thermo Scientific Sorvall RC6+ Centrifuge and the supernatant was collected. This supernatant was then run through a Nickel-binding column and the flowthrough, wash, and elution samples collected.

Protein Processing with TEV Digest and SEC Column

When the protein concentration was determined, a Tobacco Etch Virus (TEV) Digestion was done to cleave off the His-tag in the protein. TEV protease was added to the protein at a ratio of 1:25 TEV to protein. This solution was left at room temperature for ~6 hours and transferred to the cold room overnight. The protein-TEV solution was run through a nickel affinity column to separate the His-tag from the protein. An SDS-PAGE gel was run to check for the cleavage of the His-tag. After, the flowthrough and wash fractions were concentrated, and the protein was run through a Cytiva AKTA Protein Purification System with a Size Exclusion Chromatography (SEC) column in SEC buffer (25mM Tris-HCl, 300mM NaCl, 5% Glycerol, 1mM DTT, pH 7.5) and collected in different fractions. Purity and concentration were checked using a Bradford Assay as well as SDS-PAGE gels. Protein was then stored in storage buffer (100mM NaCl, 1mM DTT, 0.2mM EDTA, 30% Glycerol) until ready for next use.

Relaxation Assay

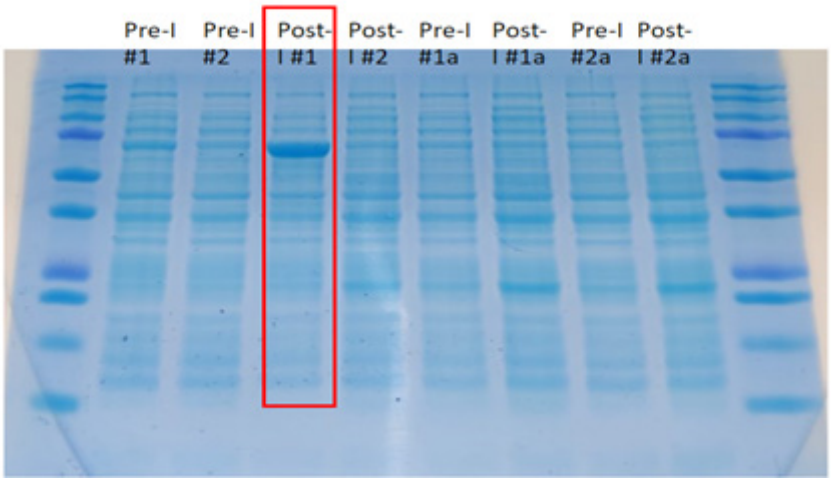
The relaxation assay was done by making serial dilutions of the enzyme. The final amounts of the enzyme from serial dilutions used in the assay were 100 ng, 80 ng, 60 ng, 40 ng and 20 ng. A DNA only control

was also included as well as a full length ETopIII control. Two buffers were also used: Stop buffer (100mM EDTA, 0.1% Bromophenol blue, water) and 10x reaction buffer (HEPES-KOH 400mM, Mg-acetate 10mM, water). The 10x reaction buffer was combined with DNA and enzyme to make the reaction happen. The mixture was incubated at 52°C for 10 minutes before adding stop buffer. After that, each 20 µl sample was loaded onto a 1% agarose, 5 µg/ml chloroquine gel, running it at 20V for 24 hours.

Results

Figure 5

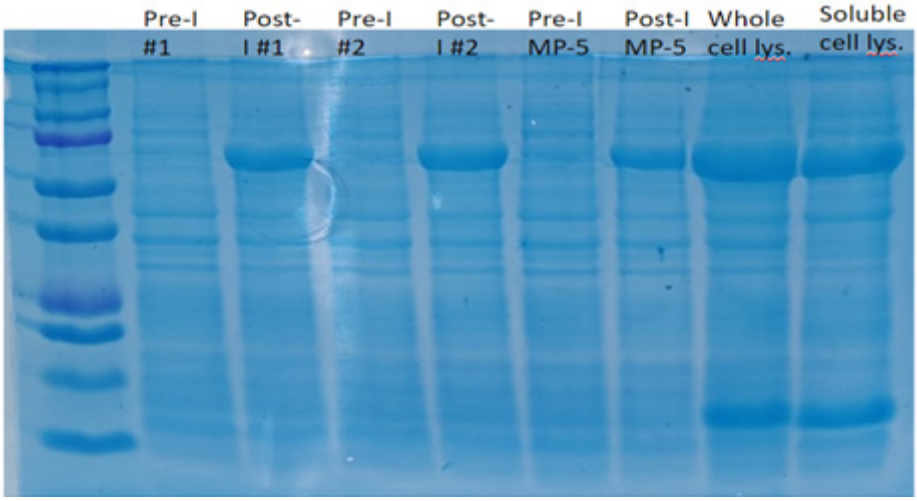
Pre- and post- induction samples SDS-PAGE gel



SDS-PAGE gel showing the pre- and post- induction samples of different colonies. Truncated ETopIII protein (621 out of 653 amino acids) was overexpressed in colony #1, with a size of ~73.2 kDa.

Figure 6

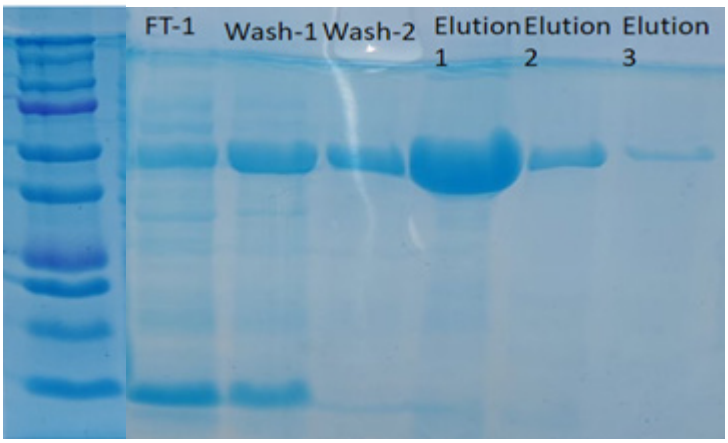
Whole cell and Soluble cell lysate samples SDS-PAGE gel



Note: Gel showing the presence of the recombinant protein in whole and soluble cell lysates after lysozyme treatment. Previous pre and post-induction samples included for reference.

Figure 7

SDS-PAGE gel showing samples after Ni-NTA column elution



Note: Gel after the different elutions with Nickel-NTA binding column (Column 1). Most protein was present in Elution 1, similar results observed for the other columns. Standard ladder from another gel included for reference.

Figure 8

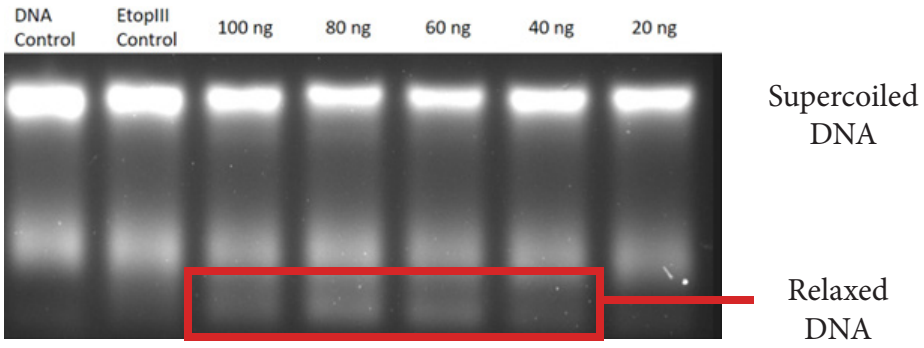
Results from Standard Bovine Serum Albumin (BSA) Curve

Curve Name	Curve Formula	A	B	R2
StdCurve	Y=A*X+B	1.46	0.0887	0.963

Note: Using relevant software, the protein was found to have a concentration of 0.75 mg/ml in pool 1, and 0.20 mg/ml for sample 2. Total amount of protein present was 28.15 mg for pool 1, and 6.42 for pool 2.

Figure 9

Relaxation Assay Results



Note: Relaxation Assay showing the activity of the truncated ETopIII protein. Relaxed DNA present as bands at the bottom of the gel.

Discussion

Overall, the results indicate that the truncated Topoisomerase III protein was successfully purified and isolated in enough concentration to send for crystallization. The total amount of protein gotten from Pool 1 was 28.15 mg (0.75mg/ml in 37.5 ml total volume), whereas Pool 2 had a total 6.42 mg of protein (0.20 mg/ml in 32 ml total volume).

Crystallization results are partially pending on the work of Dr. Kemin Tam at the Argonne National Laboratory. The substrates used for the co-crystallization of the protein were the following: Top3RNA8merC (CGCAACUU), Top3RNA8mer (AACUGUUG), Top3BDNA8merC (CGCAACTT) and Top3DNA8mer (AACTGTTG). Initial results show that there were aggregations in the RNA nucleotide Top3RNA8mer condition. These aggregations indicate that the protein did not crystallize favorably. Instead of uniform crystals, the RNA ligand solutions formed white, cloudy precipitate structures.

Given the crystallization results, it is hard to determine the three-dimensional structure of the protein, and thus the knowledge gap on the lack of inhibitors for this enzyme remains unsolved. However, the aggregation of the protein in one of the conditions indicates that the pH of the buffer close to the protein's isoelectric point (pI) might have caused some precipitation. Since the isoelectric point of the protein is around pH 7.6, a buffer that is a significantly different pH as the protein pI might result in a more stable structure in solution.

Conclusion

With the preliminary results received from Dr. Tan's Lab about the aggregation of the protein in one of the RNA substrate conditions, the need arises for more protein to be purified in order for more experiments to be run with it. The next step is to re-start the protein purification prep process with another 3L of bacterial culture. In this new preparation, the final buffer used should have a pH significantly different from 7.6.

The results from the relaxation assay indicate that the protein can still relax some DNA, this is seen in lanes 3-6. It is seen that there is a slight relaxation of the DNA, seen as the low-intensity band denoted by the red rectangle in **Figure 9**. The possible implications of this result are that even with the deletion of the 32 amino acids from the intrinsically disordered region in the C-terminus of the ETopIII protein, the enzyme was still able to bind DNA and relax it, which is a finding concordant with literature (Li et al., 2000; Zhang et al., 1994; Zhang et al., 1996). This could possibly indicate that for future preparations, extra cleaving to this part of the enzyme could still be done without complete loss of the protein's DNA-binding abilities.

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