Expression of P266L Mutant of T7 RNA Polymerase in Varying Growth Conditions
Kramer Scott Crider

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Expression of P266L Mutant of T7 RNA Polymerase in Varying Growth Conditions

by

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Kramer Crider

Student Name (printed)

Student Signature

12/01/19

Date
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Abstract

A mutation of proline to leucine at position 266 in the amino acid sequence of T7 RNA polymerase has been found to increase the efficiency of transcription in bacteria. This mutation reduces short abortive RNA molecules by stabilizing the transition from the initiation complex to the elongation complex during transcription. The purpose of this project was to express and purify P266L T7 RNA Polymerase and to observe any changes that resulted when cells were expressed in TB and LB. The results indicated successful expression and purification of the mutant protein, and no recognizable difference was observed in the purity of the protein between cells expressed in various growth media.
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Introduction

Transcription begins when an RNA polymerase binds to a DNA promoter sequence under appropriate conditions. Approximately the first 10 nucleotides that are transcribed are known as the initially transcribed sequence, and its corresponding transcribed sequence is known as the initiation complex.\(^1\) When the RNA polymerase clears the promoter region, the initiation complex undergoes a structural rearrangement to produce what is known as the intermediate complex, which further rearranges to produce the elongation complex.\(^2\) However, sometimes the initiation complex, given its instability, dissociates before the elongation complex is formed, resulting in an abortive transcript.\(^3\) Efforts to recover this dissociation are continually made by the intermediate complex, which maintains promoter contact during abortive synthesis.\(^4\) This occurs a number of times until a stable elongation complex is formed and productive transcription can begin. Research has found that a mutation in the monomeric RNA polymerase from bacteriophage T7 desensitizes the molecule’s susceptibility to this abortive cycle of transcription, resulting in a more efficient and effective RNA polymerase.\(^5\) This mutation is found at residue 266 in the amino acid sequence at which a proline is exchanged for a leucine. A 3D crystal structure of this mutant protein in complex with transcription complexes is shown in Figure 1. The purpose of this project was to express and purify P266L T7 RNA Polymerase and to observe any changes resulting from the variability of growth media.

\(^1\) Guillerez et al., “A Mutation in T7 RNA Polymerase That Facilitates Promoter Clearance.”
\(^2\) Guillerez et al.
\(^3\) Guillerez et al.
\(^4\) Durniak, Bailey, and Stetiz, “The Structure of a Transcribing T7 RNA Polymerase Complex Captured During Its Transition from Initiation to Elongation.”
\(^5\) Tang et al., “Relaxed Rotational and Scrunching Changes in P266L Mutant of T7 RNA Polymerase Reduce Short Abortive RNAs While Delaying Transition into Elongation.”
Figure 1. A 3D crystal structure of P266L T7 RNA Polymerase in complex with DNA. (A) A crystal structure of the initiation complex bound to the promoter. (B) A crystal structure of the elongation complex. (C) A crystal structure of P266L T7 RNA Polymerase intermediate complex bound to both the promoter and downstream DNA.6

6 Durniak, Bailey, and Stetiz, “The Structure of a Transcribing T7 RNA Polymerase Complex Captured During Its Transition from Initiation to Elongation.”
The expression gene of P266L T7 RNA Polymerase is buried within the lac operon of *Escherichia coli*. Normally, the transcription of the lac operon is turned on in the presence of lactose. In a laboratory setting, it is more practical to use the structural analogue of lactose, isopropyl β-D-1-thiogalactopyranoside (IPTG). The is because lactose is metabolized by the cell and is no longer able to induce transcription of the lac operon after some time. IPTG is not a part of any metabolic process that occurs in *E. coli*, so it may be used indefinitely without slowing down the transcription of the lac operon. IPTG was used in this project to induce the expression of P266L T7 RNA polymerase.

Two purification methods were used in this project: affinity chromatography and ion exchange chromatography. A HisTrap FastFlow affinity column is used to bind proteins that have an engineered histidine tag within its primary sequence. The histidine tag binds to Ni2+ ions present in the resin of the column. The bound protein is released when a buffer with a high imidazole content is introduced into the column, because the Ni2+ has a higher affinity for imidazole than for the histidine tag. A HiTrap S cation exchange column is used to isolate positively charged proteins. The resin of the column is negatively charged and is attracted to positively charged particles. The desired protein is suspended in a buffer that has a lower pH than the protein's pI, resulting in an overall positive charge on the protein. When the protein containing buffer passes through the column, the positively charged protein binds to the negatively charged resin while any non-positively charged particles flow through the column. Finally, the protein is eluted when a high salt buffer is introduced into the column and the smaller, more positively charged particles bind to the resin in the place of the protein.
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Experimental

Transformation of Competent BL21(DE3)pLysS Cells

To begin the transformation protocol for P266L T7 RNA polymerase, a sterile, disposable vial was obtained, and 2 μL of P266L plasmid was placed in the bottom of the vial. Next, 20 μL of competent BL21(DE3)pLysS E. coli cells were placed directly onto the plasmid, and the mixture was placed on ice to incubate for 30 minutes. After the incubation period, the vial containing the mixture of plasmid and competent cells was heat shocked in a 42 °C water bath for 45 seconds. The vial was immediately placed onto ice for 2 minutes following the heat shock. After 2 minutes, 180 μL of LB was added to the vial, and it was placed in a 37 °C shaking incubator for 1 hour. After 1 hour, 80 μL of the starter culture was spread onto an LB agar plate containing 20 μg/mL carbenicillin. The plate was placed in a 37 °C incubator and grown overnight for a maximum of 16 hours. The plate was then retrieved from the incubator and placed in the 4 °C refrigerator for later use.

Growth and Expression in Terrific Broth (TB)

Two starter cultures, containing 5 mL LB, 5 μL carbenicillin (from 25 mg/ml stock solution; further references to carbenicillin are from this stock solution), and one BL21(DE3)pLysS P266L colony, were expressed in a 37 °C shaking incubator for 5 hours. The starter cultures were then aliquoted into microcentrifuge tubes and centrifuged to obtain a cell pellet. The LB supernatant was discarded, and the cell pellets were resuspended in TB and transferred into a 250 mL sterile flask containing 50 mL TB and 50 μL carbenicillin (from 25 mg/mL stock). The TB contents are shown in Table 1. The cells were placed into a 37 °C shaking incubator overnight for a maximum of 16 hours. The cells were then transferred into a 4 L flask containing 1 L of TB. The optical density at 600 nm
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(OD<sub>600</sub>) was measured to obtain a baseline cell concentration. The 1 L culture was then placed into the 37 °C shaking incubator. At an OD<sub>600</sub> of 0.6, a final concentration of 0.5 mM IPTG was added to induce expression of the P266L T7 RNA polymerase. The induced cells were allowed to express at 37 °C for 4 hours. The expressed cells were harvested, and a 5.32 g pellet was obtained. The cell pellet was stored at -80 °C until later use.

<table>
<thead>
<tr>
<th>Sterilization Method</th>
<th>Contents</th>
<th>Amount</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved (900 mL volume)</td>
<td>Yeast extract</td>
<td>24 g</td>
<td>24 g/L</td>
</tr>
<tr>
<td></td>
<td>Tryptone</td>
<td>12 g</td>
<td>12 g/L</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>4 mL</td>
<td>4 mL/L</td>
</tr>
<tr>
<td>Sterile filtered (100 mL volume)</td>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2.31 g</td>
<td>0.017 M</td>
</tr>
<tr>
<td></td>
<td>K&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>12.54 g</td>
<td>0.072 M</td>
</tr>
</tbody>
</table>

*Contents were combined into sterile 1 L bottle upon completion of autoclave treatment and stored at room temperature.*

**Growth and Expression in Luria Broth (LB)**

Four starter cultures, containing 4 mL LB, 4 μL carbenicillin (from 25 mg/mL stock), and 1 BL21 P266L colony, were expressed in a 37 °C shaking incubator for 5 hours. The 4 mL starter cultures were then transferred into a 250 mL sterile flask containing 50 mL LB and 50 μL carbenicillin (from 25 mg/mL stock). The cells were placed into a 37 °C shaking incubator overnight for a maximum of 16 hours. The cells were then transferred into a 4 L flask containing 1 L of LB. The OD<sub>600</sub> was measured to obtain a baseline cell concentration. The 1 L culture was then placed into the 37 °C shaking incubator. At an OD<sub>600</sub> of 0.6, a final concentration of 0.5 mM IPTG was added to induce expression of the P266L T7 RNA polymerase. The induced cells were placed in the 37 °C shaking incubator to express for 4 hours. The expressed cells were harvested, and a 4.15 g pellet was obtained. The cell pellet
was stored at -80 °C until later use.

**Purification of P266L T7 RNA Polymerase Expressed in TB using FPLC**

The 5.32 g cell pellet was removed from the -80 °C freezer and resuspended in 50 mL buffer A as well as 1 Roche Complete EDTA-free Protease Inhibitor tablet. All nickel affinity purification buffers are shown in Table 2. The cell suspension was sonicated 5 seconds on / 5 seconds off for 10 minutes on ice at a power level of 4 to mechanically lyse the cells. The suspension containing the lysed cells was centrifuged at 19,650 x g in the Thermo Scientific™ Fiberlite™ F15 fixed angle rotor for 45 minutes. A total of 50 mL of the supernatant containing the protein was collected and prepared to load onto the HisTrap FastFlow 5 mL nickel affinity column.

The column was equilibrated with buffer A for 10 minutes at a flow rate of 2 mL/min using FPLC. After equilibration, the 50 mL sample was loaded onto the column in 20 mL increments. Once the entire sample was loaded onto the column, the column was washed with 10% buffer B, the elution buffer, until a baseline was obtained. Once the baseline was visible, the protein was eluted with 80% buffer B, and the fractions containing the protein according to the chromatograph were collected, pooled, and stored at 4 °C to use on the Hi Trap S 5 mL column the following day. A total of 18 mL of eluted protein was collected. The chromatograph from the nickel affinity purification column is shown in Figure 2.

| **Table 2. Nickel Affinity Purification Buffers** |
|----------------|----------------|---------------|
| **Contents**   | **Buffer A**  | **Buffer B**  |
| 375 mM NaCl    | 37.5 mL       | 18.75 mL      |
| 20 mM Tris-HCl pH 8 | 10 mL | 5 mL          |
| 0/200 mM Imidazole | 0.68 g | 3.4 g         |
|                |               | (from 5 M stock) |
|                |               | (from 1 M stock) |
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<table>
<thead>
<tr>
<th>β-Mercaptoethanol</th>
<th>80 µL</th>
<th>40 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total volume</strong></td>
<td>500 mL</td>
<td>250 mL</td>
</tr>
</tbody>
</table>

*Buffers used in HisTrap FastFlow 5 mL column stored at 4 °C until ready for use.*

**Figure 2.** The chromatogram generated from the nickel affinity purification of P266L T7 RNA Polymerase expressed in TB. The peaks labeled (a), (b), and (c) denote non-binding debris that passed through the column as the desired protein was loaded onto the column. The peak labeled (d) denotes non-desired protein and/or biomolecules that eluted during the washing step with 10% buffer B. The baseline is observed following this peak. Peak (e) denotes the elution of the desired protein with 80% buffer B, followed by the beginning of a new baseline due to the imidazole concentration shift.
The 18 mL eluted sample was diluted with 4 equivalent volumes of S dilution buffer (18 mL x 4 = 72 mL dilution buffer). All S buffers are shown in Table 3. The Hi Trap S 5 mL column was prepared for the sample by washing with S buffers A and B for 10 minutes at a flow rate of 2 mL/min. The column was loaded with 80 mL of sample in 20 mL increments. Once the sample was loaded, it was washed with 3 column volumes of S buffer A (5 mL column x 3 = 15 mL; 15 mL / 2 mL/min = 7.5 min.). The protein was eluted with 40% S buffer B, and 14 mL of eluted sample was collected. The chromatograph from the S column is shown in Figure 3. The purified sample was then concentrated to 2 mL followed by the addition of 2 mL of 100% glycerol. The concentrated protein sample was then aliquoted into microcentrifuge tubes at volumes of 200 μL, and the tubes were stored at -80 °C. SDS-PAGE was used to analyze the results of this purification and to confirm the desired protein was present in the eluted fractions (see Figure 4).

<table>
<thead>
<tr>
<th>Table 3. Hi Trap S Buffer Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contents</strong></td>
</tr>
<tr>
<td>0/75/1000 nM NaCl</td>
</tr>
<tr>
<td>50 mM HEPES-KOH pH 7</td>
</tr>
<tr>
<td>2 mM EDTA</td>
</tr>
<tr>
<td>5% glycerol</td>
</tr>
<tr>
<td>2 mM DTT</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
</tr>
</tbody>
</table>

Buffers used in Hi Trap S 5 mL column stored at 4 °C until ready for use.
Figure 3. The chromatogram generated from the S column purification of P266L T7 RNA Polymerase expressed in TB. The peaks labeled (a), (b), (c), and (d) denote non-binding debris that passed through the column as the desired protein was loaded onto the column. The baseline following peak (d) was generated as the column was washed with 15 mL of S buffer A. Peak (e) denotes the elution of the desired protein with 40% S buffer B, followed by a return to baseline.
Figure 4. SDS-PAGE analysis of purification of P266L T7 RNA Polymerase expressed in TB. The molecular weight (M.W.) ladder used to compare protein sizes was the Thermo Scientific™ PageRuler™ Prestained 10-180kDa Protein Ladder. (a) Supernatant of the lysed cells loaded onto nickel affinity purification column. (b) Eluted protein collected after both nickel affinity and S column purifications. (c) 2 mL concentrated protein sample. Bands in lanes (b) and (c) indicate successful purification of 99 kDa P266L T7 RNA Polymerase.
Purification of P266L T7 RNA Polymerase Expressed in LB using FPLC

The 4.17 g cell pellet was removed from the -80 °C freezer and resuspended in 20 mL buffer A as well as one Roche Complete EDTA-free Protease Inhibitor tablet. All nickel affinity purification buffers are shown in Table 2. The cell suspension was sonicated while on ice at a power level of 4 to mechanically lyse the cells. The sonication program was set to 5 seconds on / 5 seconds off for 5 minutes. The suspension containing the lysed cells was centrifuged at 19,650 x g in the Thermo Scientific™ Fiberlite™ F15 fixed angle rotor for 45 minutes. A total of 20 mL of the supernatant containing the protein was collected and prepared to load onto the HisTrap FastFlow 5 mL column. The column was washed with buffers A and B for 10 minutes at a flow rate of 2 mL/min. After the wash was complete, the 20 mL sample was loaded onto the column.

An error occurred while using the FPLC, and the sample was sent directly into the waste container rather than loaded onto the column. To recover the protein, the 300 mL of waste contents were loaded onto the column manually with a 50-mL syringe. The column was then reattached to the FPLC and washed with 10% buffer B. After a baseline was obtained, the protein was eluted with 80% buffer B. The fractions designated by the chromatograph where the protein eluted were collected, and additional fractions of question were collected and pooled, and additional fractions of question were collected as well to test for remaining presence of the desired protein. Approximately 12 mL of eluted protein was collected and stored at 4 °C. The chromatograph for this purification is shown in Figure 5. Due to the complication that occurred with the operation of the FPLC, an S column was not performed. Instead, an SDS-PAGE gel was run to confirm that the desired protein was present in the eluted fractions (see Figure 6).
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Figure 5. The chromatogram generated from the nickel affinity purification of P266L T7 RNA Polymerase expressed in LB. The peak labeled (a) denotes non-binding debris that passed through the column as the desired protein was loaded onto the column. Compared to the load peaks in Figures 2 and 3, a single peak is seen during this load due to the manual loading of the column with a 50-mL syringe. The peak labeled (b) denotes non-desired protein and/or biomolecules that eluted during the washing step with 10% buffer B. Peak (c) denotes the elution of the desired protein with 80% buffer B, followed by the beginning of a new baseline due to the imidazole concentration shift.
Figure 6. SDS-PAGE analysis of purification of P266L T7RNA Polymerase expressed in LB. The molecular weight (M.W.) ladder used to compare protein sizes was the Thermo Scientific™ PageRuler™ Prestained 10-180kDa Protein Ladder. (a) Supernatant of lysed cells that was loaded onto the nickel affinity purification column. (b) Waste sample to which protein was mistakenly sent during loading error. (c) Eluted protein collected after nickel affinity purification. The band in lane (c) indicates successful purification of 99 kDa P266L T7 RNA Polymerase.
Conclusions and Future Work

Expression of P266L T7 RNA Polymerase was successfully carried out using IPTG, a synthetic, structural analogue of the lactose molecule. It does this by binding to the lac repressor, which prevents it from binding to the transcription site, ultimately allowing the lac operon containing the P266L T7 RNA Polymerase to be continually expressed. P266L T7 RNA Polymerase has a known molecular weight of approximately 99 kDa. A band consistent with this molecular weight was found in both SDS-PAGE gels, as seen in Figure 4 and Figure 6. This is an indication that the expression of the desired protein was successful.

The protocol that was followed with modifications required that both a nickel affinity purification column as well as an S cation exchange column be used as purification methods for the desired protein. In the first prep of P266L T7 RNA Polymerase expressed in the TB medium, both of these purification methods were carried out with positive results. Due to the error that occurred during the nickel affinity purification of the protein expressed in the LB, an S column was not performed because an SDS-PAGE gel was needed to confirm that protein was recovered from the waste. Nevertheless, the comparison of the two SDS-PAGE analyses, seen in Figure 4 and Figure 6, indicate that there is little difference in protein purity in LB versus TB, as both gels have a substantial amount of protein of appropriate molecular weight. In addition, the S cation exchange column serves to wash remaining nucleotides bound to the P266L T7 RNA polymerase, so it is sufficient to compare the two bands at 99 kDa in both Figure 4 and Figure 6. Furthermore, TB would be more practical to use for expression in the future since its preparation requires fewer steps.

7 Tang et al., “Relaxed Rotational and Scrunching Changes in P266L Mutant of T7 RNA Polymerase Reduce Short Abortive RNAs While Delaying Transition into Elongation.”
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than does the preparation of TB, although both broths are sufficient if expression is desired.

The theoretical pI of the mutant protein is 6.77, according to the ExPASy Bioinformatics Resource Portal. Regarding the S column, the protocol requires a buffer of pH 7, which is higher than the mutant protein’s pI, rendering an overall negatively charged protein. However, the protein’s positive charge density remains sufficient in allowing it to bind to the column, as indicated in the results of the SDS-PAGE gel in Figure 4. Additionally, the buffer remains effective at ± 1 pH unit from the protein’s pI. Since nucleotides are substantially negatively charged, this cation exchange column is adequate for this step; however, optimized results would likely be observed if the buffer pH is below the pI of P266L T7 RNA Polymerase.

Future work regarding P266L T7 RNA Polymerase may include an analysis of its efficiency in comparison to the wild type T7 RNA Polymerase. This analysis would serve to confirm that P266L T7 RNA Polymerase is more efficient than the wild type in terms of its frequency of producing abortive transcripts. If this were confirmed, it would benefit numerous biochemical protocols that require specific expression of a protein, as P266L T7 RNA Polymerase would express desired proteins at a higher yield than its wild type counterpart. In addition, further optimization of the purification protocol for P266L T7 RNA Polymerase may be carried out.

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8 Durniak, Bailey, and Stetiz, “The Structure of a Transcribing T7 RNA Polymerase Complex Captured During Its Transition from Initiation to Elongation.”
Bibliography


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