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Purification of Histidine-Tagged T4 RNA Ligase from *E. coli*

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ABSTRACT

Here we report the construction of a histidine-tagged T4 RNA ligase expression plasmid (pRHT4). The construct, when overexpressed in BL21 (DE3) cells, allows the preparation of large quantities of T4 RNA ligase in high purity using only a single purification column. The histidine affinity tag does not inhibit enzyme function, and we were able to purify 1–3 mg pure protein/g cell pellet. A simple purification procedure ensures that the enzyme is de-adenylated to levels comparable to those found for many commercial preparations. The purified protein has very low levels of RNase contamination and functioned normally in a variety of activity assays.

INTRODUCTION

T4 RNA ligase (E.C. 6.5.1.3) catalyzes the formation of a phosphodiester bond between a 5'-terminal phosphate nucleic acid donor and a 3'-terminal hydroxyl of an acceptor RNA with concomitant cleavage of ATP to AMP and pyrophosphate (2,5,12,15). The enzyme has also been shown to join an acceptor RNA to adenylated substrates, allowing the modification of the 3'-end of RNA strands (3). This has led to the development of strategies that allow the easy isolation and sequencing of RNA strands of unknown sequence (6).

Conventional protocols for the purification of T4 RNA ligase involve the use of 3–5 columns (1,4,5,7,8,13,14) and a relatively large amount of labor to produce high-quality ligase. Moreover, commercial sources of this enzyme can be variable in quality and are relatively expensive. Since our laboratory consumes large quantities of this enzyme, we decided to generate a histidine-tagged T4 RNA ligase variant to purify the enzyme in high yield and purity using a single column. The addition of six

or more histidine residues to either the amino or carboxy terminus of a protein has proven to be a highly successful and popular way of purifying protein (9). The method relies on the high binding affinity between multiple histidine residues and nickel ions, which, when immobilized on a column [typically nickel-nitrilotriacetic acid (Ni-NTA)], allows the specific retention of histidine-tagged proteins.

We modified the T4 RNA Ligase gene 63 (10) to include six histidine (6× His) codons on the 3'-end of the gene. This modified gene was inserted into the multiple cloning site of the pRSET 6a plasmid (11) and transformed into BL21 (DE3) competent cells. Our final T4 RNA ligase construct, pRHT4, was obtained by screening constructs for the best overall expression, purification, and enzyme activity. After optimizing the wash, de-adenylation, and elution steps in our purification protocol, protein activity was compared with commercial enzyme (Amersham Biosciences, Piscataway, NJ, USA). The histidine-tagged T4 RNA ligase compared favorably in a variety of functional tests.

MATERIALS AND METHODS

Plasmid Preparation

The T4 RNA ligase gene 63 on plasmid pMG518 DNA, generated by Heaphy et al. (4), was PCR-amplified and modified using a 5' primer containing a *Nde*I restriction site (5'-CAACGTAG-TACATATGCAAGAAGCTTTTAA-CAATTTA-3'; underscore, *Nde*I site; bold residues, T4 RNA ligase sequence) and a 3' primer containing 6× His codons and a *Hind*III site (5'-CAACGTAGCCAAAGCTTAGTGATG GTGATGGTGATGGTATCCTTCTG GGATAA-3'; underscore, *Hind*III site; italics, reverse complement 6× His codons; bold, reverse complement T4 RNA ligase residues). After removing primers with a QIAquick™ spin column (Qiagen, Valencia, CA, USA), PCR product was added to the plasmid pRSET 6a (11) in the ratio of 1.3:1. This mixture was digested simultaneously with *Nde*I and *Hind*III (New England Biolabs, Beverly, MA, USA). Small DNA fragments liberated during

the digestion were removed by a second spin column. After ethanol precipitation, the cut plasmid and modified T4 RNA ligase gene were joined together using T4 DNA ligase (Invitrogen, Carlsbad, CA, USA).

Plasmid Screening

The ligated mixture was transformed into chemically competent BL21 (DE3) cells, plated onto LB plates containing 100 µg/mL ampicillin, and incubated at 37°C overnight. Sixteen colonies containing inserts were tested for their ability to induce protein, and those that showed good induction were screened for T4 RNA ligase activity after Ni-NTA (Qiagen) purification, following substantially the protocol outlined by Qiagen (9). We found that imidazole had an inhibitory effect on T4 RNA ligase function and dialyzed samples before screening for activity using assays described below. The best construct, pRHT4, was sequenced in both directions across the insertion site. It contains an intact copy of the T4 RNA ligase gene and correctly incorporates 6× His residues (Figure 1).

Expression of T4 RNA Ligase Using pRHT4

Plasmid pRHT4 DNA was transformed, and cells were plated as discussed in the plasmid screening protocol. A single colony was used to inoculate 50 mL media (LB with 100 µg/mL ampicillin), which was grown overnight at 37°C shaking at 250 rpm. Ten milliliters of the overnight culture were then added to 500 mL fresh media while continuing to shake at 37°C. When the absorbance (A_{600}) reached approximately 0.7, cells were induced by the addition of 1 mM IPTG. The culture was incubated for another 2–2.5 h until the A_{600} reached approximately 1.3, and then the cells were harvested by centrifugation at 4000× g for 20 min at 4°C. Induction was confirmed, and overexpression was estimated by SDS-PAGE. T4 RNA ligase was expressed at the level of 5–7 mg/g pelleted cells (We obtained 4–6 g cell pellet/L culture). Pelleted cells from 500 mL of culture were resuspended in 5 mL lysis buffer, and the resulting

mixture dripped into liquid nitrogen and stored at -80°C .

Purification Using a Single Ni-NTA Column

Typically, we took 1 mL resuspended pellet and added lysis buffer supplemented with 1 mM PMSF (Amersham Biosciences), 5 mM β -mercaptoethanol (Caledon Laboratories, Ontario, Canada), 1 mg/mL lysozyme (Sigma, St. Louis, MO, USA) and 5 $\mu\text{g/mL}$ DNase I (Sigma) to make up a final volume of 10 mL. The mixture was incubated on ice for 30 min. Cells were disrupted by vortex mixing vigorously for 30 s and cooling on ice for 3 min. This was repeated a total of five times. All the following steps were carried out at 4°C . The lysate was centrifuged at $10\,000\times g$ for 20 min to pellet cellular debris, and the supernatant was carefully decanted. Approximately 2 mL 50% Ni-NTA agarose slurry (Qiagen; 0.3 $\mu\text{mol/mL}$ binding capacity) was washed with lysis buffer and then added to the lysis supernatant. This solution was mixed gently for 1 h before loading onto an empty

column. The column was sequentially washed with 8 mL each of W1, W2, and W3 buffers. Bound protein was collected by passing elution buffer (E) over the column. The void volume was discarded and about 1 mL of eluate was found to contain the majority of the protein. The eluted protein was dialyzed against storage buffer overnight (dialysis tubing MWCO 12–14 kDa; VWR International, West Chester, PA, USA). We typically recovered approximately 300 μL dialyzed protein, which was stored at -20°C and used for our activity assay experiments. Figure 2 shows the course of the purification.

The following buffers were all pH 8.0 unless otherwise noted: lysis, 50 mM KH_2PO_4 , 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 300 mM NaCl, 5 mM imidazole; W1, 50 mM KH_2PO_4 , 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 300 mM NaCl, 10 mM imidazole; W2, 50 mM KH_2PO_4 , 300 mM NaCl, 10 mM imidazole; W3, 50 mM KH_2PO_4 , 300 mM NaCl, 20 mM imidazole; E, 50 mM KH_2PO_4 , 300 mM NaCl, 100 mM imidazole; storage, 25 mM HEPES (pH 7.5), 2 mM DTT, 0.1 mM EDTA, 50% glycerol. We added pyrophosphate to

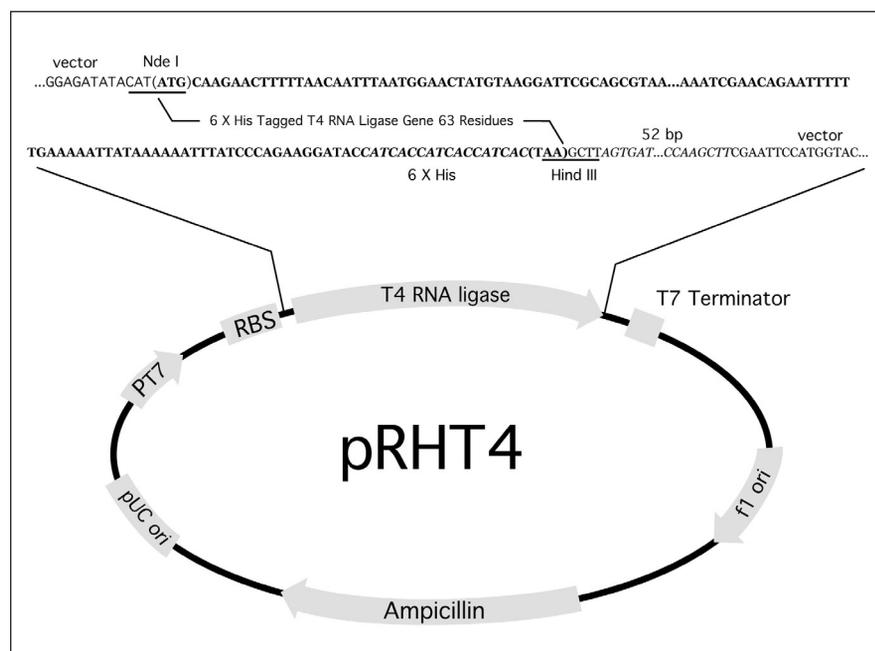


Figure 1. Map of the pRHT4 (3.98 kb). The sequence detail shows in order: part of pRSET 6a vector sequence, underscore: *Nde*I site, parentheses: start codon, bold: T4 RNA ligase gene 63 residues (1125 bp), bold italics, 6x His codons, parentheses: stop codon, underscore: *Hind*III site, italics: 52-bp fragment (see Results and Discussion section for details), followed by pRSET 6a vector sequence. The remaining 2.8 kb constitutes the pRSET 6a vector, which contains a T7 promoter (PT7), ribosome binding site (RBS), T7 terminator, f1 origin (f1 ori), ampicillin resistance gene (Ampicillin), and pUC origin (pUC ori) (Reference 11 and www.invitrogen.com).

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the lysis and W1 buffers to convert the adenylated form of T4 RNA ligase to its de-adenylated form (4,10,14).

Activity Assays

An acceptor RNA 124 bp long (ending with the sequence rAGC) was ligated to cytidine 3', 5'-[³²P] bisphosphate (*pCp, 3000 Ci/mmol; New England Nuclear, Boston, MA, USA) in the presence of ligase buffer [50 mM HEPES, pH 8.3, 10 mM MgCl₂, 3.3 mM DTT, 10 μg/mL BSA, 8.3% glycerol (3)], 5 μM ATP, 3 μM RNA acceptor, 0.04 μM *pCp, and variable amounts of enzyme. We titrated the activity of our enzyme using 0.8 U/μL Amersham enzyme (supplied at 40 U/μL) as a reference (Figure 3a). The same acceptor RNA was also ligated to a 5'-[³²P]-labeled 21-bp RNA (sequence: 5'-rU-UUGCUAUAAGAUGGGUGGCA-3') by incubating the enzyme in the presence of ligase buffer, 3 μM acceptor RNA, 0.2–0.8 μM donor RNA, 1 mM ATP, and enzyme. Here we used 1.6 U/μL Amersham enzyme as a reference (Figure 3b). RNase activity was tested by incubating a 0.35 μM solution of a 281-bp internally labeled RNA in the presence of ligase buffer and 1.6 U/μL enzyme in a time course lasting 6 h. De-adenylation was determined by performing a *pCp ligation assay as described above with or without ATP, the fraction reacting without ATP being proportional to the amount of adenylated enzyme in the reaction.

All time courses were carried out at room temperature (23°C), and time points were stopped by the addition of an equal volume of gel loading buffer (97% formamide, 10 mM EDTA, bromophenol blue). Samples were separated on 6% or 12% polyacrylamide gels and analyzed using a Storm™ 820 Phosphorimager (Amersham Biosciences). One unit of enzyme is defined by Amersham Biosciences as the amount of enzyme required to convert 1 pmol *pCp into acid insoluble form in 10 min at 5°C using (A)_n as substrate.

RESULTS AND DISCUSSION

Sequencing pRHT4 through the ligase insert showed that our construct

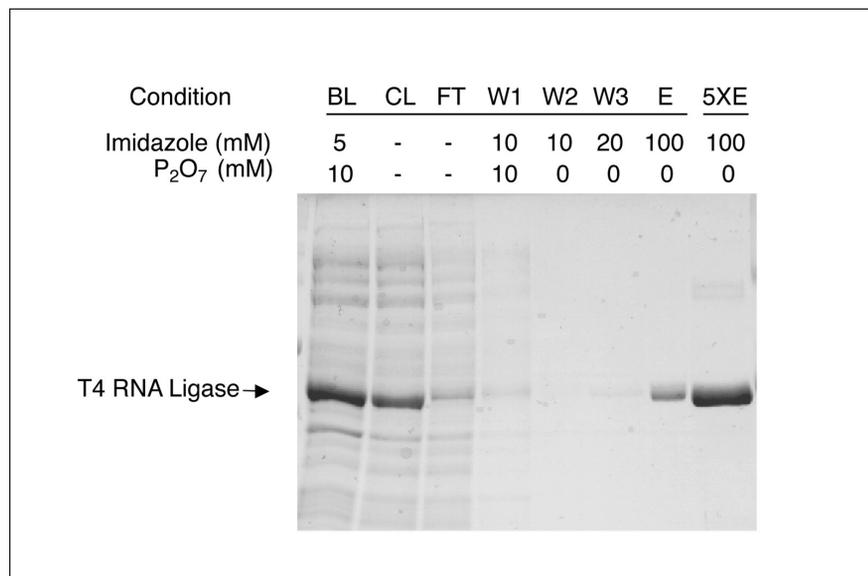


Figure 2. Nickel affinity purification of protein expressed by pRHT4 plasmid. BL21 (DE3) cells were transformed with pRHT4 plasmid and induced. The frozen cells were resuspended in lysis buffer (BL), and the supernatant resulting from lysis (CL) was mixed with Ni-NTA agarose and packed onto a column. The flow through (FT) was collected, and the column was washed with steadily increasing amounts of imidazole (W1, W2, and W3). Pyrophosphate was added to the lysis (BL) and the first wash (W1) buffers to de-adenylate the enzyme. Bound protein was eluted (E) by addition of 100 mM imidazole. After normalizing for sample volume, proteins were visualized on a 10% SDS polyacrylamide gel stained with Coomassie Blue® using standard protocols. A final lane was loaded with five times the normalized amount of elutate (E) to access purity.

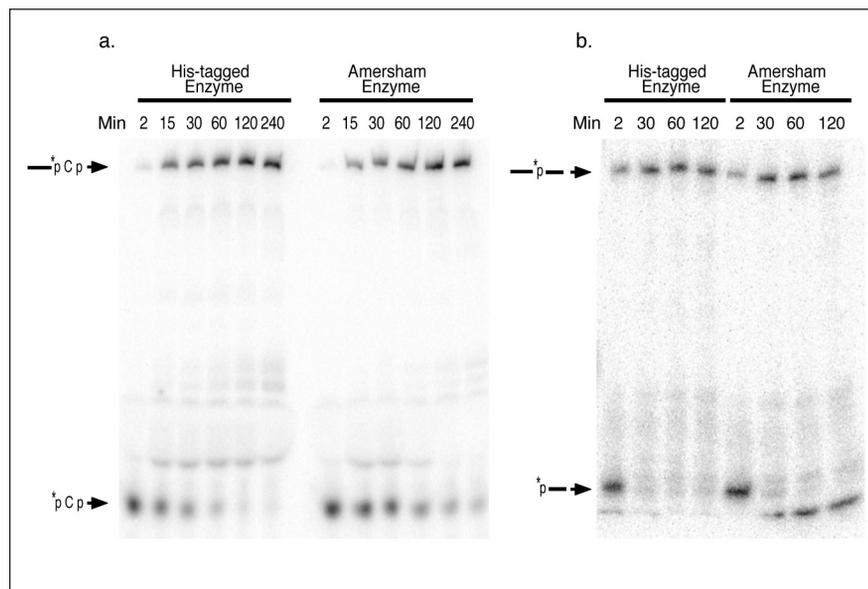


Figure 3. Activity comparison of histidine-tagged T4 RNA ligase to commercial enzyme. (a) Time courses showing the ligation of a 124-bp acceptor RNA to *pCp donor at room temperature, comparing approximately equal amounts of histidine-tagged T4 RNA ligase and Amersham enzyme (0.8 U/μL). Quantification indicates that 74% and 78% of the *pCp (histidine-tagged and commercial, respectively) are incorporated into full-length RNA after 240 min incubation. The band immediately above *pCp (which forms in the presence of high ATP concentrations) is most probably adenylated *pCp, while the remaining bands are degradation products of the acceptor RNA generated either before or during the ligation reaction. (b) Time courses showing the ligation of the same acceptor to a 5'-[³²P] 21-bp RNA donor at room temperature with approximately equal amounts of histidine-tagged T4 RNA ligase and Amersham enzyme (1.6 U/μL). The lower arrows indicate the radiolabeled donor species for each reaction, while the upper arrows indicate the ligated RNA product. The specific activity of our enzyme was 8–24 × 10³ U/mg protein.

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contained an intact copy of the T4 RNA ligase gene and 6× His residues as expected (Figure 1). Somewhat surprising was the discovery of a 52-bp insert immediately after the *Hind*III site of our construct. The sequence shown here in bold [...*CATCAC*(TAA)*GCTTAGTG-ATGGTGATGGTGATGGTATCC-TTCTGGGATAACAACGTAGCC-AAGCTTCGAATT*... (italics, part of histidine tag; parentheses, stop codon; underscore, *Hind*III site; bold, 52-bp fragment, followed by the pRSET 6a vector sequence)] is due to an unusual ligation event that occurred during the preparation of this construct. The insertion likely has little effect on the over-expression and activity of T4 RNA ligase because of its presence after the stop codon for the gene.

The recombinant gene expressed well in *E. coli*, and we successfully obtained 1–3 mg pure T4 RNA ligase (estimated by SDS-PAGE) with a total activity of approximately 24 000 U from 1 g cell pellet using a single Ni-NTA column purification. This recovery compares favorably with the best yields found by conventional purification methods (1,4,5,7,8,13,14) and is nearly completely free of RNase contamination. A low concentration of internally labeled RNA (0.35 μM) when incubated in the presence of approximately 1.6 U/μL enzyme degraded at a rate of 0.4%/h. This was only 2.5 times the background rate of hydrolysis measured in the absence of enzyme.

The addition of a histidine affinity tag does not inhibit enzyme function, but it is critical that imidazole be removed by dialysis before using the enzyme. Enzyme activity was almost not evident in the presence of 250 mM imidazole but could be rescued by dialysis into storage buffer overnight.

Normally, T4 RNA ligase requires the pre-adenylation of the enzyme using ATP before it can join together phosphorylated donor and acceptor strands (15) and is often partially pre-adenylated inside bacteria. Therefore, purification of the enzyme typically results in both the adenylated and unadenylated enzyme being recovered. The de-adenylated form of the enzyme is desirable, as it allows the reaction of pre-adenylated substrates with an acceptor strand, and so provides a flexible

method of adding modified nucleotides to the 3'-end of RNA (3,16). By simply adding pyrophosphate to the lysis and W1 buffers, we found that the enzyme was de-adenylated 5-fold (a level comparable to the Amersham enzyme). This enzyme efficiently ligated adenylated 4-thiouridine monophosphate onto an acceptor RNA in the absence of ATP, again confirming that the enzyme was substantially de-adenylated.

In summary, we have generated a histidine-tagged T4 RNA ligase construct, allowing the easy purification of large amounts of the enzyme using a single purification column. The purified protein contains negligible amounts of RNase contamination and allows the efficient ligation of phosphorylated and adenylated constructs to RNA.

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