Expression and Purification of the *Thermus thermophilus* Argonaute Protein

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[Abstract] The Argonaute protein of *Thermus thermophilus* (*Tt*Ago) has recently been studied in detail. For its *in vitro* characterization, *Tt*Ago was purified after heterologous expression in *Escherichia coli* (*E. coli*). As *Tt*Ago expression is toxic, a tightly controlled system was used for protein expression. The expression strain *E. coli* KRX carries a chromosomal T7 RNA polymerase gene under control of a rhamnose promoter. The *ago* gene is expressed via an IPTG-inducible T7 promoter. This allows for tightly (double) controlled expression of (toxic) *Tt*Ago. Here, we describe the steps required for controlled expression and purification of this toxic protein.

Materials and Reagents

A. Expression of *Tt*Ago in *E. coli* KRX
   1. Glycerol stock of *Escherichia coli* KRX (*Promega corporation, catalog number L3002*) [encodes a T7 RNA polymerase gene under control of a rhamnose promoter], transformed with plasmids pRARE (*EMD Millipore, plasmid of Rosetta™ (DE3) Competent Cells, catalog number: 70954-3*) [encodes tRNAs for rare codons to enhance protein translation efficiency] and pWUR702 [pCDF-1b derivative, with *T. thermophilus* HB27 TT_P0026 *ago* gene insert fused to an N-terminal strep(II)-tag, expression under control of an IPTG-inducible T7 promoter] (*Addgene, catalog number: 53079*).
      Note: The plasmid pWUR703 (Addgene, catalog number: 53082) can be used for expression of TtAgoDM (Double Mutant, D478A, D546A). Expression and purification of this protein is identical to TtAgo expression and purification.
   2. 1000x Chloramphenicol solution (34 mg/ml) dissolved in 100% ethanol
   3. 1000x Streptomycin solution (50 mg/ml) dissolved in MilliQ H_2O
   4. 20% D-glucose solution dissolved in MilliQ H_2O (sterile filtered)
   5. 20% L-rhamnose solution dissolved in MilliQ H_2O (sterile filtered)
   6. 1 M IPTG dissolved in MilliQ H_2O (sterile filtered)
   7. LB medium (see Recipes)

B. Purification of *Tt*Ago
   1. Cell pellet from o/n *Tt*Ago expression (see above)
   2. Strep-Tactin Sepharose® 50% suspension (IBA, catalog number: 2-1201-XXX)
   3. 1.2 ml Bio-Spin® Chromatography Columns (Bio-Rad Laboratories, catalog number: 732-6008)
   4. Buffer I (see Recipes)
   5. Buffer II (see Recipes)
   6. Buffer III (see Recipes)

Equipment

A. Expression of *Tt*Ago in *E. coli* KRX
   1. 50 ml Greiner tube
   2. 2.5 L Erlenmeyer flask
   3. Centrifuge
   4. 37 °C shaker incubator
   5. 20 °C shaker incubator
   6. Ice-water bath (water and ice mixed)

B. Purification of *Tt*Ago
   1. French-Press, sonicator, or equivalent for cell disruption
   2. Centrifuge

Procedure

A. Expression of *Tt*Ago in *E. coli* KRX
   1. Take 10 ml LB medium in a 50 ml Greiner tube.
   2. Add 10 µl 1,000x chloramphenicol solution.
   3. Add 10 µl 1,000x streptomycin solution.
   4. Add 200 µl 20% D-glucose solution.
   5. Inoculate the culture from the glycerol stock using a sterile pipette tip.
   6. Incubate the culture o/n at 37 °C in a shaker incubator at 180 rpm.
   7. After o/n incubation, centrifuge culture for 10 min at 4,000 *x g* at room temperature.
   8. Remove the supernatant to remove any traces of D-glucose - this is important as D-glucose traces can prevent L-rhamnose uptake (and thereby represses *Tt*Ago expression).
   9. Resuspend the cell pellet in 1 ml LB medium.

10. Take a 2.5 L Erlenmeyer containing 1 L LB medium.
11. Add 1 ml 1,000x chloramphenicol solution.
12. Add 1 ml 1,000x streptomycin solution.
13. Add resuspended cells.
14. Incubate the culture at 37 °C in shaker incubator at 140 rpm.
15. Monitor the OD_{600nm} of the culture. If an OD_{600nm} of 0.7-0.8 is reached (this takes ~3-4 h), transfer the culture to an ice-water bath to cold-shock it for 15 min. This step slows down the metabolism of *E. coli* and triggers expression of cold-shock proteins which may aid *Tt*Ago folding during expression.
16. Add 1 ml 1 M IPTG and 5 ml 20% L-rhamnose solution to the culture to induce expression.
17. Transfer the culture to a 20 °C shaker incubator at 140 rpm for o/n (+/-16h) expression.
18. Harvest the cells by centrifuging culture for 15 min at 6,000 *x g* at 4 °C.
19. Remove the supernatant. You should typically end up with 4-5 grams of cell pellet.

B. Purification of *Tt*Ago

1. If using a French Press for cell lysis: Resuspend cell pellet in buffer I (5 ml Buffer I per gram of cell pellet) and pass cell suspension through French press two times at 16,000 psi (see Note c).

   If using a sonicator for cell lysis: Resuspend cell pellet in Buffer I (3 ml buffer I per gram of cell pellet), and lyse the cells by sonication. Use a tip and sonication protocol suitable for lysis of large volume cell suspensions.
2. Centrifuge for 30 min at 30,000 *x g* at 4 °C.
3. Transfer the supernatant (cell free extract) to a clean tube.
4. Prepare a Strep-Tactin Sepharose column by loading 500 µl of the 50% suspension in a 1.2 ml Bio-Spin chromatography column (final column volume is 250 µl).
5. Wash column by loading the column three times with 500 µl buffer I.
6. Load the cell free extract on the column.
7. Wash the column by loading it three times with 750 µl buffer I.
8. Wash the column by loading it three times with 750 µl buffer II.
9. Elute the protein by loading the column three times with 250 µl buffer III, collect the flow through. Typical yields of *Tt*Ago elution fractions are in the 0-2.5 µM range for fraction 1 and 3 and in the 5-10 µM range for fraction 2. Typical yields of *Tt*AgoDM are in the 0-2.5 µM range for fraction 1 and 3 and in the 2.5-7.5 µM range for fraction 2.

   Notes:

   a. *Depending on experiments, $MgCl_2$ and $MnCl_2$ concentrations can be lowered to 0.5 mM, or left out. NaCl concentration can be lowered to 0.5 M, however, TtAgo*

(especially at higher protein concentrations) is more stable at 1 M NaCl. At lower concentrations salt (especially bellow 250 mM), TtAgo is instable at protein concentrations higher than 5 µM).

b. *The protocol above describes purification of guide-free TtAgo. If guide co-purification is desired, replace all buffer I in this experiment with buffer II.*

c. *Using a French Pressure cell or sonicator gives more or less the same yield – TtAgo is very stable and little to no protein will be lost during sonication. Keep in mind however, that sonication usually is less suitable for large volumes, and a protocol suitable for lysis of a large volume of cell suspension should be applied.*

Representative data

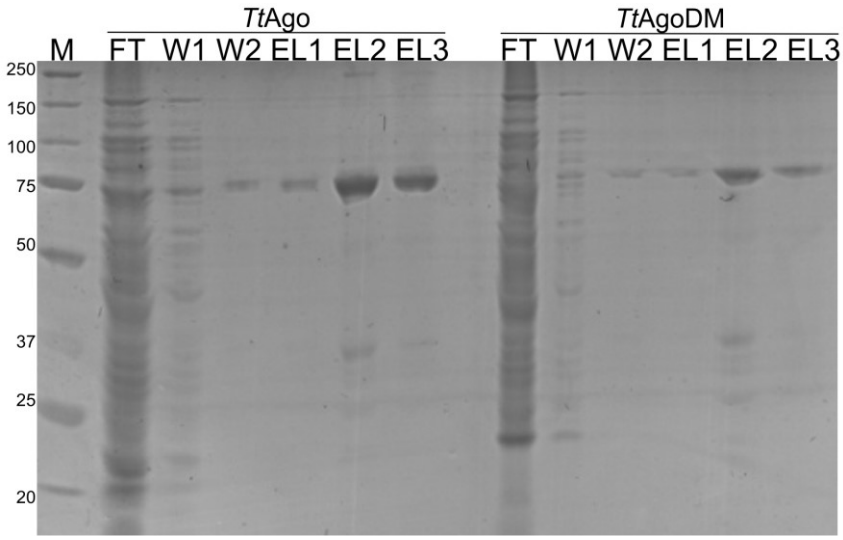

Figure 1. Coomassie Brilliant Blue stained 12% SDS-PAGE gel of *Tt*Ago (left) and *Tt*AgoDM (right) purification samples. M: Bio-Rad precision plus protein marker. Marker band sizes are indicated in kDa. FT: Column flow through fraction of loaded cell free extract. W1: Column wash fraction with buffer I. W2: Column wash fraction with buffer II. EL1-EL3: Elution fractions 1-3. The protein appears as a band with a size of +/-75kDa.

Recipes

1. LB medium (1 L)
   10 g tryptone
   5 g yeast extract
   10 g NaCl
   Fill up to 1 L with demiwater

Set pH to 7.5 with NaOH

2. Buffer I
   20 mM Tris-HCl (pH 8)
   1 M NaCl
   2 mM $MgCl_2$
3. Buffer II
   20 mM Tris-HCl (pH 8)
   1 M NaCl
   2 mM $MnCl_2$
4. Buffer III
   20 mM Tris-HCl (pH 8)
   1 M NaCl
   2 mM $MnCl_2$
   2.5 mM Biotin
   Note: Biotin can be replaced with d-Desthiobiotin, which will allow for column regeneration.

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References

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