Laboratory of Dr. Wayne L. Hubbell
Protocol adapted by Carlos J. López, PhD
Protocol can be downloaded from: http://www.biochemistry.ucla.edu/biochem/Faculty/Hubbell/

Expression and purification of T4 lysozyme mutants from inclusion bodies

***Published in López et al.*[1]**

* Most T4L mutants can be isolated from the soluble fraction of the crude extract as described by Mchaourab et al.[2] The protocol described below has been adapted from *Liu et al*.[3] to isolate unstable mutants that do not express in the soluble fraction.

1. **Protein expression**: Start a 20ml overnight LB Amp culture from a BL21 (DE3) colony. Add the 20ml of starter culture to 1L of LB Amp media and incubate at 37˚C. Add IPTG to a final concentration of 1mM once the OD_{600} has reached ~ 1AU to induce expression and incubate for 3 hours. Spin down the cells at 6,000 rpm and resuspend the pellet in lysis buffer consisting of 25mM Tris-base, 25mM MOPS, 0.1mM EDTA at pH 7.6. Freeze the cells at -20˚C.
2. **Isolation of inclusion bodies:** Thaw the cells and sonicate the crude extract 2 times (5 minutes of sonication each time) and centrifuge for 15 minutes at 15,800 rpm. **Keep the pellet**! **Note**: Most of the T4L should stay in the pellet (inclusion bodies), while the supernatant should contain mostly soluble contaminants, however some soluble T4L may also be present in the supernatant, which can be isolated using the conventional purification protocol.
3. Wash the inclusion bodies by resuspending the pellet in 20ml of lysis buffer (For the first wash, Triton X should be added to a final concentration of 2% v/v in order to remove weakly bound contaminants). Break the clumps with a glass rod and sonicate the sample for 5 minutes. Incubate the solution in the nutator for 1-2 hours at 4˚C. Centrifuge for 15 minutes at 15,800 rpm. Discard the supernatant. Keep the pellet.
4. Resuspend the collected pellet in 25mL of wash buffer #2 consisting of 50mM Tris-Cl and 2.5% n-octyl-β-D-glucopyranoside at pH 8, sonicate for 5 minutes and incubate in the nutator for 3 hours at 4˚C. **Note**: The purpose of the 2.5% n-octyl-β-D-glucopyranoside is to resolubilize membrane-bound protein contaminants.
5. Centrifuge for 15 minutes at 15,800 rpm and keep the pellet. The inclusion body pellet has been washed 2 times at this point to remove any soluble and membrane-bound contaminants and it is ready for solubilization. This final inclusion body pellet can be frozen and stored until needed.
6. **Resolubilization of inclusion bodies:** To solubilize the inclusion bodies, resuspend the pellet in 25ml of 10mM glycine. Use a glass rod to break the clumps and sonicate for 5 minutes. Add solid urea to a final concentration of 4M under intense stirring. Titrate the solution with phosphoric acid to pH 3.0 (only 1-2 drops is needed to reach pH 3). **Note**: the solution should turn translucent when the pH is lowered to 3. Centrifuge for 15 minutes at 15,800 rpm. Dialyze the supernatant overnight against 2L of a solution of 10mM sodium citrate, 15% glycerol, 1mM β-cyclodextrin, 5mM DTT pH 3.0. Follow by one-day dialysis against 2L of buffer consisting of 10mM sodium citrate, 15% glycerol, 1mM β-cyclodextrin, 5mM DTT pH 5.0. There will be a significant amount of insoluble precipitate following dialysis, which should be removed *via* centrifugation for 15 minutes at 15,800 rpm.
7. **Purification of resolubilized protein:** Filter the supernatant through a 0.2μm membrane. Load the solution into a CM FF Sepharose column and elute the protein with a linear gradient of 0 - 400mM NaCl in 10mM sodium citrate, 5mM DTT at pH 5.0 There is no flow through peak. The T4L fraction will elute at a conductivity of 25mS/cm or 240 mM NaCl. I usually do a 2 CV gradient from 0 to 240 mM NaCl (5ml/min) and leave it at that concentration of salt until the

protein elutes (see figure shown below). From this point on, keep protein on ice at all times since certain mutants are unstable.

8. **Spin labeling**: Filter the solution through a 0.2 μm membrane and remove DTT by using HiTrap desalting column (GE Healthcare) equilibrated with 50mM MOPS, 25mM NaCl at pH 6.8. Incubate the eluted protein overnight with 5 to 10-fold molar excess of MTSL.
9. Some mutants may form high molecular weight oligomers during the *in-vitro* refolding step, thus an additional gel filtration step (Superdex 75) is required to remove any soluble oligomeric species in the final sample. For the gel filtration step, use the following buffer: 50mM MOPS and 25mM NaCl at pH 6.8. The monomeric peak should have a retention volume of ~15.1ml. This is the final step in the isolation of T4 lysozyme from inclusion bodies.
10. Concentrate the spin-labeled protein using the Amicon 10,000 MWCO to about 400μM for EPR studies.

T4L: ε_{280} = 24.750 M^{-1} cm^{-1}; MW = 18,600 Da (1 AU = 0.75 mg/ml)

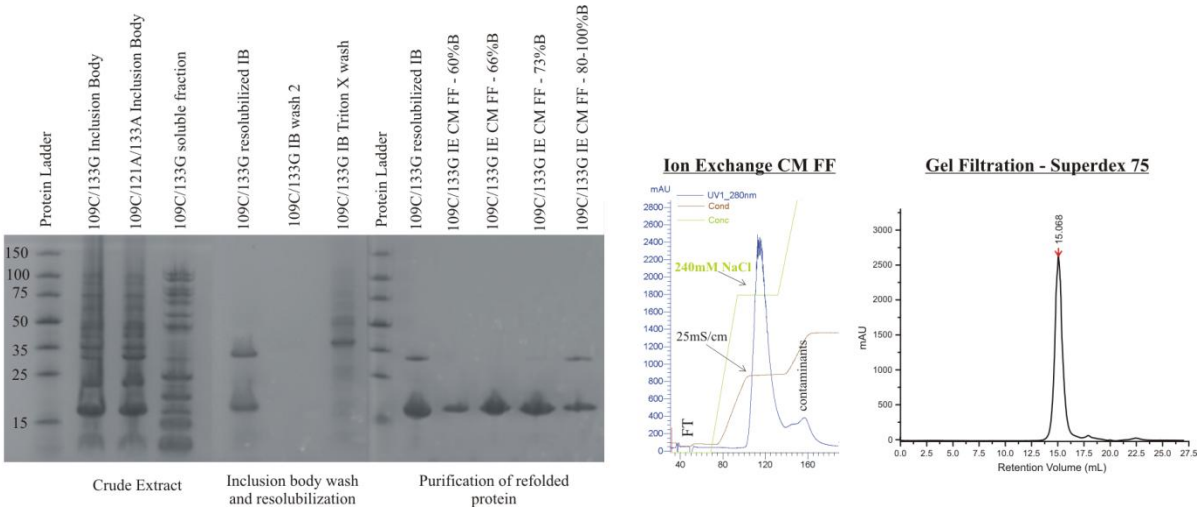

Figure 1: Isolation of T4L proteins expressed in inclusion bodies. Example of purification of two mutants (109C/121A/133A and 109C/133G) is shown. SDS-PAGE of indicated fractions is shown (left panel). Typical elution profile from ion exchange column (CM FF) and gel filtration profile are shown in the middle and right panel, respectively.

References:

(1) Lopez, C. J.; Yang, Z.; Altenbach, C.; Hubbell, W. L. *Proceedings of the National Academy of Sciences of the United States of America* 2013.

(2) Mchaourab, H. S.; Lietzow, M. A.; Hideg, K.; Hubbell, W. L. *Biochemistry* 1996, *35*, 7692.

(3) Liu, L.; Baase, W. A.; Michael, M. M.; Matthews, B. W. *Biochemistry* 2009, *48*, 8842.