Refolding Thrombomodulin EGFs 4-6 in *E. coli*

Avery M. Bonner, Lindsey D. Handley, Kyle N. Stearns and Elizabeth A. Komives

Department of Chemistry & Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California

ABSTRACT

Thrombomodulin is the protein that regulates the formation of blood clots by changing thrombin's function. Our lab has discovered a truncation of thrombomodulin called TM456m, which contains EGFs 4-6 and has complete thrombin activity. Our lab is trying to refold this protein from *E. coli* in order to expedite its purification. I transformed *E. coli* with the TM456m TrpLeader vector, expressed protein and purified inclusion bodies, and resolubilized these inclusion bodies and refolded the protein on a Ni column. We assayed the Ni column fractions using a PC assay, SDS PAGE gel, and S75 size exclusion column. The Ni column fractions have high activity, suggesting some of the protein was folded correctly.

The Coagulation Cascade

When the body experiences trauma, releasing tissue factor to the blood stream, a series of proteases become activated, which ultimately results in clot formation. This pathway is called the coagulation cascade. One of the most important proteases in the coagulation cascade is thrombin, which specifically cleaves fibrinogen into fibrin. However, thrombin's function is regulated by thrombomodulin– when bound to thrombomodulin, thrombin no longer cleaves fibrinogen, but cleaves protein C, which shuts down the coagulation cascade.

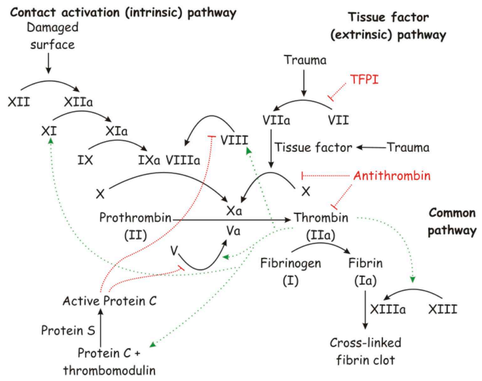

Figure 1

Thrombomodulin

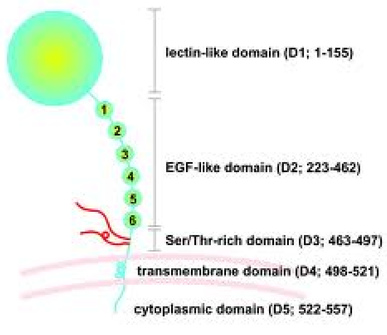

Figure 2

Thrombomodulin is a large multi-domain protein expressed on the endothelial layer of blood vessels. However, only Epidermal Growth Factor-like repeats 4-6 of thrombomodulin have thrombin activity.

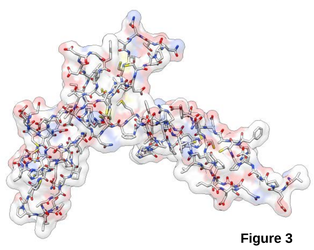

Figure 3

Figure 4

The sequence of the TM456m TrpLeader vector has several unique characteristics, beginning with the 9x His Tag, which binds to a Ni column and aids purification. TM456m has 8 cysteines, which form 4 disulfide bonds. These disulfide bonds are the biggest obstacle in correctly forming the native protein.

The TrpLeader sequence targets the entire protein for inclusion bodies in *E. coli.*

The molecular weight of the full protein is 28,000 Da.

Inclusion Body Purification Protocol

1. Growth and expression of TM456m in *E. coli*
2. Lyse cells, sonicate, add triton X-100 and PMSF
3. Isolate inclusion bodies by centrifugation
4. Wash with imidazole and re-isolate by centrifugation

Refolding Protocol

1. Resuspend inclusion bodies and solubilize in 8M urea
2. Add GSH/GSSG and mix overnight
3. Load onto Ni-NTA column
4. Wash with resolubilizing buffer with 20 mM imidazole
5. Add refolding buffer (6% sarkosyl, 50 mM Tris, pH 8.2, 100 mM NaCl, 10 mM $CaCl_2$, 1mM GSH/ 0.1 mM GSSG)
6. Wash with 25 mM imidazole, 1x TBS, elute with 250 mM imidazole, 1x TBS and collect 16 2ml fractions

PC Assay Protocol

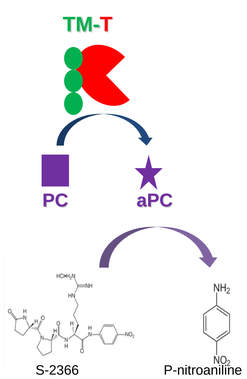

Figure 5

1. Add 20 µL of BSA/Ca^{2+} to each well
2. Add 100 µL of 1x TBS to the negative control, 90 µL to the others
3. Add 15 µL of Th solution to each well
4. Add 10 µL of TM samples to each well, wait 10 minutes
5. Set timer for 22 minutes, add 20 µL PC to first well, start timer, and finish adding
6. Set timer for 12 minutes, after 22 minutes is over, add 40 µL HAT to the first well, start timer, and finish adding
7. Add 20 µL of 100mM Tris to each well and then add 15 µL of S2366 (the chromogenic substrate) to each well
8. Start the run in the plate reader

PC Assay Results

-	+	1	2	3	5	6	7	9	11	13	15
0.7	37.9	74.4	61.2	138.1	119.1	45.9	2.24	3.29	2.24	1.36	1.10

Table 1

PC assay of the Ni-NTA fractions shows high activity of fractions 1-5.

Gel Results

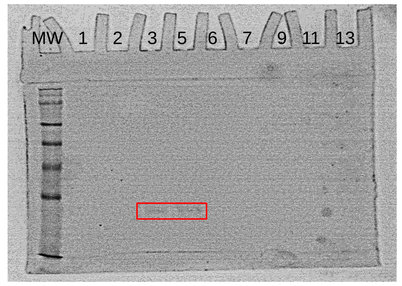

Figure 6

This is a 13% SDS PAGE gel of refolded thrombomodulin fractions collected off of the Ni column.

Lane 1: Molecular weight marker
Lanes 2-10: Ni fractions

The fractions with the highest activity had the highest concentrations of protein.

S75 Results

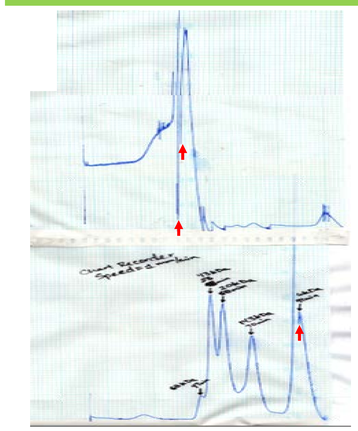

Figure 7

The S75 size exclusion column separates proteins based on size. We ran Ni fractions 1-5 (shown on the top graph), as well as a set of molecular weight standards (shown on the bottom graph).

Standards used:
BSA – 68,000 Da
Ovalbumin– 43,000 Da
Trypsin Inhibitor– 20,000 Da
Lysozyme– 14,300 Da
Insulin– 6,000 Da

The arrows indicate where we changed the range on the detector.

A lot of the refolded protein contained intermolecular disulfide bonds, making them elute from the column faster.

CONCLUSIONS AND FUTURE DIRECTIONS

- We were able to refold and get samples of active TM456m.
- In the future, the lab will pursue refolding this protein at a larger scale and will experiment with cleaving the TrpLeader sequence from the active protein.
- In addition, the lab will work to reduce the amount of intermolecular disulfide bonding that occurs during refolding.

References

- (Figure 1) http://chemwiki.ucdavis.edu/http%3A%2F%2Fchemwiki.ucdavis.edu/Wikitexts/Truman_Chem_421%3A_Nagan/Blood_Clotting_Cascades
- (Figure 2) www.readcube.com/articles/10.1186/1423-0127-19-34?locale=en
- (Figure 5) Figure created by previous Komives Lab member Nick Treuhiet