Coagulation Cascade

Hemostasis is the halt of blood flow through a damaged blood vessel. Trauma that causes hemostasis occurs through the exposure of tissue factor to factor VII, which activates factor X, activating prothrombin. When hemostasis occurs, thrombin cleaves fibrinogen into fibrin which promotes clot formation (the coagulation cascade). When thrombin binds thrombomodulin, on the surface of cells, thrombin’s activity changes from procoagulant to anticoagulant, resulting in the activation of protein C. Protein C is an inhibitor of the coagulation cascade, thus stopping the formation of clot.

Thrombomodulin

Thrombomodulin is a protein with 557 amino acids that is present on the endothelium of all cells lining blood vessels. It binds to thrombin, a protease that, unbound, promotes coagulation by cleaving fibrinogen to make clots. Thrombomodulin has five domains; the tail has an unknown function, the transmembrane domain anchors the protein, the EGF-like repeats change the function of thrombin, and the lectin domain is an anti-inflammatory molecule.

TM456t

It binds to thrombin, a protease that, unbound, promotes coagulation by cleaving fibrinogen to make clots. Thrombomodulin has five domains; the tail has an unknown function, the transmembrane domain anchors the protein, the EGF-like repeats change the function of thrombin, and the lectin domain is an anti-inflammatory molecule.

TM456t

Coagulation Cascade

Method

Large Scale Refolding

- Growth and expression of TM456t in E. coli
- Induction body prep
- Load resolubilized inclusion bodies in Ni column
- Refold protein solution days in refolding buffer
- Elute with imidazole

Small Scale Refolding Screen

- Growth and expression of TM456t in E. coli
- Induction body prep
- Load 8M urea inclusion bodies in Ni column
- Refold protein in epp tube for three days
- Elute with imidazole

Results

Figure 1. 15% reducing SDS-PAGE gel of our N364D TM456t prep.

Lane 1 is the molecular weight ladder.

Lane 2 is the pre-induction sample of our 1L E. coli growth.

Lane 3 is the post-induction sample of our 1L E. coli growth.

Lane 4 is the supernatant after the first sonication from the inclusion body prep.

No TM should be present because TM should be in the inclusion bodies in the pellet.

Lane 5 is the supernatant after the second sonication from the inclusion body prep.

Lane 6 is the TM eluted with 25mM imidazole after washing the column with 1x TBS 25mM imidazole.

Figure 2. Relative activities of N364D TM456t refolded at each of the given conditions. Activity of the protein was divided by the activity of the positive control in each PC assay. Each of the refolding buffer conditions also contained 50mM Tris pH 8.2, 200mM NaCl, 10mM CaCl2, 0.1mM ox glutathione, and 1mM red glutathione.

- 0%: 0.89 ± 0.09
- 1%: 0.89 ± 0.09
- 10%: 0.89 ± 0.09
- 20%: 0.89 ± 0.09

Results for N364D/N391D TM456t Large Scale Refolding

- 15% reducing SDS-PAGE gel of our N364D/N391D TM456t prep.
- Lane 1 is the molecular weight ladder.
- Lane 2 is the pre-induction sample of our 1L E. coli growth.
- Lane 3 is the post-induction sample of our 1L E. coli growth.
- No TM should be present because TM should be in the inclusion bodies in the pellet.
- Lane 5 is the supernatant after the second sonication from the inclusion body prep.
- Lane 6 is Ni fraction 6 from N364D/N391D prep.

Figure 3. 15% reducing SDS-PAGE gel of our N364D/N391D TM456t Ni fractions.

Lane 1 is the molecular weight ladder.

Lane 2-6 correspond to Ni fractions 4-8 eluted from our column refolding prep.

Figure 4. Results of the PC Assay of 2mL fractions eluted from the Ni column of the N364D/N391D large scale refolding prep. Refolded protein was eluted with 1x TBS 25mM imidazole after washing the column with 1x TBS 25mM imidazole.

Figure 5. 15% reducing SDS-PAGE gel of our N364D/N391D TM456t Ni fractions.

Lane 1 is the molecular weight ladder.

Lanes 2-6 correspond to Ni fractions 4-8 eluted from our large scale N364D/N391D on-column refolding prep.

Figure 6. 15% non-reducing SDS-PAGE gel of our N364D/N391D TM456t Ni fractions.

Lane 1 is the molecular weight ladder.

Lanes 2-6 correspond to Ni fractions 4-8 eluted from our large scale N364D/N391D on-column refolding prep.

Figure 7. Ni fractions 4-11 of the N364D/N391D large scale on column prep were concentrated and loaded onto an S75 iexclusion column. The S75 trace was overlaid onto the traces of previous wild type and N364D TM4565 S75 purifications.

Figure 8. 15% reducing SDS-PAGE gel of our N364D/N391D TM456t prep. Refolded protein was eluted with 1x TBS 25mM imidazole after washing the column with 1x TBS 25mM imidazole.

Conclusions

- Low glycosyl, low CHAPS refolding buffers better promote the refolding of N364D TM456t.
- N364D/N391D oligomerizes to a similar extent as the wild type TM456t. Therefore, the N364D mutant will likely be used in further refolding studies.

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References