Characterization and Quantification of Fibrinogen and TLT-1 Binding Properties

Charles Russell[1], Valance Washington[2], Angelia D. Gibson[1]

[1]Maryville College, Maryville, TN, USA, [2]University of Puerto Rico, Rio Piedras, Puerto Rico, USA

Abstract

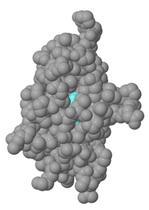
Triggering receptor expressed on myeloid cells (TREM)–like transcript 1 (TLT-1) is a platelet protein that has been linked to the progression of diseases like sepsis and acute respiratory distress syndrome (ARDS). TLT-1 is stored in the α-granules of platelets and is released to the surface as a transmembrane protein as well as in a soluble form into the plasma upon activation. Initial characterizations of TLT-1 have shown that it binds to fibrinogen. Fibrinogen is the precursor protein of fibrin, which polymerizes to form the mesh-like network of a blood clot when in the presence of thrombin. Studying the binding properties of TLT-1 and fibrinogen is crucial due to the role that TLT-1 has played in the outcomes of patients diagnosed with sepsis, as well as the overall role of fibrinogen in clot formation and immuno-response. The purpose of this study was to determine whether binding of TLT-1 and fibrinogen could be measured using fluorescence spectroscopy. To accomplish this goal, an optimized method for generating TLT-1 in our laboratory was needed. Immobilized-metal affinity chromatography (IMAC) was used to purify recombinant human soluble TLT-1 (sTLT-1) from *E. coli* transformed with a plasmid encoding the protein. Previous methods for purifying sTLT-1 required a separate refolding step after purification from inclusion bodies. We refolded the protein on nickel beads, switching from denaturing to non-denaturing buffers before elution, and successfully eluted the protein in non-denaturing buffer, thereby reducing the number of steps for purification. We then used UV-induced fluorescence spectroscopy measured using a SpectraMax Gemini multimode plate reader interfaced with Softmax Pro in a Corning black 96-well fluorescence plate to determine if changes in tryptophan fluorescence could be used to measure interaction of TLT-1 with fibrinogen. TLT-1 contains a single tryptophan residue and reaches a peak fluorescence emission intensity of less than 1000 at 320 nm at a concentration of 4.5 ***μ***M in tris-buffered saline, pH 7.4. Fibrinogen contains 72 tryptophan residues scatted among its alpha beta and gamma chains and reaches a peak fluorescence emission intensity of 27,413 at 345 nm at the same concentration. This would imply that any change in fluorescence between TLT-1 and fibrinogen would be due to conformational changes in fibrinogen. When maintained at a concentration of 4.5 ***μ***M, the fluorescence of fibrinogen decreases as it is titrated with increasing concentrations of TLT-1. Due to this drop in overall fluorescence, one can conclude that in the presence of TLT-1, fibrinogen exhibits conformational changes due to binding. Studies are ongoing to determine proper buffer and concentration ranges to obtain saturation binding curves.

Rationale

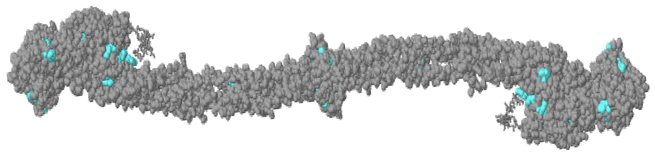
- TLT-1, a platelet protein, binds to fibrinogen[2], however little is known about their degree of interaction
- Fibrinogen contains 72 tryptophan residues[3], while TLT-1 contains 1. This suggests that intrinsic fluorescence may be a possible means of studying their binding characteristics.
- TLT-1 has successfully been purified using a method described by Gattis et al.[1]

Goals & Hypothesis

- Functional recombinant human sTLT-1 will be refolded and purified with IMAC from insoluble bacterial lysate.
- Fibrinogen fluorescence should be affected by concentration-dependent additions of TLT-1 due to conformational changes.



Structure of the immunoglobulin like domain of sTLT-1 highlighting the tryptophan residue contained in the sequence. Rendered by CR in Jsmol



Jsmol rendered image of human fibrinogen, highlighting the tryptophan residues in cyan. Rendered by CR

Expression & Purification

- BL21(DE3)pLysS cells harboring a plasmid encoding human sTLT-1 were grown to mid-log phase in LB containing 0.143 mM ampicillin.
- Cells were induced thru the addition of IPTG and glucose and harvested 8-16 hours post-induction.
- Cells were lysed with incubation with lysozyme in PBS followed by sonication.
- Insoluble cell lysates were mixed with BioRad IMAC (nickel) resin and allowed to bind by inversion.
- Beads were washed and centrifuged three times.
- Protein was eluted in PBS imidazole through centrifugation for 1 minute at 8000 RPM in elution buffer.
- SDS-PAGE and Western blotting was utilized to confirm the identity of TLT-1.

Fluorescence Assays

- Samples of fibrinogen in Tris-buffered saline or Tyrodes HEPES were titrated with increasing concentrations of TLT-1in PMMA cuvette or corning black 96-well plate
- Samples were scanned for fluorescence a Perkin– Elmer LS-5B Luminescence Spectrometer or a M2 SpectraMax Spectrophotometer
- Samples were excited at 290 nm and fluorescence was collected between 300 and 420 nm.
- Data were analyzed and plotted in Excel,

Results

- **sTLT-1 was successfully enriched utilizing IMAC eluting with non-denaturing buffers, eliminating the need for a refolding step. More stringent washing or a gel filtration is warranted.**

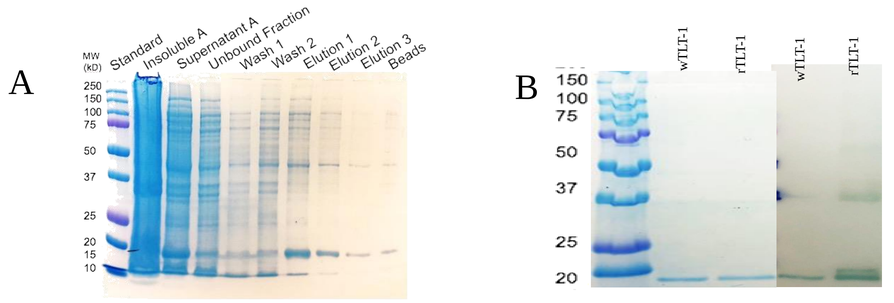


Figure 1. From left to right: SDS-PAGE of successful TLT-1 (~20 kDa) purification using non-denaturing buffers. B) SDS-PAGE of TLT-1 provided by Washington (wTLT-1) and TLT-1 purified by Russell (rTLT-1) confirming purification of TLT-1. Western Blot of wTLT-1 and rTLT-1 probed with MCab69, which is specific to TLT-1 (1:1000 in 2% nonfat dry milk in PBS/Tween), followed by and HRP-goat anti-mouse IgG (1:100000) and developed in TMB.

- **The purified refolded TLT-1 exhibits concentration-dependent binding to immobilized fibrinogen.**
- **Fibrinogen or heparin in solution inhibits TLT-1 binding to immobilized fibrinogen.**

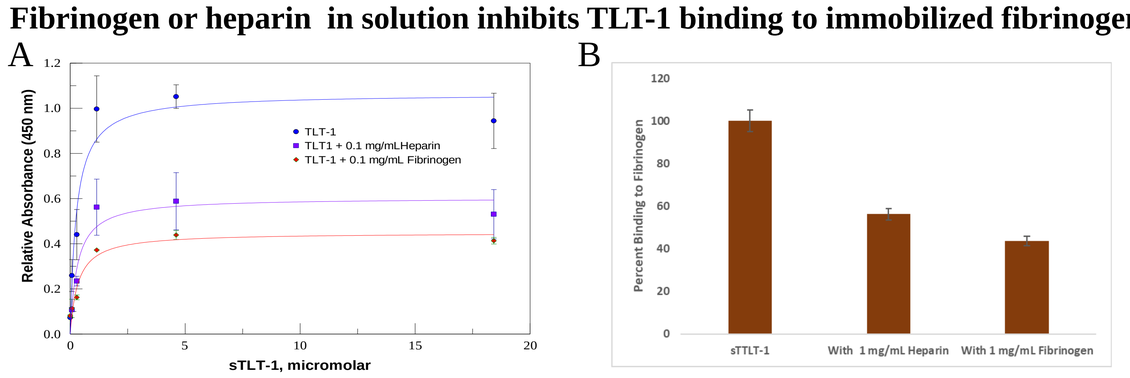


Figure 2. ELISA type assay demonstrating concentration-dependent TLT-1 binding to fibrinogen. TLT-1 was detected with MCab69 (1:10000 PBS/Tween), followed by and HRP-goat anti-mouse IgG (1:1200000) and developed in TMB A) TLT-1 was added to a plate containing immobilized fibrinogen in Tyrodes buffer or Tyrodes buffer containing fibrinogen or heparin. Heparin lowering binding suggests that it blocks the interaction between TLT-1 and fibrinogen. B) Quantification of changed in maximum binding with competing fibrinogen or heparin.

- **Fibrinogen experiences decreased fluorescence when titrated with increasing amounts of sTLT-1 (Washington), but impurities in column-refolded sTLT prevent quantification in plate-reader.**
- **The fluorescence intensity of fibrinogen peaks at 345 nm and is much 16 times higher than sTLT-1.**

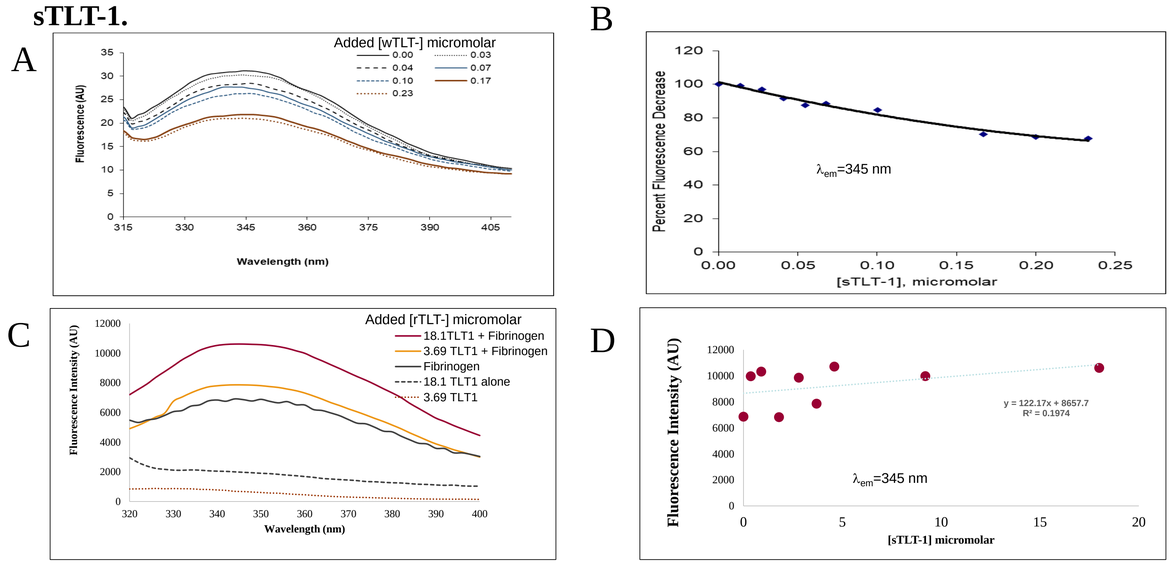


Figure 3. A-B shows fluorescent emission spectra of 4.5 μM fibrinogen titrated with increasing amounts of TLT-1, excited at 290 nm, collected on a Perkin-Elmer LS-5B Luminescence Spectrometer. C-D illustrates fluorescent emission spectra of samples of 3.9 μM fibrinogen, TLT-1, and fibrinogen bound to TLT-1 collected with a M2 SpectraMax Spectrophotometer

Summary and Future Work

- TLT-1 can be refolded during IMAC, eluted, and greatly enriched in non-denaturing buffer.
- The refolded protein binds TLT-1 specific antibodies and retains fibrinogen-binding function.
- Fibrinogen experiences decreased fluorescence when titrated in increasing TLT-1 concentrations and exhibits inhibitory behavior when heparin in introduced.
- Future work requires additional purification steps, further testing of function, and refinement of fluorescence assays in plate reader.

Acknowledgements & References

This project was funded in part by the Scots Science Scholars program

1. Gattis *J. Biol. Chem* 2006 2. Washington *J. Clin. Invest* 2009 3. Zhang *J. Biomat. Sci.* 2013