Hydrogen-Deuterium Exchange on Murine Urokinase-type Plasminogen Activator (muPA)

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Abstract and Background

Murine urokinase-type plasminogen activator (muPA) is a trypsin-like serine protease and is instrumental in tissue remodeling processes as well as being heavily implicated in tumor metastasis. The protein has a zymogen form, which is converted to an active enzyme when cleaved by another another enzyme. Figure 1 depicts a linear diagram of the protein illustrating the two cleavage sites, the his-tag, and the zymogen form. A his-tag is the chain of six histidines at the N terminus of the protein. TEV cleaves off this his-tag of muPA, which converts the protein into the zymogen form. Plasmin cleaves at a different location which converts the zymogen into the active form of the protein. Previous studies have alluded to the dynamic properties of muPA. Through the use of gels, absorbance readings, activity assays, and hydrogen-deuterium exchange, this research set out to examine these properties and investigate whether the zymogen form of the protein is also capable of activity.

ZYMOGEN

Figure 1

muPA Purification Protocol

1. Transformed BL21 (DE3) *E.coli* cells with the muPA plasmid
2. Inoculation was done in 2xTY media with ampicillin

3. Resuspended frozen cell pellet in sonication buffer, and sonicated on ice
4. Split sonication buffer, and centrifuged in 3 different washes with varying percentages of Triton X

5. After washes, pellets were resuspended in denaturing buffer
6. Took an A280 to determine concentration of the supernatant, which was adjusted to 0.2 mg/mL

7. Dialyzed against new denaturing buffer, with 10x less BME, overnight
8. Dialysis buffer switched to a tris and glycerol one for 8-10 hours, before switching to fresh batch

9. Centrifuged and then filtered supernatant to get rid of any precipitate
10. Washed previously prepared nickel sepharose slurry with nickel binding and nickel wash buffers

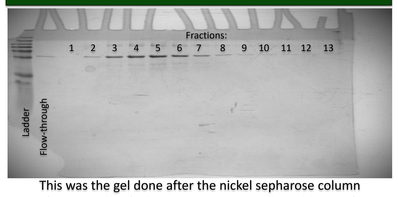
11. Eluted the bound fraction with nickel elution buffer and collected in 2 mL fractions
12. Analyzed fractions on 13% SDS-PAGE gel, and then pooled muPA containing fractions

13. Since resulted with a small volume of muPA, only activated with TEV
14. Took an A280 to determine what concentration of TEV to add

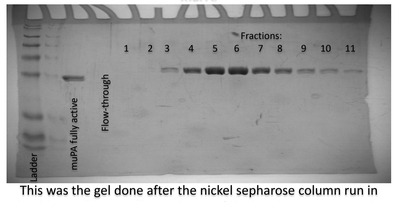
15. Filtered through a syringe, filtered muPA through another nickel column to dislodge TEV
16. Used a Superdex 75 size exclusion chromatography (SEC) column

Did an activity assay with the activated muPA from 7/6/17
Did an activity assay with elution from second Ni column to see if protein was stuck there – see assay graphs

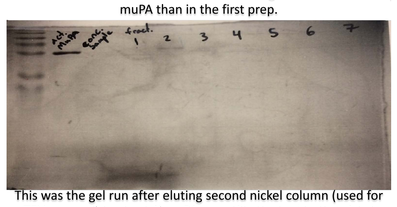
Gels



This was the gel done after the nickel sepharose column purification in the first protein prep done. The lightness of the bands in the picture demonstrate the weak concentration of the muPA.



This was the gel done after the nickel sepharose column run in the third muPA prep. The darkness of the bands compared to the active muPA at 8.8 uM demonstrated a stronger concentration of muPA than in the first prep.

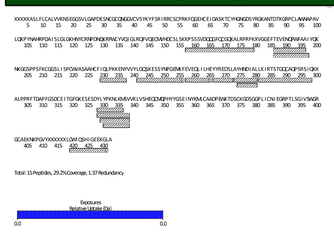


This was the gel run after eluting second nickel column (used for TEV removal) to check if muPA was still attached to the nickel beads.

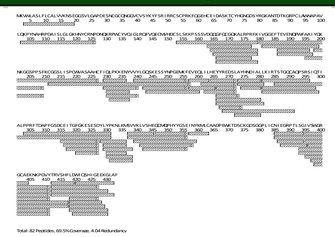
Absorbance Readings at 280 nm

Step in Protocol	A280 Reading and Analysis
Refolding Day: Determined the concentration of the supernatant in order to do a 1/20 dilution	A280 = 0.21 Concentration of protein using Beer's law = 2.69 mg/mL
Dialysis Day: Determined the absorbance of the protein in order to see if it changed during dialysis	A280 = 0.36056
TEV Activation Day: Took an absorbance measurement to see the molarity of the protein we have, to determine how much TEV to add	A280 = 0.4356 Molarity of muPA = 9.08 uM
SEC and 2nd Nickel Column Day: Did a Ni column to get rid of the TEV and took an A280 before concentrating the muPA	A280 = 1.2866E-2 Too low! Based on the first protein prep done, this shows no protein, so the Nickel column was then eluted, a gel was run, and then a chromogenic assay
SEC and 2nd Nickel Column Day: After concentrating, we took two more A280s to see if the absorbance had increased	A280s: First = -5.1951E-3 Second = 1.2865E-3 Too low!

Hydrogen-Deuterium Exchange Results

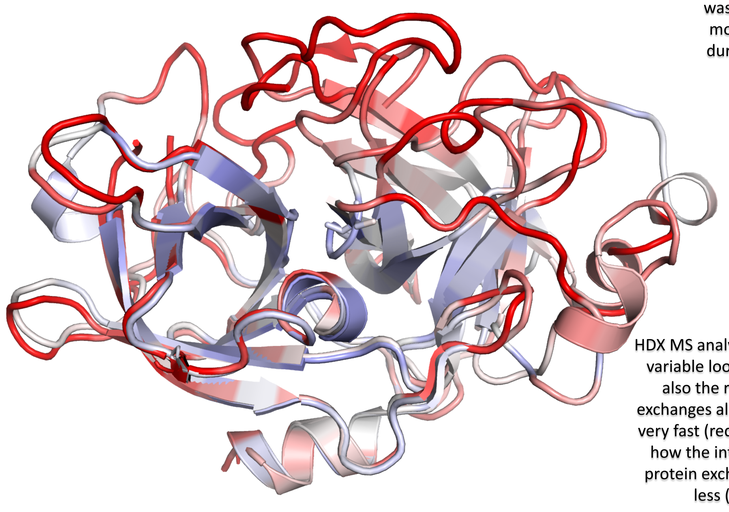


Coverage from TCEP with Guanidine Quench



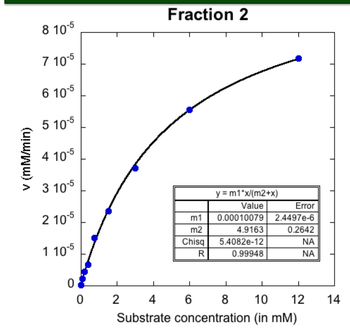
Coverage from TCEP Quench

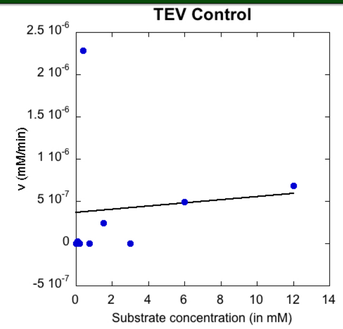
Plasmin activated muPA was run through the HDX machine to determine which quench solution produces more amino acid sequencing coverage. The quench solution without guanidine was found to be more effective during these ID runs.

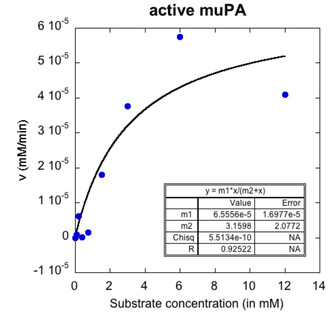


HDX MS analysis shows the variable loop in muPA is also the region that exchanges all of its amides very fast (red). Also notice how the interior of the protein exchanges much less (blue).

Chromogenic Substrate Assays







This chromogenic substrate assay was done after eluting the second nickel column (used to remove TEV) and after seeing that fraction 2 on the gel had some protein. The assay was to determine if there was any muPA left on the column, or if the protein shown on the gel was actually TEV, which is why 3 rows of reactions were done — fraction 2, TEV, and active muPA. The stark difference in the TEV graph and the fraction 2 graph allude to the protein being muPA.

Conclusions and Discussion

In the span of three weeks, three attempts at muPA protein purification were made. The first's yield was too low, prompting a different approach. Instead of using 2xTY media, ZN media was utilized due to the presence of lactose in 2xTY media. IPTG is an analog of lactose and was used to induce protein production in the *E. coli* grown. Since lactose could have potentially induced protein production before the optimal time where the IPTG works, the ZN media was used. This ultimately failed due to too rapid growth. The third protein growth was again done in 2xTY media. The protein yield was too low again, prompting elution of the nickel column used to remove TEV to determine if muPA was stuck on there. The results of the activity assay done and gel run demonstrated that there was another protein on the column that was not TEV, and can be seen in the respective graphs. The protein that was on the column appears to be muPA. This prompts further questioning of whether the zymogen form of muPA is capable of activity without cleavage by plasmin. Tissue plasminogen activator, the other clot busting protease, has this property, so it may be general. Hydrogen-deuterium exchange was done to examine which parts of the protein were exchanging more in the active form. This would be interesting to compare to the zymogen form once the purification process is refined.

References

Kromann-Hansen T, Lange E, Sørensen H, Hassanzadeh-Ghassabeh G, Huang M, Jensen J, Muyldermans S, Declerck P, Komives E, Andreasen P (2017) "Discovery of a Novel Conformational Equilibrium in Urokinase-type Plasminogen Activator" *Scientific Reports* (2017)

Acknowledgments

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