Comparison of the Formation of Polymeric Chains Between Ubiquitin and the Ubiquitin-like Proteins Rub1 and Nedd8

Amanda Chow
achow@umd.edu
Life Sciences
Cell Biology and Molecular Genetics
Biochemistry

Introduction

Ubiquitin is a small eukaryotic protein (8.5 kDa) made up of 76 amino acid residues. Its function is signal substrate proteins for degradation. Ubiquitination of a substrate protein is a modification that involves two enzymes, E1 and E2-25K. These enzymes will form bonds between ubiquitin and the substrate protein. The enzymes will also form polyubiquitin chains by linking individual ubiquitin molecules together.

Two proteins that are structurally very similar to ubiquitin is Rub1 (in *Saccharomyces cerevisiae*) and Nedd8 (in humans). These two proteins undergo a metabolism that is very similar to that of ubiquitin. Rub1 and Nedd8 are homologous proteins both show a high degree of similarity (60% similar sequence) with ubiquitin. Despite their similarities, Nedd8 and Rub1 is not activated by the E1 enzyme and therefore does not form polymeric chains in vivo.

There are also seven regions of dissimilarity between Nedd8/Rub1 and ubiquitin. We wish to create mutant proteins involving insertion of one of the dissimilar regions of ubiquitin into both Nedd8 and Rub1 in order to analyze the mutant proteins' ability to form polymeric chains. It has been found previously that mutating residue 72 from alanine to arginine significantly increased the amount of activated Nedd8. Therefore, we also made Nedd8 mutants that included the A72R mutation.

We hope to find whether the dissimilar regions have important effects on the formation of polymeric chains in Nedd8 and Rub1, and how certain regions of ubiquitin allow its normal function. This could lead to future research on the importance of the sequence of ubiquitin for its regulation of protein degradation for the normal functioning of the overall cell.

Size Exclusion Column on the FPLC system used for purification

Growth and Purification Methodology of Rub1/Nedd8 mutants

In total, there will be twenty-one mutants that need to be expressed and purified, seven of which are Rub1 mutants, and fourteen Nedd8 mutants. Three Rub1 mutants and wild type Rub1 have been expressed and purified so far. The Rub1 mutants I expressed and purified are Rub1 + Ub region II, Rub1+ Ub region III, and Rub1 + Ub region V.

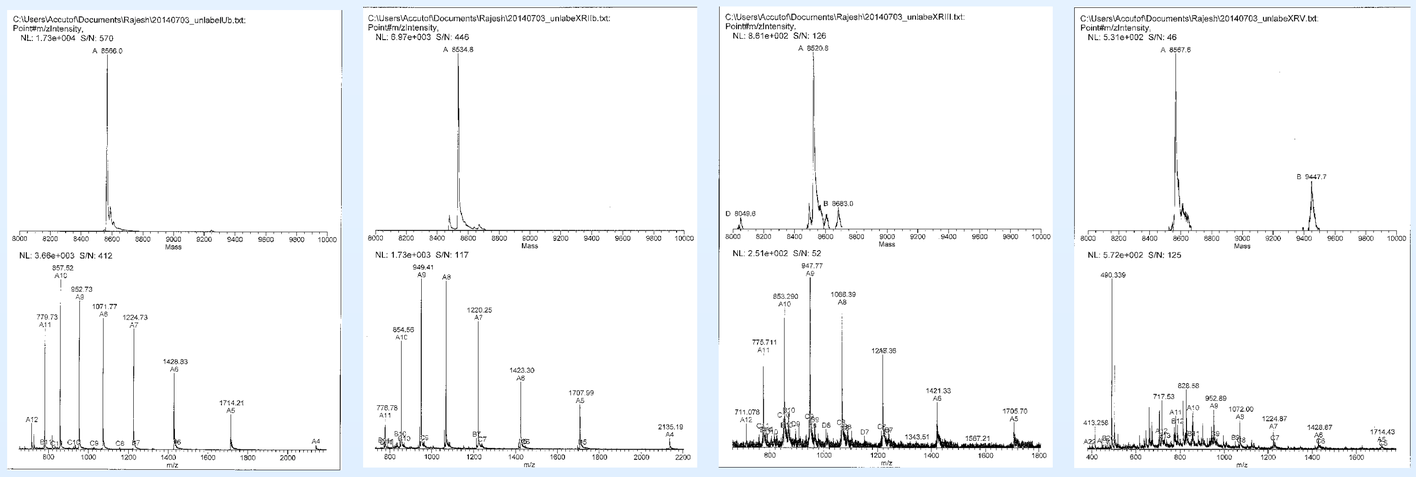
The Rub1 mutants were created using PCR site-directed mutagenesis with a wild type Rub1 template inserted into a pET3a plasmid containing ampicillin resistance. This plasmid was transformed into competent Rosetta BL21 cells, and grown in LB at 37°C and induced with IPTG at OD_{600}. After lysing, the protein was run through a chitin column and size exclusion column. Mass spectrometry was performed to verify the mass of the protein.

Nedd8 mutants were also created and grown using a similar protocol as above. After lysing, the soluble portion was heated to precipitate unwanted proteins. It was further purified and concentrated, then run through a size exclusion column. They will also be analyzed with mass spectrometry. All of the Nedd8 mutants are currently still being purified.

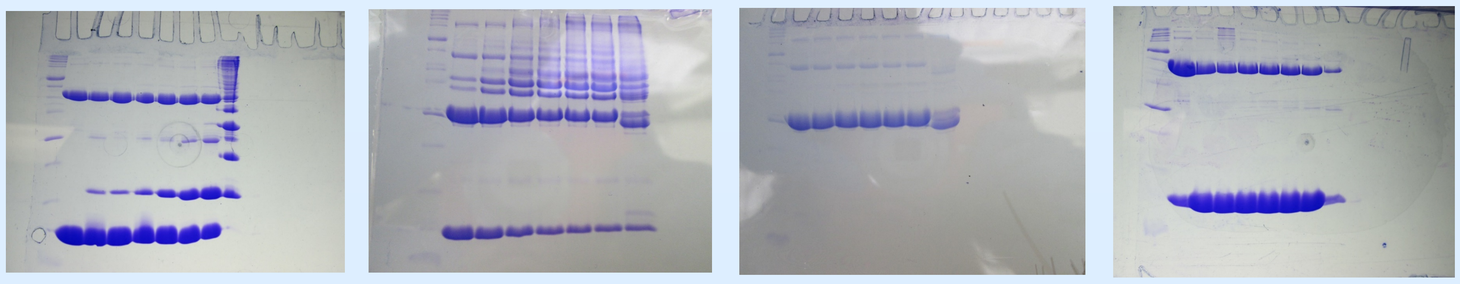
```
U: MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
R: MIVKVKTLTGKEISVELKESDLVYHIKELLEEKEGIPPSQQRLIFQGKQIDDKLTVTDAHLVEGMQLHLVLTLRGG
N: MLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQMNDEKTAADYKILGGSVLHLVLALRGG
     I          II        III   IV                              V      VI    VII
```

Seven major regions of difference

Data and Results

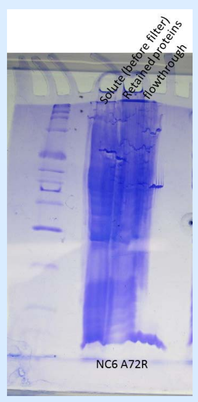


Spectra from mass spectrometry verifies the mass of our desired Rub1 mutants at 8.5 kDa .

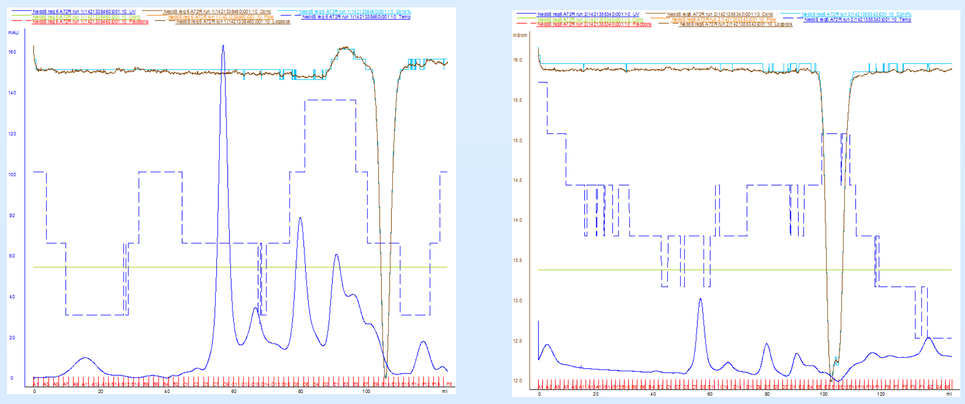


Wild type Ub | Rub1 + Ub region II | Rub1 + Ub region III | Rub1 + Ub region V

Samples of the polymeric chain being formed at time points taken from 0 hours to 24 hours.



Left: The SDS-PAGE shows Nedd8 + Ub region 6 +A72R before and after purification, with the purified protein showing up in the flowthrough.



Middle and Right: These two chromatograms from the size exclusion column show Nedd8 + Ub region 6 + A72R being separated from other proteins based on its size. The UV peak at 80 mLs was found to contain the mutant.

Formation of Polymeric Chains

The mutants were added to enzymes E1 and E2-25K along with other necessary components at concentrations that simulated the in vivo environment, in order to induce chain formation. 2 µL samples were taken at 30 min, 1, 2, 3, 4, 6, 8, and 24 hours then added to SDS to stop chain formation. The samples were then analyzed by SDS-PAGE.

Discussion

The mass spectrometry spectrums verifies that there was protein of the correct masses in each of the protein samples, at 8.5 kDa.

The catalytic reaction shown on the SDS-PAGE reveals that wild type ubiquitin and Rub1 + Ub region II formed polymeric chains as seen by presence of dimers, trimers, and tetramers on the SDS-PAGE. However, Rub1 + Ub region III and Rub1 + Ub region V SDS-PAGE did not show any polymers so there was no chain formation. We expected the Rub1 mutants to not produce any chains, as Rub1 + Ub region III and Rub1 + Ub region V showed. Therefore, regions III and regions V may not be involved in the formation of chains. However, Rub1 + Ub region II showed chain formation, so region II of ubiquitin may be involved in the formation of Rub1 chains. Wild type ubiquitin functioned as the control and it formed polyubiquitin chains as expected.

Nedd8 + Ub region 6 + A72R has been purified and will be tested for formation of polymeric chains in the near future. The peak around 80 mL was found to contain our desired mutant.

Future Work

We wish to finish purifying and analyzing the remaining eighteen Rub1 and Nedd8 mutants with one region of Ub inserted in them. Specific sections that seem to be influential in the formation of polymeric chains will be of interest. More Rub1 and Nedd8 mutants containing two regions of Ub inserted in them can be analyzed for their ability to form polymeric chains to test whether these different sequences work in tandem.

Acknowledgements

Special thanks to Dr. David Fushman and Dr. Rajesh Singh of the Fushman Lab in the College of Chemistry and Biochemistry at the University of Maryland.