TEV Protease Cleavage of MBP and His Affinity Tags in *de novo* Fusion Proteins

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Abstract

Polyhistidine (His) and Maltose Binding Protein (MBP) tagged proteins were successfully expressed in *Escherichia coli*. TEV protease cleavage and MBP-free cloning were pursued as strategies for obtaining pure, MBP-free protein variants for further research. A protocol for TEV cleavage was developed, and methods of subsequent purification or alternative cloning were explored.

Introduction

Recent work by the Seelig Lab built on the work of Newton *et al.*[1] to explore random, 83 residue proteins, using RNA display to select for ATP/GTP binding proteins. Subsequent sequencing identified the top binders in each library. Around five proteins with high expression and purity from *E. coli* cultures were selected from each library.

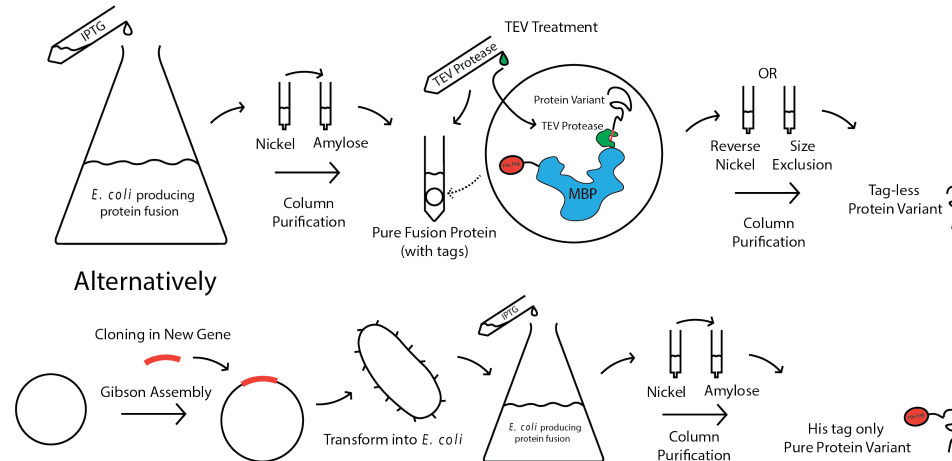
In order to characterize the structures of those proteins, x-ray crystallography was chosen. But to produce protein crystals for x-ray crystallography, pure and free protein is required. Initially, MBP was chosen as a tag to aid in protein solubility and expression. However, it is also bulky and affects crystallization. Thus, this project aimed to produce sufficient amounts (1-2 mg) of pure and MBP-less (only HIS-tagged) proteins through the methods of TEV cleavage and MBP-less cloning.

TEV protease cuts a protein only at a highly specific sequence of amino acids. Our initial protein was tagged with both MBP and His tags, which were separated from the protein by the short recognition sequence at which TEV protease cuts.

Methods

In order to produce protein for TEV cleavage and purification, fusion protein was expressed in RosettaTM(DE3)pLysS *E. coli* using a pET expression vector with IPTG induction of the lac operon. Cells were lysed by sonication and centrifuged at 30 thousand x g. Protein fusion, containing His and MBP tags, was purified by a Ni-NTA column followed by an Amylose resin column. Purify and yield were assessed with A280 UV absorbance and SDS-polyacrylimide gel electrophoresis (PAGE).

Methods cont.



After purified protein was obtained in sufficient quantity, TEV cleavage was performed. Optimization of the recommended protocol yielded near complete cleavage with 100 U of TEV protease per mg of protein at a concentration of 5 mg/mL in our amylose elution buffer.

Subsequent purification of the TEV cleaved protein was attempted with a reverse phase nickel column. Analysis of cleavage efficiency and purification effectiveness were measured with SDS-PAGE gels stained with Coomassie Brilliant Blue dye and Pierce silver stain (Fig 1&2).

Results

A protocol was developed that led to consistent >90% cleavage of fusion protein with limited exceptions. Protein variants (5C, 16D, 16E, 20A) were screened at a small scale for effective cleavage (Fig 1). 5A, 9B, 9C, 9D, 20C, 20E, and 20F (not pictured) were all cleaved with >88% efficiency. Reverse nickel purification of the TEV cleaved protein did not separate the tag-less protein in any variants tested (5A, 9B, 9C, 9D, 16D, and 16E). Further investigation of the reverse nickel purification with silver stained SDS-PAGE revealed that at least one variant, 16D, bound to the nickel column without a His tag attached, circumventing purification (Fig 2).

Fig. 1

MW	5C 2 hr	5C ON	16D 2 hr	16D ON	16E 2 hr	16E ON	20A 2 hr	20A ON
% Cleavage	83	100	49	84	88	100	98	100

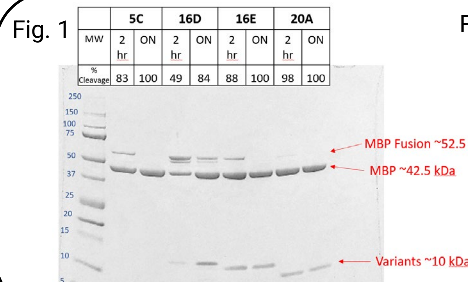


Fig. 2

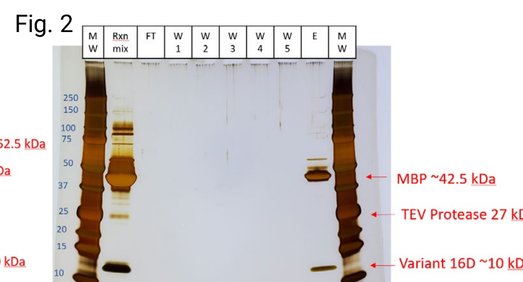


Figure 1. SDS-PAGE gel of protein fusion cleavage with TEV Protease stained with Coomassie Brilliant Blue dye. 5C tag-free variant does not stain well and is thus not visible. For each treatment, a 2 hour room temperature incubation and additional overnight lanes are shown. Approximately 5 ug of total protein was loaded in each well.

Figure 2. SDS-PAGE gel of attempted reverse nickel-NTA column purification of cleaved 16D protein. Rxn mix= TEV ON treatment mixture, FT= Flow-through of column, WX=Wash number X, E=Elution. Tag-free 16D can be seen as a band in the elution fraction.

Future Directions

- Since Ni-NTA purification of cleaved protein variants clearly failed for at least one variant, it may merit additional investigation with other protein variants. Future work will also attempt to purify the cleaved protein through FPLC size exclusion, where eluting tag-less protein could be detected with a refractive index (RI) detector.
- The alternative method of cloning the tag-free protein variants with only a His tag began with backbone PCR of a pET28a plasmid. Since the His tag alone is much less bulky, this is a potential alternative to completely tag-less variant.
- The next steps include the insertion of custom gene blocks using a Gibson assembly process and forming a Gibson assembly product for each variant. Each product will be confirmed via sequencing prior to testing expression. Extra care will be taken to optimize purification efficiency of the His tag only variant with only a nickel column.
- In either case, once pure, tag-free protein is obtained, the first objective will be to better characterize its independent solubility and other biophysical properties.

Acknowledgements

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Citations:

[1]Newton, M. S., Morrone, D. J., Lee, K., & Seelig, B. (2019). Genetic Code Evolution Investigated through the Synthesis and Characterisation of Proteins from Reduced-Alphabet Libraries. *ChemBioChem*, *20*(6), 846–856. https://doi.org/10.1002/cbic.201800668