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14. ABSTRACT

This project focuses on the development of highly effective non-chromatographic methods for purifying recombinant proteins and enzymes. This report covers work at Ohio State University, which focuses on the further optimization of the purification technology in the PI's new laboratory. A key aspect of the technology involves the use of a self-cleaving protein module, which can be used to make any purification tag self-cleaving. By combining this module with a variety of purification tags, we have generated a number of simple, inexpensive and highly

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Report Title

Final Report: Production of Self-Purifying Proteins in a Variety of Expression Hosts with Focus on Organophosphorus Hydrolase

ABSTRACT

This project focuses on the development of highly effective non-chromatographic methods for purifying recombinant proteins and enzymes. This report covers work at Ohio State University, which focuses on the further optimization of the purification technology in the PI's new laboratory. A key aspect of the technology involves the use of a self-cleaving protein module, which can be used to make any purification tag self-cleaving. By combining this module with a variety of purification tags, we have generated a number of simple, inexpensive and highly effective methods for protein purification. We have applied these methods to the production of several proteins, including organophosphohydrolase (OPH), which can be used to degrade nerve agents and environmentally persistent insecticides. We have further demonstrated these methods in high cell-density fermentation at laboratory scale, and have provided evidence of their effectiveness. Our most recent work has been on the optimization of the fermentation process itself, as well as a more biochemical optimization of the expression system. Overall, the ARO support on this project has yielded two patent applications, seven peer-reviewed papers and one book chapter. In addition to these, three manuscripts are in late stages of preparation.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Non-Antibody Protein Production
Informa Life Sciences, Prague, Czech Republic, May 24-25, 2012,
"Next-generation inteins for applications in mammalian cells"

The Bioprocessing Summit: Affinity Tag Protein Purification
Cambridge Healthtech Institute, Boston, MA, August 22-23, 2011,
"Tag Technologies: Opportunities and Challenges in the Biopharmaceutical Industry."

BioProcess International
Informa Life Sciences, Nice, France, April 6-8, 2011,
"Self-cleaving Non-chromatographic Purification Tags: Current Results and Future Prospects."

Number of Presentations: 3.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received	Paper

TOTAL:

Number of Manuscripts:

Books

Received	Paper

TOTAL:

Patents Submitted

None

Patents Awarded

None

Awards

None

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Michael Coolbaugh	1.00	
FTE Equivalent:	**1.00**	
Total Number:	**1**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
David W. Wood	0.00	
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Robert Wensing	0.10	Chemical Engineering
FTE Equivalent:	**0.10**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	1.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:	1.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:	1.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	1.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	1.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attached report.

Technology Transfer

"Production of Self-Purifying Proteins in a Variety of Expression Hosts with Focus on Organophosphorus Hydrolase"

ARO Small Research Grant 60034LS
PI: David Wood
April 1, 2011 - March 31, 2012

Final Report

I. Foreword

The importance of proteins and enzymes in medicine, industry, and defense is very well established. A particular focus of this work is those proteins and enzymes that are required in very large quantities, at minimal expense, for various military and civilian applications. Most prominent among the military applications are highly specialized enzymes that can be used to degrade chemical and biological weapons, as called for by several treaties[1]. These enzymes can also be used for biosensors and for protective measures against potential attacks[2, 3]. A critical bottleneck in the development and application of these technologies is a lack of high-yielding, inexpensive manufacturing processes for commodity-scale amounts of the required proteins and enzymes.

The primary goal of our first ARO proposal was to develop non-chromatographic methods for recovering, concentrating and purifying proteins of military interest - most notably, the organo-phosphohydrolase (OPH) enzyme. This work focused on the creation of self-cleaving purification tags that can be used in a variety of protein expression hosts for the production of arbitrary "self-purifying" proteins. These methods are reminiscent of conventional affinity methods, wherein the DNA encoding a recombinant target protein is fused to the DNA of a convenient "affinity tag" for expression and purification[4]. Our methods retain the power and generality of the conventional affinity methods, allowing the target to be expressed in an optimal host organism for simple purification, but have the added advantage of a self-cleaving tag (triggered to cleave by mild pH and/or temperature changes) that allows a general non-chromatographic separation. Therefore, processes associated with these tags are general, simple, trivial to scale up, and cost a very small fraction of conventional affinity methods.

The core technology of this work is a self-cleaving elastin-like protein (ELP) tag. This tag was created by combining an ELP with an engineered self-cleaving intein, which was developed in previous work. The ELP tag very selectively precipitates in response to salt and temperature, and can therefore be used a genetically fused tag to selectively precipitate any target protein. In this case, the ELP acts similarly to a conventional affinity tag, where the tag is added to any target protein by simple DNA fusion. Thus, the ELP tag can provide a strong, non-chromatographic method for purifying any target protein, while the self-cleaving function of the intein provides a very simple method for tag removal after purification of the target. Overall, this method

provides a very convenient and inexpensive method for purifying target proteins at a variety of scales, although some additional development remains to be done. In particular, the self-cleaving function of the intein is not compatible with mammalian cell culture and other expression hosts, primarily due to premature cleaving of the tag during protein expression. The ELP protein is also quite large, which decreases the overall efficiency of the process. Further, the ELP tag method has not been demonstrated at large scale in high-density fermentation in microbial hosts, which is a critical gap in its appeal.

The overall goals of our first renewal proposal were primarily to generalize these methods for a wide variety of expression hosts, and to find an optimal approach for the inexpensive expression and purification of the OPH enzyme at high activity and yield. Over the course of the renewal grant, the PI (David Wood) moved his laboratories from Princeton University to Ohio State University, and the renewal effort was effectively split into two years and Princeton followed by one year at OSU. This report focuses on the single year of support at OSU. Briefly, the work at Princeton went far to achieve the research goals of the renewal, with one publication that specifically includes scale-up analysis of a proposed OPH production process[5]. In addition, we developed a number of supplementary tools to be used with these systems, both in research and at production scales, which were published in several additional papers. These tools, based on rapid recombination-based cloning methods, will accelerate research on the expression of new proteins and enzymes.

At OSU, the work shifted to focus on the development of a high-yielding and inexpensive production process for the OPH enzyme, as well as further optimization of the purification tag and intein that are enabling for this process. In particular, we worked to develop a high-yielding fed-batch fermentation process, using inexpensive raw materials, that can produce a tagged OPH enzyme for simple purification. Although we encountered some technical issues, we are confident that this work will produce at last one publication in the next year. A second research area included the optimization of our purification tag for optimal expression and recovery yield, and this work has been largely completed. A manuscript on this work is also in late stages of publication. Finally, a third area of work is the development of a new self-cleaving intein module, which could be used with transgenic and mammalian expression hosts. This is a critical goal for this technology in general, and we have had some very exciting success in this area. The results of these three approaches are the main topics for this report.

The ability to rapidly clone, express, purify and evaluate new enzymes and proteins will increase the capability of the military to respond quickly to new chemical and biological threats. Although this proposal is primarily focused on products of military relevance, it should be noted that general methods for rapidly and reliably expressing and purifying proteins will find countless applications in the private and academic sectors as well, with far-reaching benefits to medicine, the chemical industry, pure research, the environment, and other biotechnology areas.

II. Table of Contents

III. List of Appendices, Illustrations and Tables.

Appendices

Illustrations

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IV. Executive Summary

This work focused on the further development and optimization of processes based on self-cleaving tag technologies for the purification of arbitrary proteins at large scale. The goals also included optimization of the technology at a molecular level.

The first specific goal of this work was to develop an optimized small-scale fermentation process for producing OPH enzyme from inexpensive raw materials, and show that the enzyme can be easily and cheaply purified using a self-cleaving ELP precipitation tag. This work was somewhat delayed by equipment failures and training issues, and as of the writing of this report, this work is still in progress.

The second primary goal of the work was to optimize the ELP tag length for high expression and high recovery during purification. This optimization was based on the expectation that long ELP tags would lead to relatively low expression of the target protein, but high yields during purification. Shorter ELP tags, on the other hand, would lead to higher expression yields, but lower recovery during purification. It was further expected that the optimal ELP tag length would be target dependent; larger target proteins might require larger ELP tags for reasonable purification yields. The primary conclusion of these studies is that, indeed, there is an optimal size of ELP for final target protein yield, and it is significantly smaller than the tag we have been using in all of our previous work. This work is also continuing with additional target proteins and conditions, and we anticipate at least one publication in the next year or so.

The third goal, which was added during the course of the work, was the development of a highly controllable intein that might be used with mammalian cell expression systems. Although this was suggested in this proposal, it was not initially included as a specific aim. This is a critical goal for the overall development in intein technology, however, and the graduate student supported by the grant made some excellent progress in this area as well. In particular, we have developed a yeast surface display method for screening intein activity via flow assisted cell sorting (flow cytometry). This methods provides a unique ability to screen intein cleaving activity under various non-native conditions, and we believe that it will eventually provide a means for generating a wide range of controls for intein cleaving activity. These mutants will then find uses in a wide variety of expression hosts, allowing them to make a significant impact in a much wider range of biotechnology applications.

Because the period of support was one year, and it started as the PI moved to OSU, no graduate students supported by the grant have yet finished their Ph.D. programs. One student, Michael Coolbaugh, was fully supported for the year and is currently entering his fourth year of study as a Ph.D. student at OSU. Support from this grant will be recognized in his publications and dissertation as they appear.

It is anticipated that the work supported will lead to a number of additional publications, particularly in the development of the optimized ELP tag, the development of the yeast surface display system, and the development of an additional cloning method for intein use (the LIC intein method). Although these manuscripts are in preparation, the ELP optimization is at a very late stage and should appear within the next few months.

V. Statement of the Problem Studied

Difficulties with large-scale protein production can typically be broken into those associated with high-level expression of a given product (typically in a recombinant organism), and those associated with the complexity and expense of downstream processing and purification. In general, the cost of recovery and downstream processing represents the bulk of the overall price for a given product[6, 7], although the required purity can also have a major impact. In many cases, significant optimization is required for even laboratory-scale purification, and scale-up of the laboratory process can introduce additional complexity. Although the downstream costs can be substantial, the overall process costs can be decreased if the active target protein can be highly overexpressed in a simple production organism. Thus, optimization of target protein expression is also an important step in process development - even at laboratory scale. A well-designed large-scale enzyme production processes will therefore include high product expression in a well-behaved host, coupled with a simple, general and inexpensive purification method. Ideally, the purification method will be at least partly non-chromatographic, and will allow the product to be purified at high concentration with minimal buffer use via an optimized platform technology.

The primary goal of our first proposal was to develop non-chromatographic methods for recovering, concentrating and purifying proteins of military interest - most notably, the organo-phosphohydrolase (OPH) enzyme. This work focused on the creation of self-cleaving purification tags that can be used in a variety of expression hosts for the production of arbitrary "self-purifying" proteins. These methods are reminiscent of conventional affinity methods, wherein the DNA encoding a recombinant target protein is fused to the DNA of a convenient "affinity tag" for expression[4]. The conventional affinity tag allows easy purification of the target via a well-established and optimized method, and the tag is typically removed by protease treatment once the target is pure. Our methods retain the power and generality of conventional affinity methods, allowing the target to be expressed in an optimal host organism for simple purification, but have the added advantage of a self-cleaving tag (triggered to cleave by mild pH and/or temperature changes) that allows a general non-chromatographic separation. Therefore, processes associated with these tags are general, simple, trivial to scale up or down, and cost a very small fraction of conventional affinity methods.

The overall goals of this grant were to generalize these methods for a wide variety of expression hosts, and to find an optimal approach for the inexpensive expression and purification of the OPH enzyme at high activity and yield. Many of these goals were achieved at Princeton, although some work remained to be done after the Wood laboratory moved to Ohio State University. The work at OSU focused on broad applications of intein technology, with the more specific goal of demonstrating an optimized system for producing the highly stable OPH-S5 mutant in fermentation with an ELP-based purification. In particular, the research focused on three areas of intein purification technology development. These included the following:

Fermentation development. In previous work, we showed that E. coli cells can be used with inteins in a high cell-density, fed-batch configuration (Fong and Wood 2010). In our published work, we used common laboratory reagents for the fermentation,

which can be expensive. Thus, we suggested that the cost of the fermentation could be greatly decreased by using commodity-scale nitrogen sources, such as agricultural and food wastes, as well as tap water and food-grade corn syrup as a carbon source. This would be accomplished through the development of an optimized fed-batch fermentation at bench scale, using highly inexpensive nutrients.

ELP tag development. In much of our published work, we purified various target proteins by using a fused, self-cleaving ELP precipitation tag. The ELP tag allows a non-chromatographic purification of the target protein by a highly selective precipitation step. Our tag is quite large, however (550 amino acids), which increases the effectiveness of the purification step, but likely decreases the efficiency of protein expression. We have hypothesized that we might achieve a higher overall efficiency by optimizing the purification with smaller ELP tags, but the optimal size has not been determined. Thus, we conducted experiments on smaller tags with a variety of product proteins to determine the optimal ELP tag size for efficient expression with a high yielding purification process.

Intein development. All of our previous work has relied on the ΔI-CM intein, which is triggered to cleave by a combination of pH and temperature change. We have shown extensively that this intein is highly effective in recombinant protein production in E. coli, and have published several papers on this. Unfortunately, the pH and temperature activity profile for this intein is not appropriate for expression in mammalian, yeast or transgenic hosts. In these hosts, the intein will cleave prematurely, rendering the purification method ineffective. Although intein development was not a primary goal of this grant, it is critical that inteins be compatible with additional hosts before the technology will find wide spread acceptance. Thus, we have included this as additional work, and focused on the development of a yeast-based selection system for identifying new and improved inteins.

VI. Summary of Most Important Results

Brief Summary of Results from First Grant

Our first proposal successfully combined self-cleaving affinity tag technology with polyhydroxybutyrate (PHB) production in bacteria to generate a radical breakthrough in protein production and purification (Figure 1). More specifically, we combined a self-cleaving protein element with a naturally occurring PHB-binding protein to produce a self-cleaving PHB-binding affinity tag[8]. Target proteins fused to this tag are expressed in an engineered strain of *Escherichia coli* that also produces small (100-200 nm) intracellular granules of PHB. The PHB-binding affinity tag immobilizes the target protein onto the intracellular PHB granules. The granules can then be easily recovered using a variety of established mechanical methods, and the attached target protein is then purified by simple washing. Once the contaminant proteins are washed away, the target protein is self-cleaved from the granules by a mild pH shift (from pH 8.5 to 6.5) and released into solution in a highly purified form as shown in Figure 1. The PHB granules can then be discarded. By

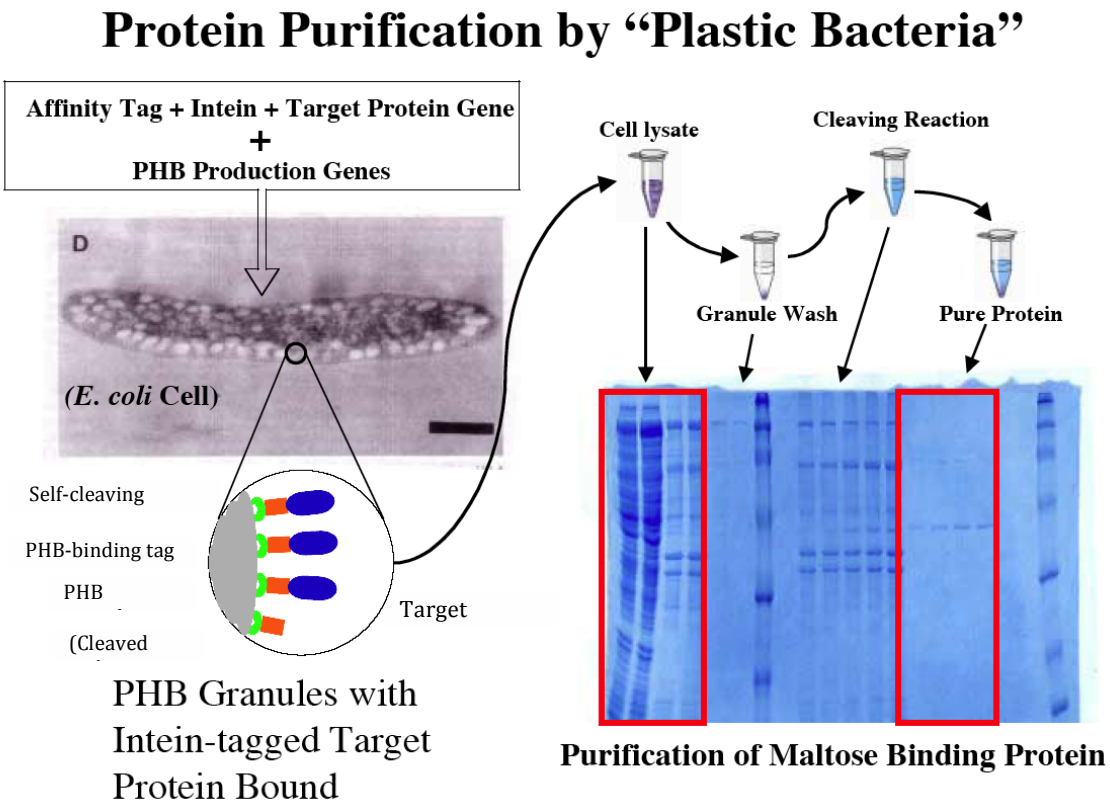

Figure 1: Purification of a target protein by immobilization on PHB granules using a self-cleaving PHB-binding tag. In this system, *E. coli* cells are transformed with a plasmid that causes them to make large, intracellular PHB granules (left). On a second plasmid, a protein is expressed that is tagged with a self-cleaving PHB-binding tag. The target remains bound to the granules for easy purification, and is then self-cleaved for recovery from the soluble phase. Example purification of maltose-binding protein is shown at right.

allowing the bacterial cells to effectively produce both the affinity resin (PHB) and the tagged protein, the cost of the process is greatly reduced[9]. Furthermore, the need for chromatographic equipment is eliminated. This combination of improved economics and simplicity constitutes a significant breakthrough in the large scale production of purified proteins and enzymes for industrial and military use.

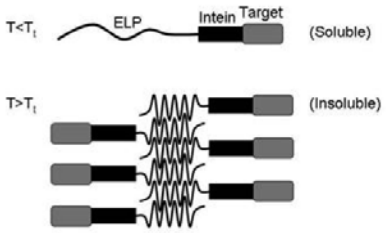

Figure 2: At temperatures below the ELP transition temperature (T<T$_t$, top), the ELP is unstructured and soluble in buffer. At temperatures above the transition temperature (T>Tt, bottom), the ELP proteins form a higher order structure and self-associate, precipitating along with any protein fused to them.

The project was expanded to include the use of reversibly precipitating elastin-like peptide (ELP) tags (Figure 2). ELP tags have the remarkable property that they reversibly self-associate and precipitate in response to small changes in temperature and salt concentration[10-12]. By combining ELP tags with a self-cleaving protein element, a given target can be easily and reversibly precipitated via the ELP, allowing it to be purified by simple centrifugation or filtration[13-15]. Once the protein is purified, it can be resuspended and cleaved, at which point the cleaved ELP tag can be removed by an additional round of thermal precipitation (Figure 3).

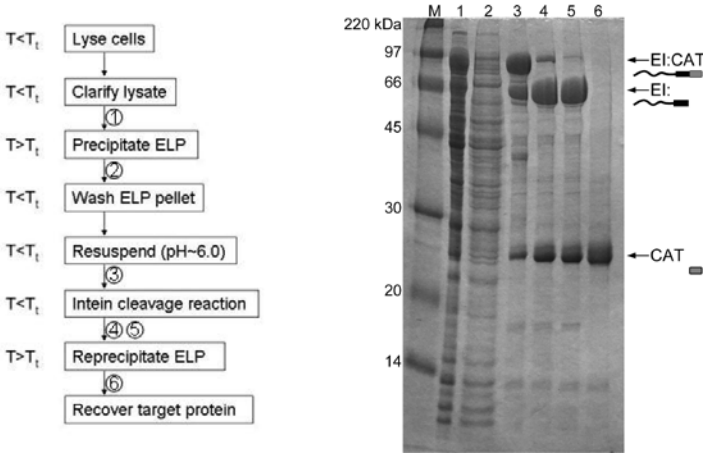

Figure 3: ELP-intein fusion purification method (left) and typical purification result (right). Cells are lysed and the lysate is clarified at a temperature below the transition temperature of the ELP tag (tagged protein stays in soluble fraction). Once insoluble cell debris is removed, salt is added and the temperature raised to precipitate the ELP. Centrifugation recovers the insoluble ELP fusion, and the soluble contaminants are washed away. The ELP fusion is then re-dissolved at low temperature in a low pH buffer, triggering the intein-ELP tag to self-cleave. Once the cleaving is complete, the temperature is again raised to remove the cleaved tag, leaving the purified protein in the soluble phase. SDS-PAGE Protein gel lanes at right, lane 1 = clarified lysate; lane 2 = soluble contaminants removed; lane 3 = recovered, uncleaved ELP fusion protein (some cleaving has started); lane 4 = cleavage products after 8 hours at room temperature; lane 5 = cleavage products after overnight at room temperature; lane 6 = recovered, soluble product protein after precipitation of ELP tag.

In many ways this system is more attractive than the original PHB system, and provides and excellent alternative in many cases. One specific advantage is that the ELP tag can significantly increase soluble expression and activity of "difficult" proteins. When compared to the work of previous investigators, these results suggest that the ELP purification method will almost certainly be able to replace the initial capture and first column steps of conventional protein purification strategies, and can be used with difficult and poorly soluble proteins as well. The ELP system also has the advantage of being a cleaner process, and (in principle) being compatible with expression systems for secreted proteins. These aspects make this system particularly attractive for

development in mammalian and yeast-based expression systems, including those for antibodies and complex glycoproteins. Further, a rough economic analysis of these systems predicts that they will likely have a major impact on purification costs for protein and enzyme products - decreasing expenses by as much as 100 fold compared to conventional affinity tag methods with proteolytic tag removal, and as much as an order of magnitude compared to other intein processes[9, 16, 17].

The development of both of these systems led to several publications, patents and presentations, as well as one PhD dissertation. In particular, the "Plastic Bacteria" system, based on PHB-binding, was published in *Protein Science*[8], while the ELP system was initially published in *Nature Methods*[13]. A follow up paper on the economic performance of these systems was subsequently published in *Microbial Cell Factories*[9], an invited ELP paper was published in *Nature Protocols*[14] and a PHB methods chapter is in press in *Methods in Molecular Biology*. In addition to these papers, two patent applications were filed, and this work was included in a chapter on industrial applications of inteins[16]. A review paper with an undergraduate first author was also published[18]. Notably, this research has generated quite a lot of attention, and has led to a commentary on the PHB system by George Georgiou in *Protein Science*[19], as well as short blurbs on the ELP system in *C&E News*[20], *The Scientist*[21], *Chemistry World*[22], and several online research discussion groups. It has also been presented in several invited talks at industrial conferences as an emerging "disruptive" technology with the potential to radically impact the pharmaceutical industry in the next 10 years. Further, the DNA and expression strains associated with this work have now been requested by over 150 academic and industrial researchers worldwide, many of which are trying the system with a wide variety of expression hosts, target proteins and mechanical separation configurations.

Brief Summary of Results from Princeton Work

The first two years of this grant supported the development of simpler and less expensive operating modes for these novel purification methods, as well as their demonstration in high cell-density *E. coli* fermentation and with more complex protein products. Perhaps the most important development of the this work was the substitution of ammonium sulfate for sodium chloride in the ELP precipitation process[23]. This substitution allows the precipitation to take place at room temperature, and at an 80% lower salt concentration (Figure 4). These combined advantages greatly simplify the instrumentation requirements for the method, making it much more attractive for scale up. These results have been published in the journal *Protein Expression and Purification*[23], and the modifications to the methods are now described in our subsequent review and methods papers[24, 25].

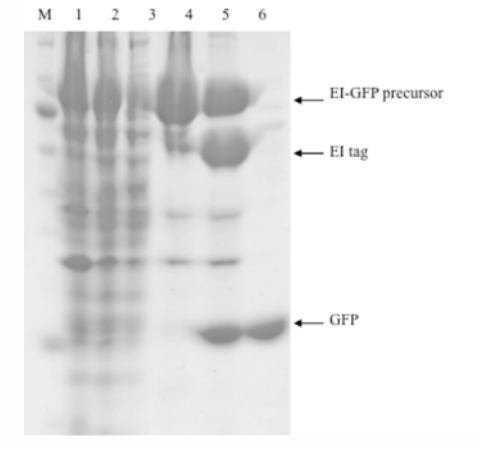

Figure 4: ELP-intein purification of the Green Fluorescent Protein using ammonium sulfate precipitation at room temperature.

Two additional demonstrations of the ELP-intein method with more complex products

were completed, including its use for purifying complex multi-subunit proteins and its use for purifying disulfide bond-containing proteins secreted in *E. coli*. The multi-subunit protein purified was the *E. coli* RNA polymerase holoenzyme, which consists of six subunits. The assembled enzyme was selectively precipitated by tagging the alpha subunit with a self-cleaving ELP tag (Figure 5)[26]. This work underscores the gentle nature of this purification method, and suggests its use in experiments aimed at identifying protein-protein interactions through protein fishing experiments. This work was published in *Protein Science*[26]. In our other demonstration, the secreted target proteins included a single-chain antibody and an antibody fragment, both of which require proper disulfide bonding for activity. These proteins were secreted in several *E. coli* strains for comparison, and were purified in an active form using our self-cleaving intein combined with a conventional chitin-binding tag. This work clearly demonstrates the advantages of the pH and temperature control of our intein, which allows efficient cleaving to take place without interfering with the native disulfide bonds of the targets. This work has been recently published in *Protein Expression and Purification*[27].

At laboratory scale, we combined advanced recombination-based cloning methods with intein-based purification methods, to produced purified ELP-intein tagged targets in high cell-density *E. coli* fermentation. The first recombination method that we used is based on a commercially available system from Invitrogen, known as the Gateway™ system. This method allows the rapid insertion of a given target gene into a variety of expression vectors for rapid optimization of expression and purification[28]. We have now made available a series of four expression vectors to the research community, each of which includes a different purification tag, and have had several requests for the set. As our intein capabilities increase, this set will be expanded to include additional promoters and tags to be used in alternate expression hosts. This paper received an Editor's Choice award in *Biotechnology and Bioengineering*[28]. Our second paper in this area demonstrates the use of inteins with very convenient and rapid Topo® cloning, also available from Invitrogen. In this case, PCR products are directly ligated into expression vectors in a five-minute room-temperature reaction, greatly decreasing the time required to fabricate expression plasmids. This paper also required significant modification of the intein, and was published very recently in *Biotechnology Progress*[29].

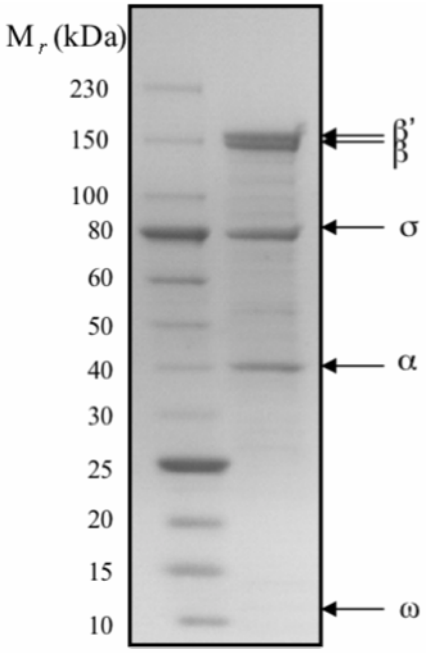

Figure 5: ELP-intein purification of the *E. coli* RNA polymerase holo-enzyme. The α-subunit was tagged and allowed to assemble with the remaining subunits upon over-expression. The assembled enzyme could be recovered active and intact via a single round of ELP precipitation.

In our initial attempts to produce proteins at large scale, we developed an efficient fed-batch fermentation process using a supplemented minimal medium. This method produces extremely high cell densities of *E. coli*, and

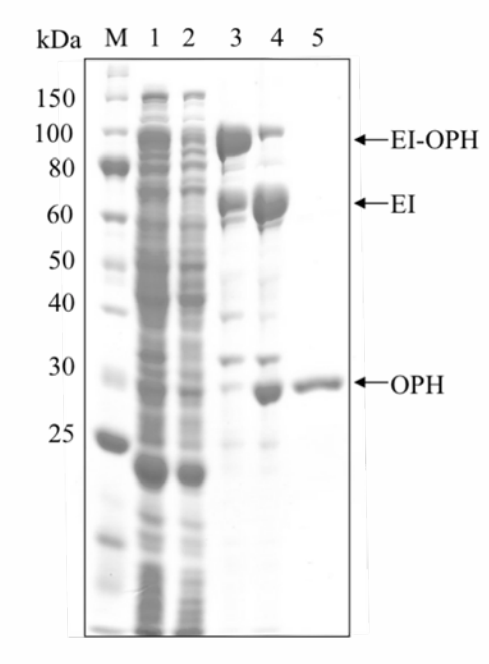

Figure 6: ELP-intein purification of OPH from a high cell-density *E. coli* culture using two rounds of ELP precipitation. M = molecular weight markers; lane 1 = clarified lysate; lane 2 = soluble contaminants removed; lane 3 = recovered, uncleaved ELP fusion protein (some cleaving has started); lane 4 = cleavage products after overnight at room temperature; lane 5 = recovered, soluble product protein after precipitation of cleaved ELP tag.

provides efficient expression of uncleaved precursor proteins for purification. A single round of optimization produced highly active and pure OPH at a yield of close to 100 mg per liter, without any conventional chromatographic processes (Figure 6). This work was recently published in *Microbial Cell Factories*[5].

In summary, our work at Princeton during the first two years of the grant led to several publications, patents and presentations, as well as three PhD dissertations. Our work on enhancing the method through the use of ammonium sulfate for precipitation appeared in *Protein Expression and Purification*[23], while our demonstration of the system for purifying the *E. coli* RNA polymerase holoenzyme though tagging a single subunit appeared in *Protein Science*[26]. Our efforts to simplify cloning of new targets into intein-based expression vectors have appeared in *Biotechnology Progress*[29] and have received an Editor's Choice award in *Biotechnology and Bioengineering*[28]. This work has also been the topic of reviews in the *Chemical Engineering Journal*[18] (with an undergraduate first author), *Trends in Biotechnology*[24], *Separations Science and Technology*[30], and chapters in *Current Protocols in Protein Science*[25] and *The Encyclopedia of Industrial Biotechnology*[31]. Most recently, two additional research papers have been published, including the aforementioned work on secreted protein targets in *E. coli*[27], and on high cell-density fermentations with products expressed in *E. coli*[5].

Brief Summary of the Results from the Current Grant (at OSU)

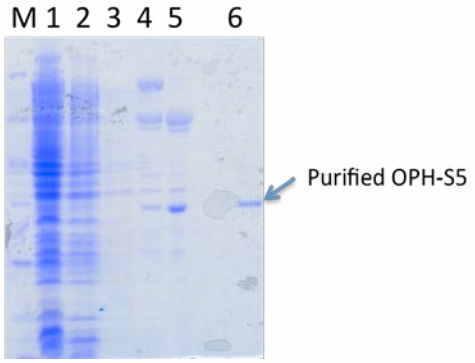

Figure 7: Recent ELP-intein purification of OPH from shake-flask *E. coli* culture (carried out by new personnel at OSU). M = molecular weight markers; lane 1 = clarified lysate; lane 2 = soluble contaminants removed; lane 3 = contaminant proteins from second wash; lane 4 = recovered, uncleaved ELP fusion protein (some cleaving has started); lane 5 = cleavage products after overnight at room temperature; lane 6 = recovered, soluble product protein after precipitation of cleaved ELP tag.

Fermentation Development. As explained above, our previous work has generally been carried out at laboratory scale, using research-grade reagents for cell culture and purification. These reagents are generally expensive, and a simple analysis can demonstrate that cheaper growth media components are necessary before this process will be economical[32]. Due to a series of technical issues, we have not been able to complete work on the fermentation development. These difficulties have been somewhat compounded by a change in laboratory personnel due primarily to graduating Ph.D. students at the beginning of the funding period (Baley Fong and Wan-Yi Wu), and the main focus of work in this area has actually been primarily on training new people. This has been successful, however, and we have been able to reproduce shake-flask work from Baley and Wan-Yi (Figure 7). Because this is a critical goal for the development of this technology in general, we are continuing this work, and have started an informal collaboration with Sanofi-Pasteur (Lyon, France), to evaluate the technology with industrial targets.

Tag Development. 2) Studies on ELP tag length are ongoing and have been nearly completed. This work was completed primarily by an undergraduate student (Robert Wensing), who was supervised by Michael Coolbaugh, and is continuing with a new undergraduate (Brian Novi), with support from several additional undergraduates. This work generated a senior thesis project by Robert, who is now pursuing a PhD in chemical engineering at the University of Illinois. His final presentation is appended to this report at Appendix A. Although he was not paid during his work, his materials and supplies were provided by the grant, and he is therefore listed in the report as being supported at 10%. His preliminary studies showed that indeed, the intein can be significantly shorted without a major impact on the purification efficiency. The impact on the protein expression levels was not as clear. He carried out his studies on two test proteins, Carbonic Anhydrase (CA) and beta-Galactosidase (Bgal). The most critical results for CA are summarized in Figure 8. In particular, we observed that the overall yield of the CA target protein initially increases as the ELP tag size goes from 50 to 400 amino acids, but then the decreases as the ELP tag goes to the currently used 550 amino acids. We believe that the increase in yield at low ELP size is due to the greater purification efficiency of larger ELP tags, and this is supported by the data shown on slides 11-13 of the attached presentation (Appendix A). The decrease in overall yield at the largest ELP sizes is likely due to losses of expression efficiency arising from the

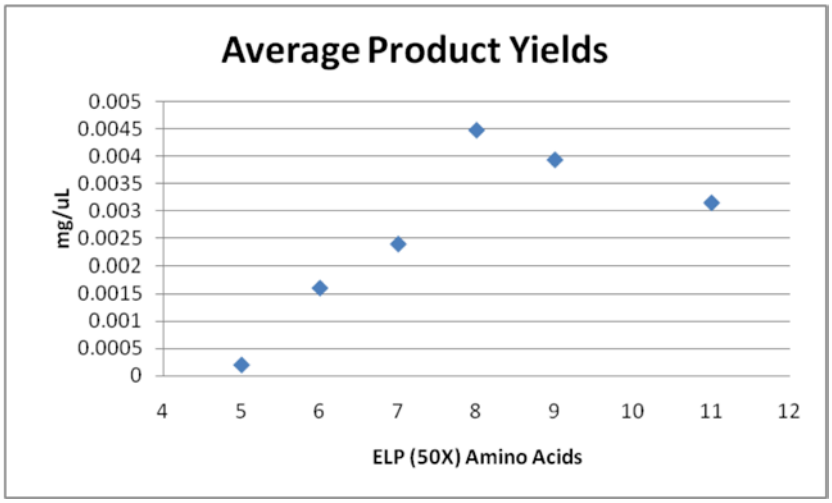

Figure 8: Average product yields vs. ELP tag length for *Neisseria gonorrhoeae* Carbonic Anhydrase (NGCA). As the ELP length decreases from 11 repeats (550 total amino acids) to 8 repeats (400 amino acids) the overall yield increases. We hypothesize that this is due to higher expression levels of the tagged protein due to a decrease in metabolic burden of its expression. As the ELP size decreases below 8 repeats, we observe that the overall yields decrease. We hypothesize that this is due to inefficient precipitation of the ELP tag at these sizes, leading to decreases in overall recovery of the final product.

metabolic burden of expressing the large tag. Somewhat surprisingly, the Bgal product yield decreased monotonically from low to high ELP size, which was initially somewhat puzzling. However, we noted that the Bgal protein seems to precipitate very efficiently, even with a very small tag, which effectively decouples the purification efficiency from the expression efficiency. Thus, the decrease in overall yield as the tag increases in size is primarily due to decreases in expression efficiency with larger tags. Previously reported data (from another group) on Bgal precipitation in the absence of an ELP tag is shown in slide 24, which somewhat supports our hypothesis. We are currently seeking to produce highly quantitative data on expression and purification efficiency. For this reason, Brian Novi has expanded this work to include several additional test proteins (beta-lactamase, OPH, a second CA and an antimicrobial peptide), and we have generated non-cleaving control fusions of different sized ELP tags with each target. The non-cleaving control tags will allow us to directly measure the impact of ELP tag length on expression, as well as on initial purification and recovery. All of the required clones have been completed, and initial data is being generated now. We are quite certain this this work will produce at least one publication, as comprehensive data of this type has never previously been generated.

Intein Development. 3) We have been quite successful in developing a novel genetic selection system for modified inteins using yeast-surface display (Figure 9). This work has been completed by Michael Coolbaugh, and was recently presented at the Annual Meeting of the American Institute of Chemical Engineers (AIChE). His presentation has been included as Appendix B. In the yeast display system, the intein is inserted into a fusion protein and displayed on the surface of yeast cells. Two fluorescent labels are used to distinguish cleaving inteins from non-cleaving inteins, where cleaving by the intein releases one of the labels. Mutant libraries can then be easily screened by fluorescence activated cell sorting (FACS). We have constructed the system, and validated it with several control inteins. Further, we have started library construction based on three different mutagenic strategies, and have shown that we can isolate

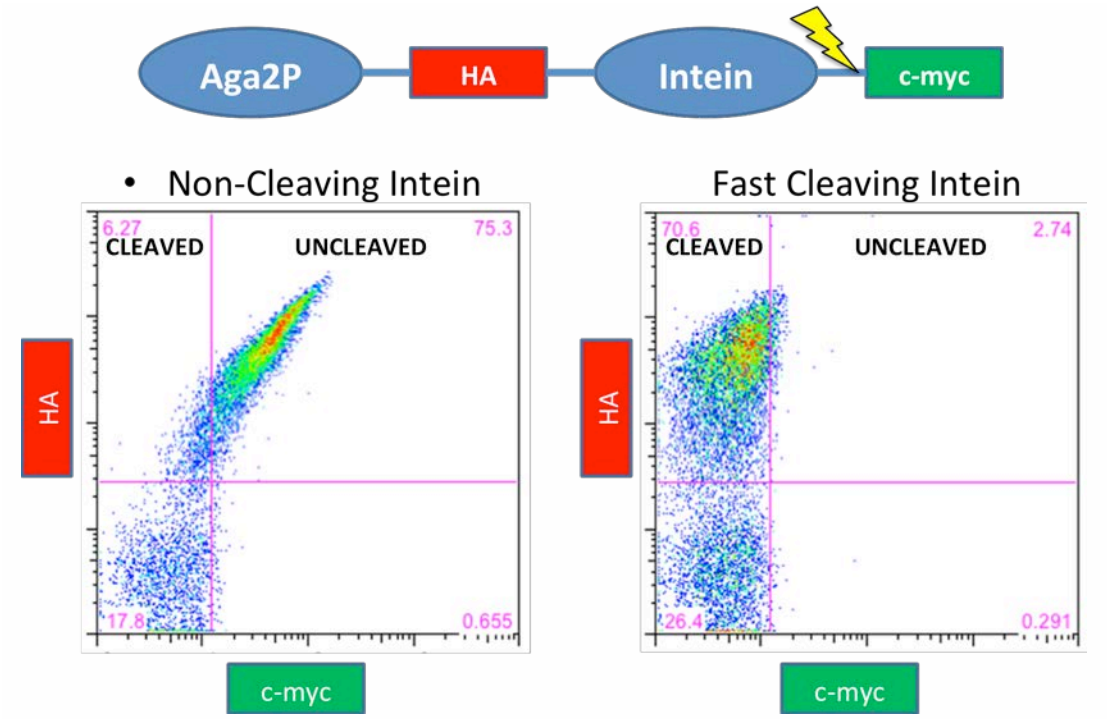

Figure 9: Schematic representation of the yeast display screening system for intein function. At top is shown the displayed protein, which includes HA (red) and c-myc (green) epitopes. If the intein is not cleaved, then both signals remain (lower left: non-cleaving control intein). If the intein cleaves, then the c-myc signal is lost, leaving only the HA signal (lower right: fast leaving control intein). The inclusion of both signals allows a simultaneous evaluation of expression level and cleaving, which provides a method for screening cleaving rates under a wide range of conditions. These screens can be combined with evolutionary or rational approaches to develop inteins with highly practical characteristics.

mutants with increased cleaving controllability. Our primary hurdle up to now has been the tendency of the yeast cells to continue to express surface proteins after they have been removed from growth media and placed in buffer. Put simply, the FACS system cannot distinguish well between newly synthesized active inteins that have not had a chance to cleave, and inteins that have been around for a while but are inactive. We are currently working on a variety of tuning strategies to allow us to select between these two. Notably, the introduction of surface kinetics of this type on a yeast-display system is new, and we have encountered a variety of interesting challenges. We are confident that this work will yield at least one additional publication in the coming year, and it has been presented at the most recent AIChE meeting in Minneapolis. As of this report, we have completed several screens, and have developed two libraries of mutants with altered temperature optima for cleaving. We are currently working to characterize these inteins.

Brief Summary of Continuing Intein System Development Work

In our most recent work, we have developed a new intein with increased sensitivity to zinc, which has the potential to allow intein technology to be used in mammalian cell hosts. We are currently working to demonstrate this intein with CHO, and have had some results indicating that we are able to make uncleaved precursors, and recover them from CHO cell culture.

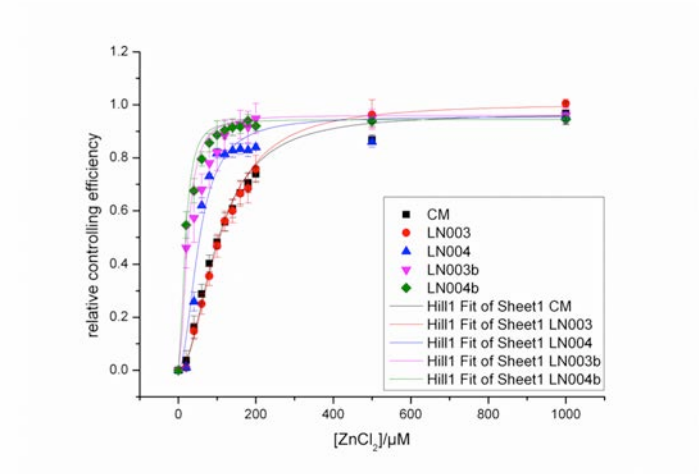

Figure 10: Modified zinc-sensitive intein with increased cleaving control. Plot shows relative control of intein cleaving versus zinc concentration. Our original ΔI-CM intein requires close to 600 µM zinc for suppression of cleaving, while the new inteins (LN004b) are suppressed at less than 100 µM. The lower zinc concentration is compatible with mammalian (CHO) cell culture, which suggests that this intein will be useful in this context.

An additional recent development has been the introduction of a previously reported choline-binding module to the self-cleaving intein system. Notably, choline possesses a quaternary amine group, which makes it highly similar to commonly used quaternary amine anion exchange resins (e.g., Q-sepharose). Because these resins are very commonly used in the biopharmaceutical industry, they are freely available, and have been accepted by the FDA for biopharmaceutical production. Our initial work with this binding module have been started with our *E. coli* cell expression system and ΔI-CM intein. The results have been very promising (Figure 11), and we have been able to purify several proteins. At this point, we are receiving resin samples from several vendors who have shown interest in evaluating the self-cleaving choline tag with their own resins. Our primary ongoing work in this area has been to improve the column binding capacity though salt and pH adjustment, and of course to demonstrate the tag in mammalian cell culture with the new zinc-sensitive intein.

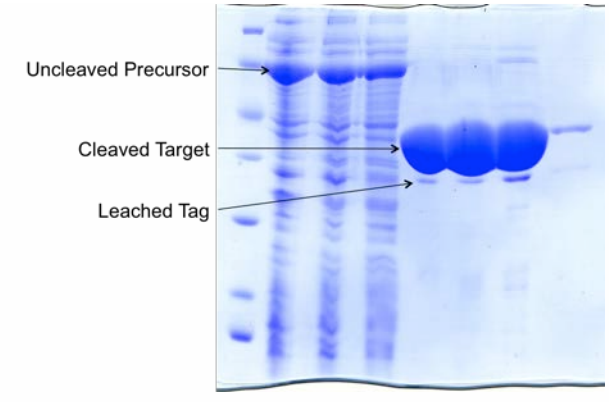

Figure 11: Example purification using choline-binding tag and commonly used Q-sepharose resin.

Brief Summary of Current IP and Commercialization Status

Our early intein work led to the filing of two patents at Princeton. One of these was based on the "plastic bacteria", where the intein was combined with a protein that selectively binds to poly-hydroxybutyrate granules inside of *E. coli* cells. In effect, this allowed the cells to produce both the affinity resin and tagged protein, and thus provided a non-chromatographic means for protein

purification. In this case, the cells were lysed, and granules with bound tagged proteins were washed. The self-cleaving reaction then releases the target protein from the clean granules. We also applied for a patent on the self-cleaving ELP tag, which also gained quite a lot of interest from industry and academic researchers. Both of these patents were eventually dropped after several rounds of office actions. In both cases, the examiners argued that the addition of a new tag to the existing intein system did not provide sufficient novelty for the patent to be issued. Although the PHB-binding tag had not been reported previous to our work, and therefore we argued that the tag itself had novelty, the examiners disagreed, and the patents were eventually abandoned.

Despite this disappointment, we are continuing to work with a number of companies on the development of intein technologies, and our work has been included in several grant proposals from various collaborative groups for protein purification. Overall, our research on intein purification system development has generated quite a lot of attention, and has led to a commentary on the PHB system by Professor George Georgiou in *Protein Science*[19], as well as noteworthy mention of the ELP system in *C&E News*[20], *The Scientist*[21], *Chemistry World*[22], and several online research discussion groups. This work has also been presented in several featured and keynote talks at industrial conferences as an emerging "disruptive" technology with the potential to radically impact the pharmaceutical industry in the next 10 years, and has spawned new conference tracks on Tag Technologies in Cambridge Healthtech Institute Bioprocessing Summit meetings (Cambridge, MA, August 24-27, 2009 and Cambridge, MA, August 23-24, 2010), both of which included Feature Presentations by David Wood on the future of self-cleaving tag technologies. In all, this work has been presented in well over a dozen invited presentations at major international industrial conferences in the last several years. Further, the DNA and expression strains associated with this work have now been requested by over 150 academic and industrial researchers worldwide, many of which are trying the system with a wide variety of expression hosts, target proteins and mechanical separation configurations.

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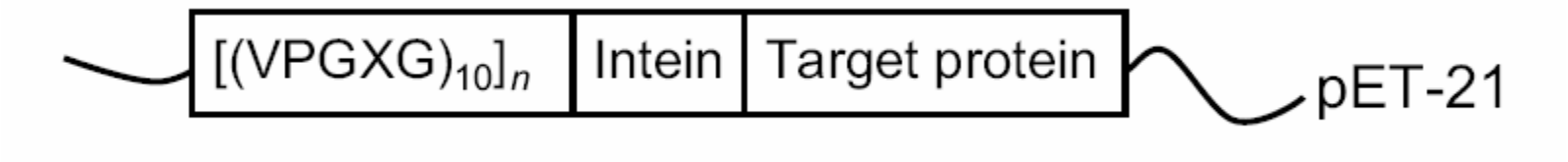
Investigation of Elastin-like Polypeptide Tag Length to Target Protein

By Rob Wensing

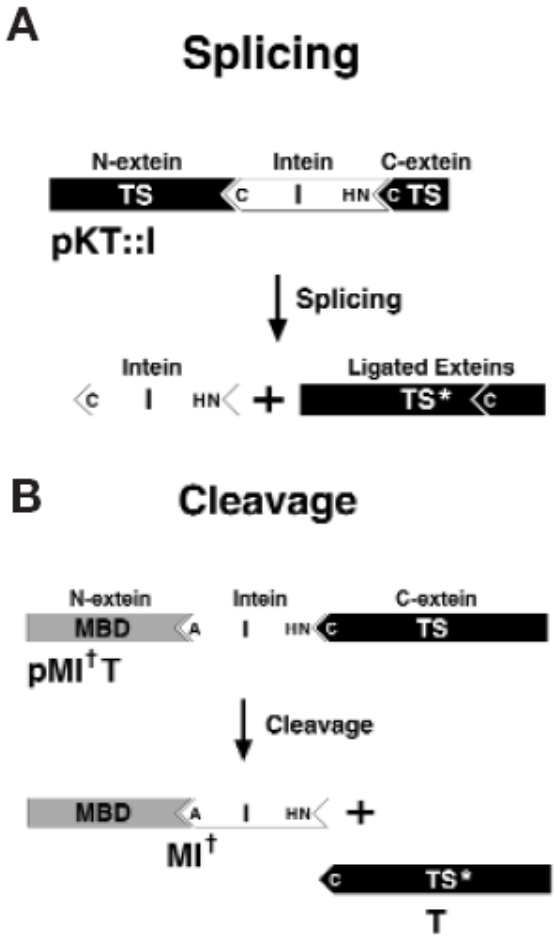


Overview

- Background
- Hypothesis
- Methodology
- Results
- Conclusions

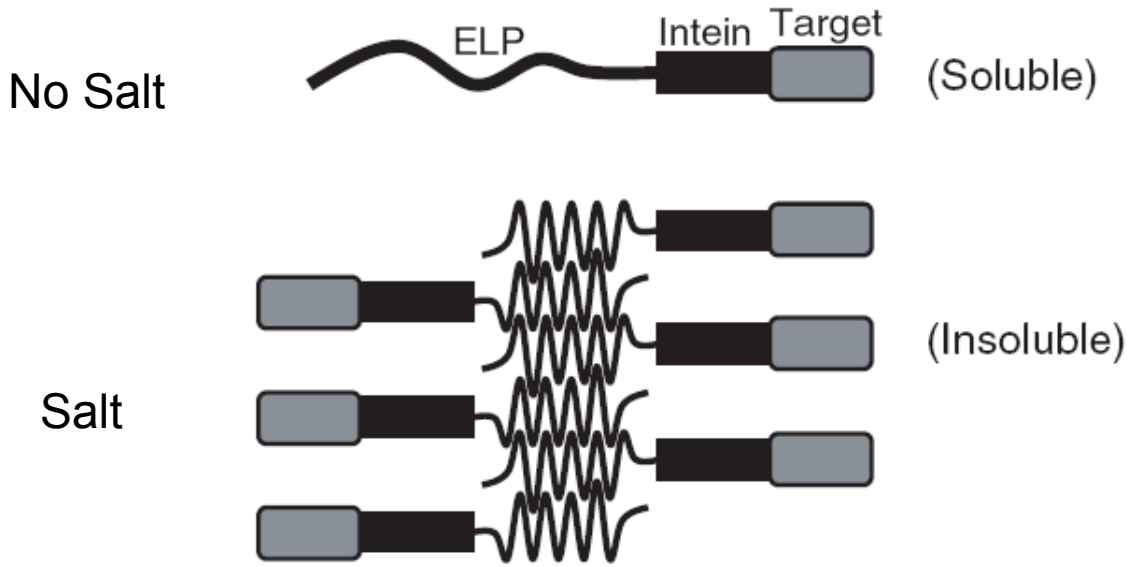
Background

- **Intein**-self-splicing protein shown in figure on right
- Shortened 441→ 168 amino acids long
- Mutation introduced to suppress N-terminal cleavage



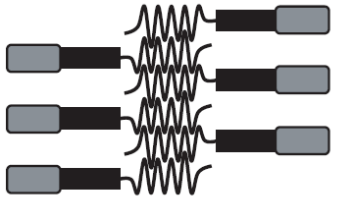
Background

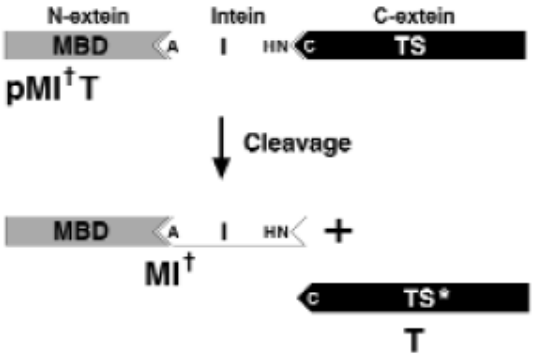
- **Elastin-like Polypeptide** (**ELP**)-Repetition of 5 hydrophobic amino acids currently 550 amino acids long



Background

- Lysed cells
- Added salt to precipitate E-I-TP
- Centrifuged and removed supernatant
- Shifted pH to 6.2 for 20 hours
- Added salt to precipitate E-I and leave TP in solution
- Centrifuged and kept supernatant with product





Background

- ELP tag has been successfully shortened when fused with thioredoxin(12 kDa) down to 100 amino acids using 1.5 M NaCl
- Decrease in ELP tag size increased expression by 4-fold
- Enhanced media yielded 36-fold increase from LB

Table 1. Amino Acid Compositions of ELP[$V_5A_2G_3$]-90, GFP-ELP, and *E. coli* Cellular Proteins

	% weight		
amino acid	ELP[$V_5A_2G_3$]-90	GFP-ELP	*E. coli* protein *(17)*
glycine	32.10	20.39	7.81
valine	35.74	23.29	5.99
proline	23.95	15.26	4.56
alanine	3.39	3.25	13.02
other	4.82	37.81	68.62

Background

- Concentration and temperature needed to precipitate ELP was reduced by using ammonium sulfate in place of sodium chloride

  1.5 M to 0.4 M 37°C to room temperature

	Kosmotropic						*Chaotropic*
Anions:	SO_4^{2-}	> HPO_4^{2-}	> CH_3COO^-	> Cl^-	> Br^-	> NO_3^-	> ClO_4^- > SCN^-
Cations:	NH_4^+	> K^+	> Na^+	> Mg^{2+}	> Ca^{2+}	> Mn^{2+}	> Cu^{2+}

Fig. 1. The Hofmeister series.

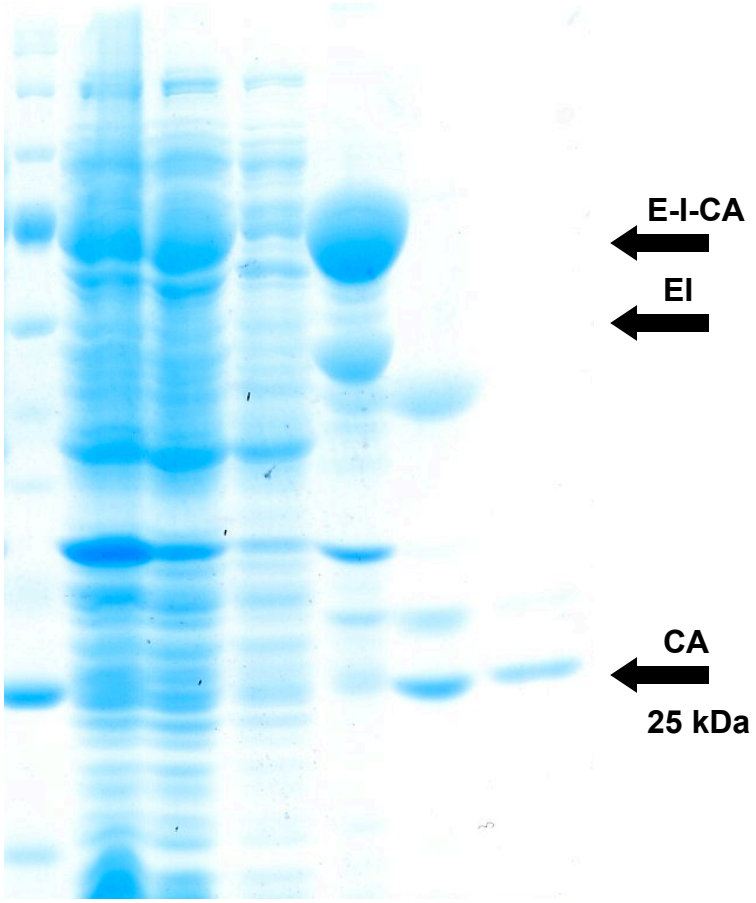
Hypothesis

- Determine relationship of target protein to ELP tag length and salt conditions needed to yield a successful purification at room temperature

Results

SDS-PAGE Analysis of CA

Lane	Contents
1	Ladder
2	Whole Lysate
3	Clarified Lysate
4	Contaminants
5	Start of Cleaving
6	End of Cleaving
7	Product



SDS-PAGE Analysis of CA

Lane	Contents
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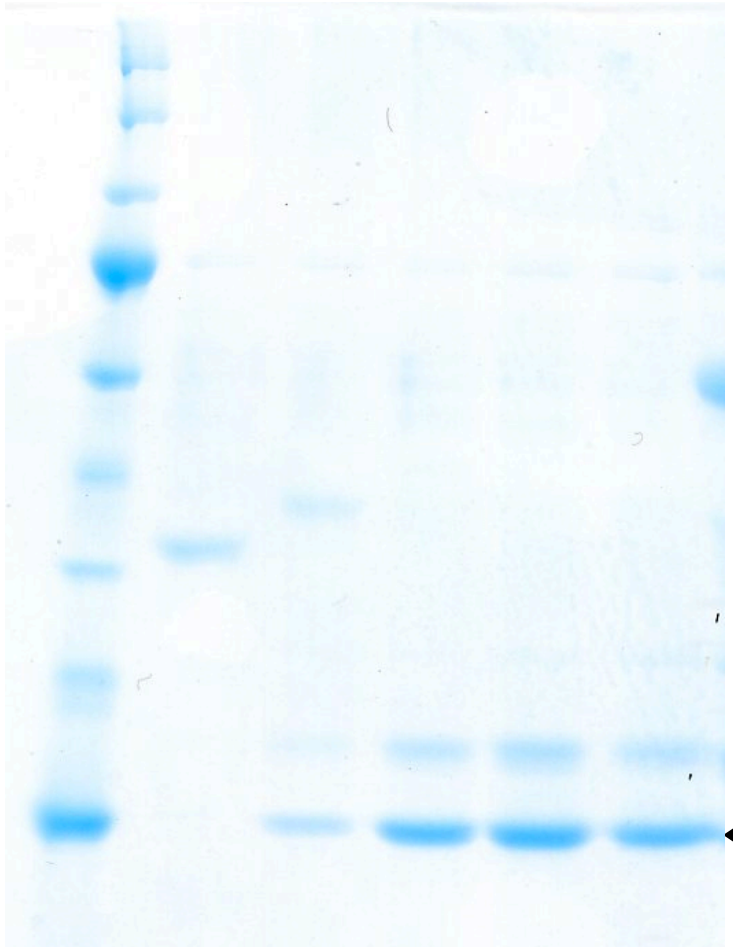
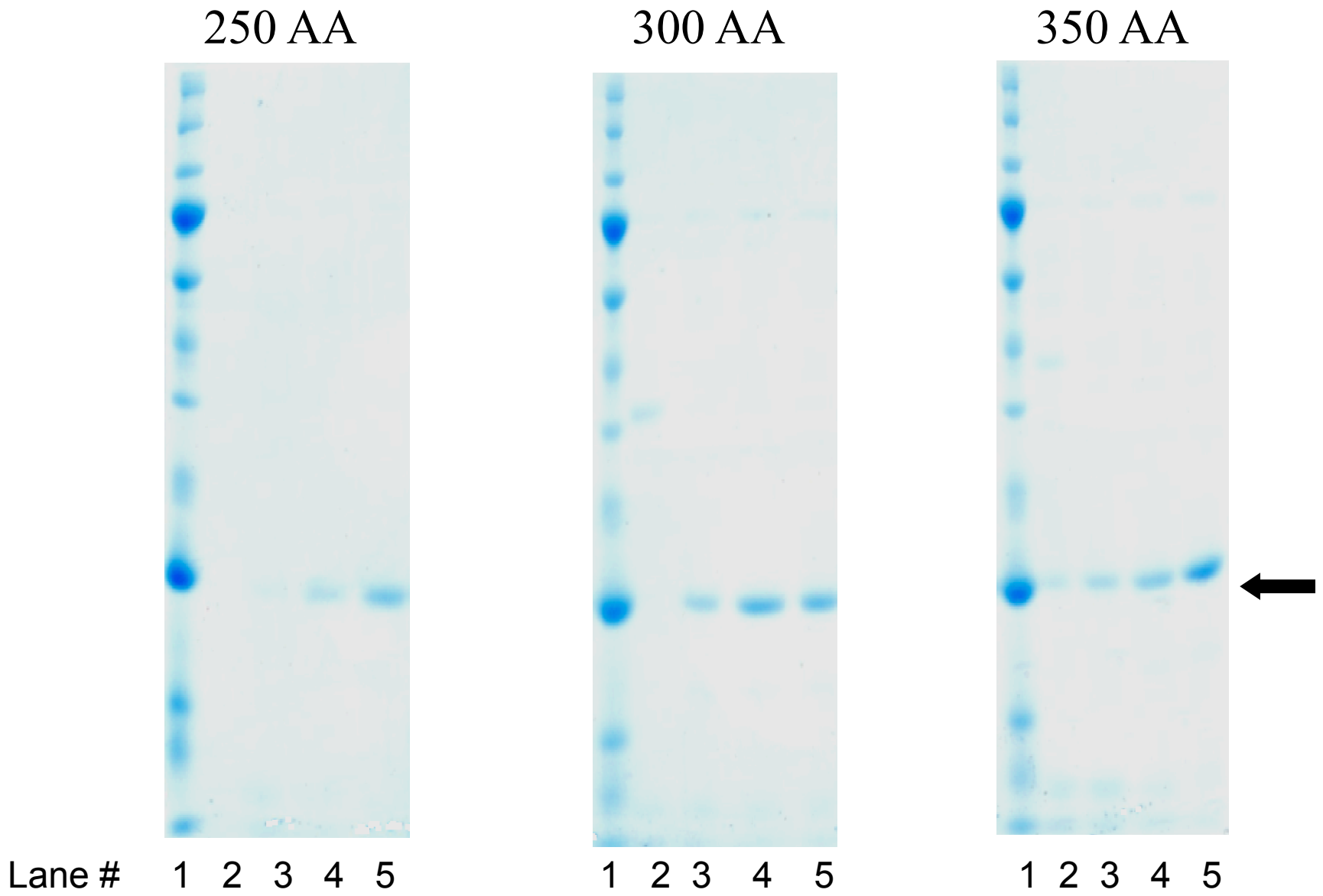
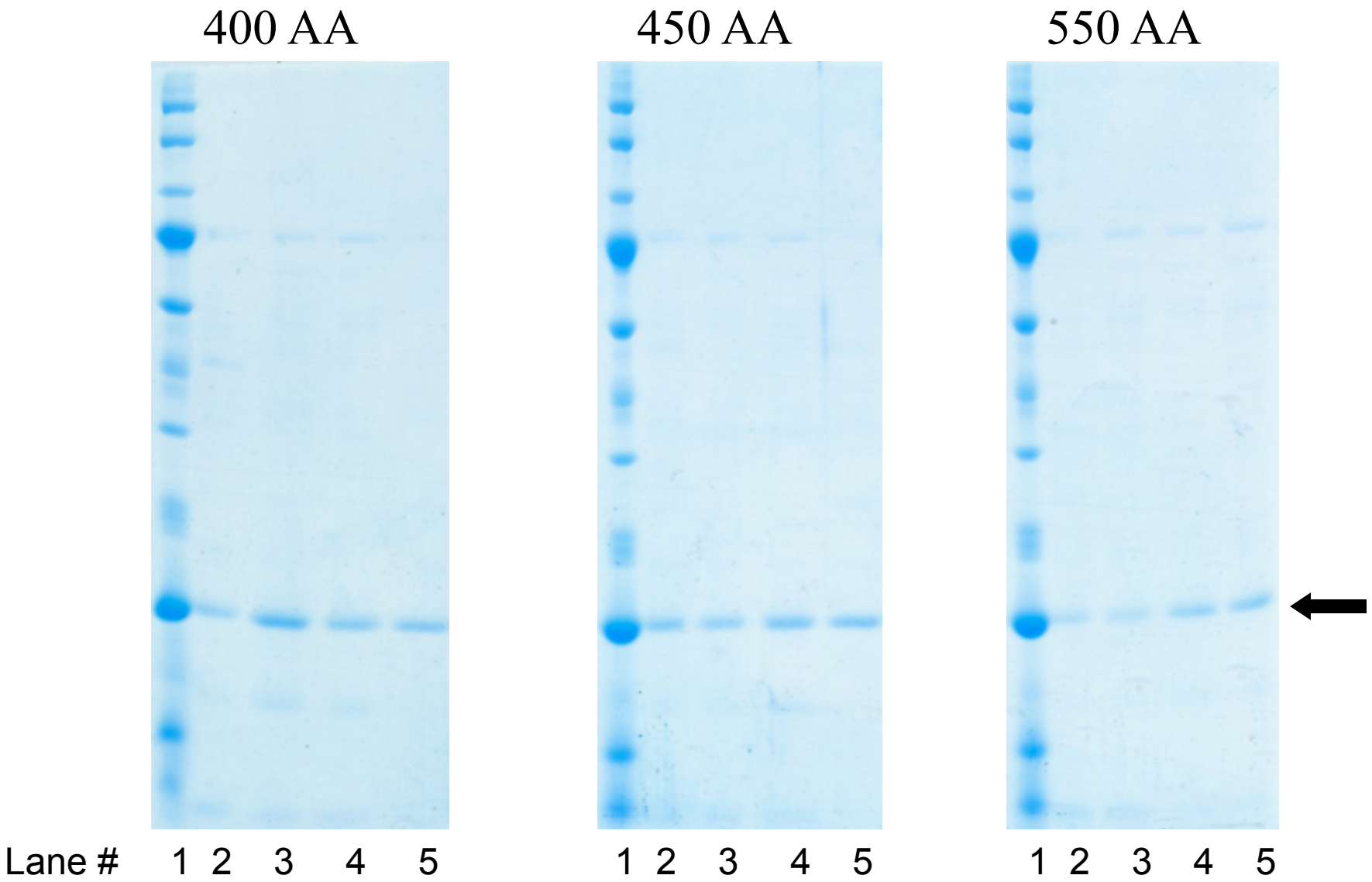
2	ELP 300 AA
3	ELP 350 AA
4	ELP 400 AA
5	ELP 450 AA
6	ELP 550 AA (Original)

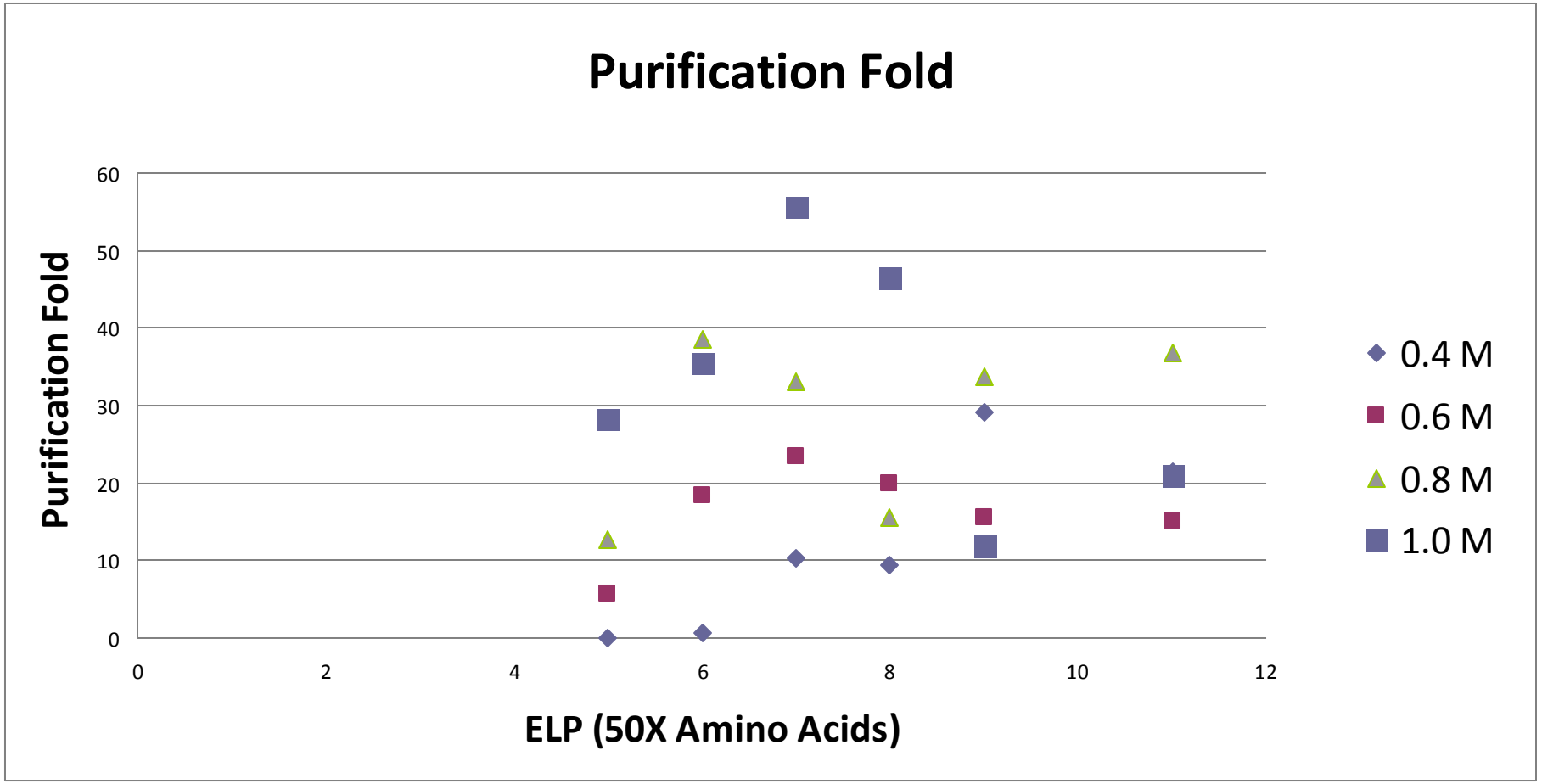


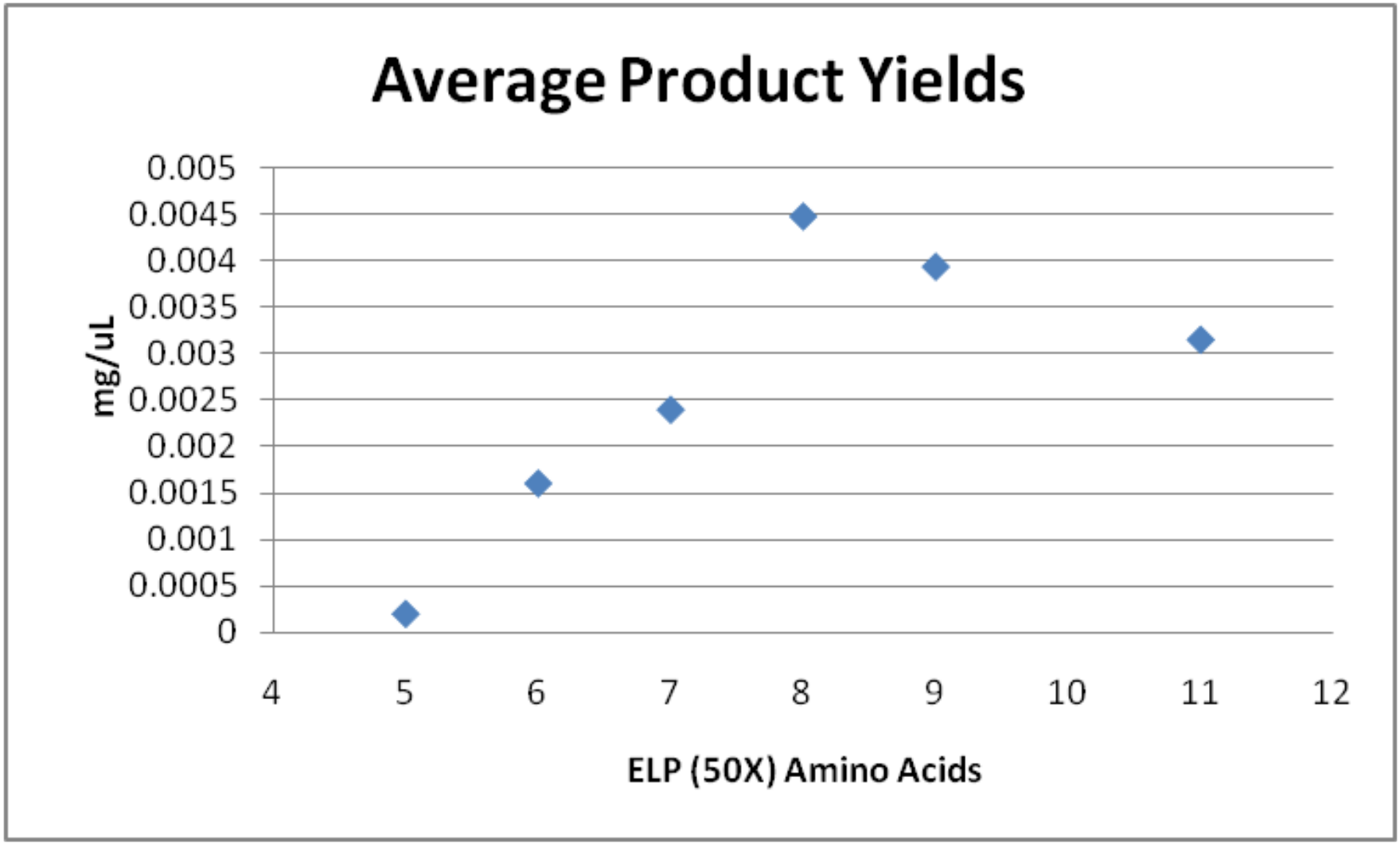


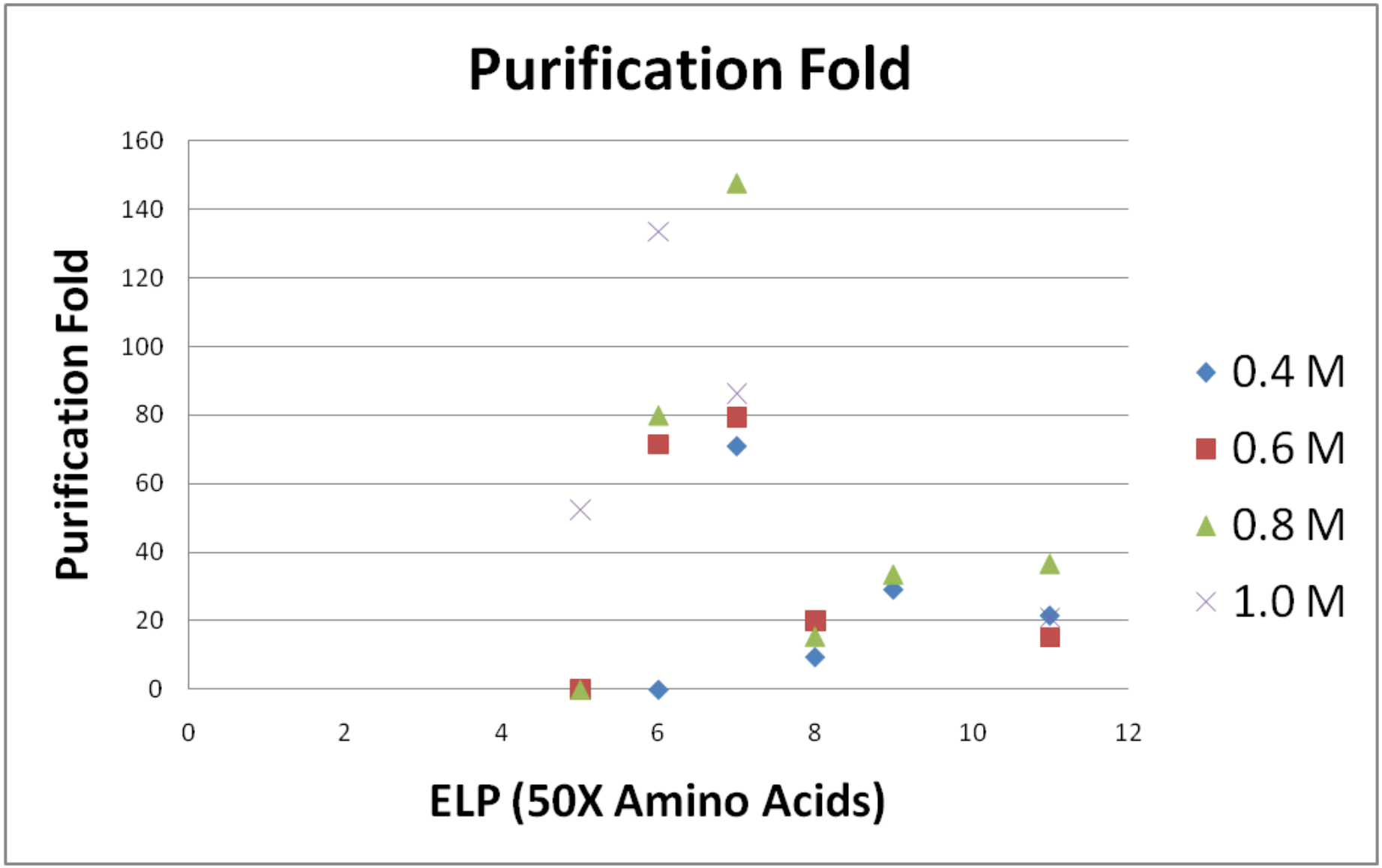
1-Ladder 2-0.4M 3-0.6M 4-0.8M 5-1.0M



1-Ladder 2-0.4M 3-0.6M 4-0.8M 5-1.0M

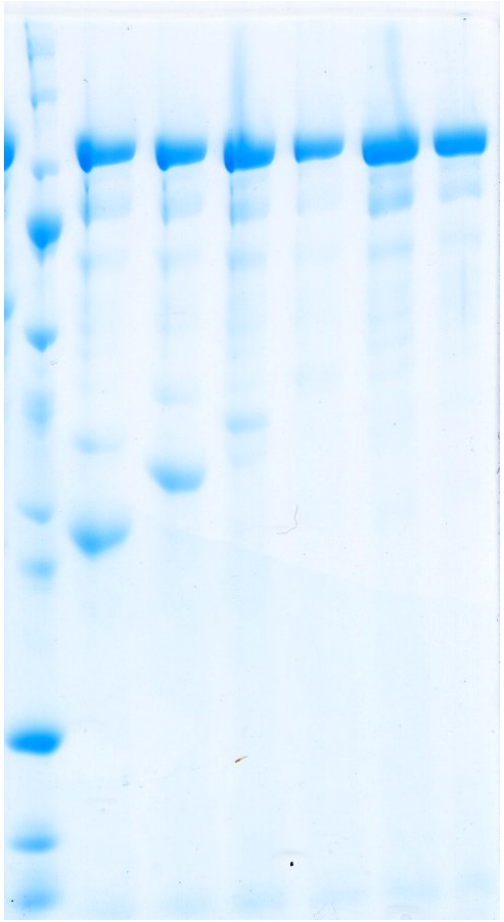

Purification Fold
Purification Fold
60
50
40
30
20
10
0
0
2
4
6
8
10
12
ELP (50X Amino Acids)
0.4 M
0.6 M
0.8 M
1.0 M


Average Product Yields
mg/uL
0.005
0.0045
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
4
5
6
7
8
9
10
11
12
ELP (50X) Amino Acids

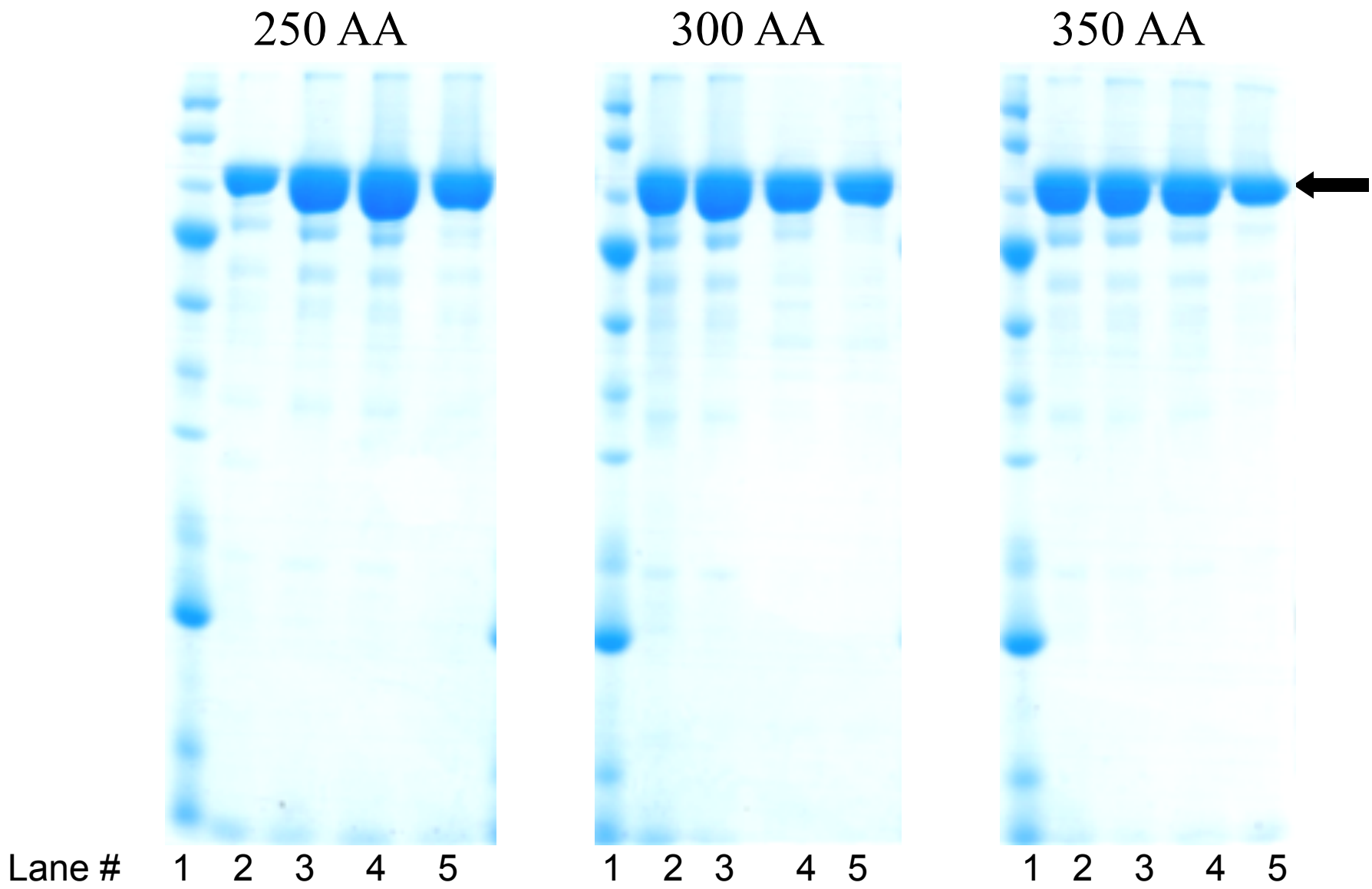

Purification Fold
Purification Fold
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
ELP (50X Amino Acids)
0.4 M
0.6 M
0.8 M
1.0 M

SDS-PAGE Analysis of Bgal

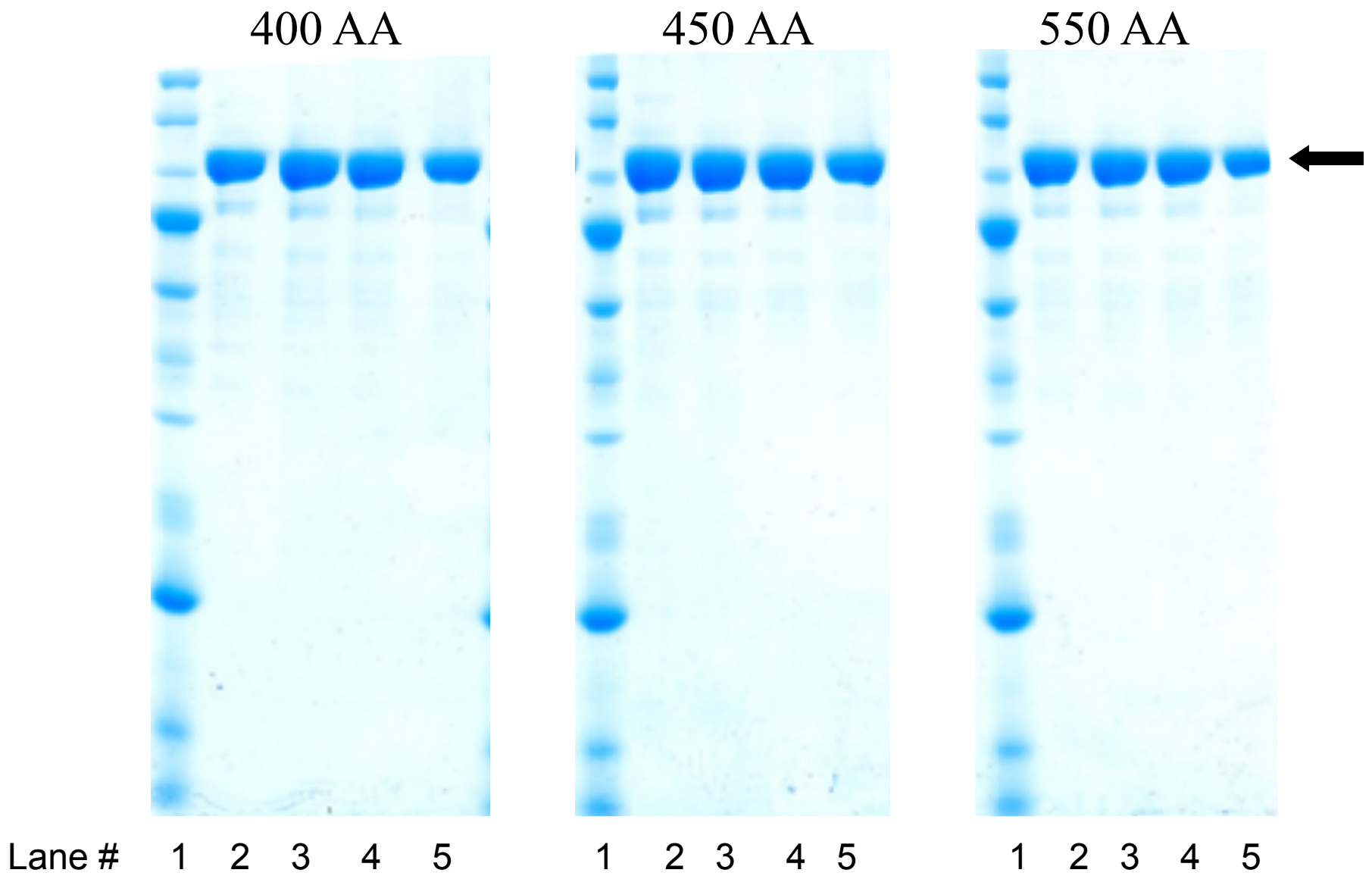
Lane	Contents
1	Ladder
2	ELP 250 AA
3	ELP 300 AA
4	ELP 350 AA
5	ELP 400 AA
6	ELP 450 AA
7	ELP 550 AA (Original)



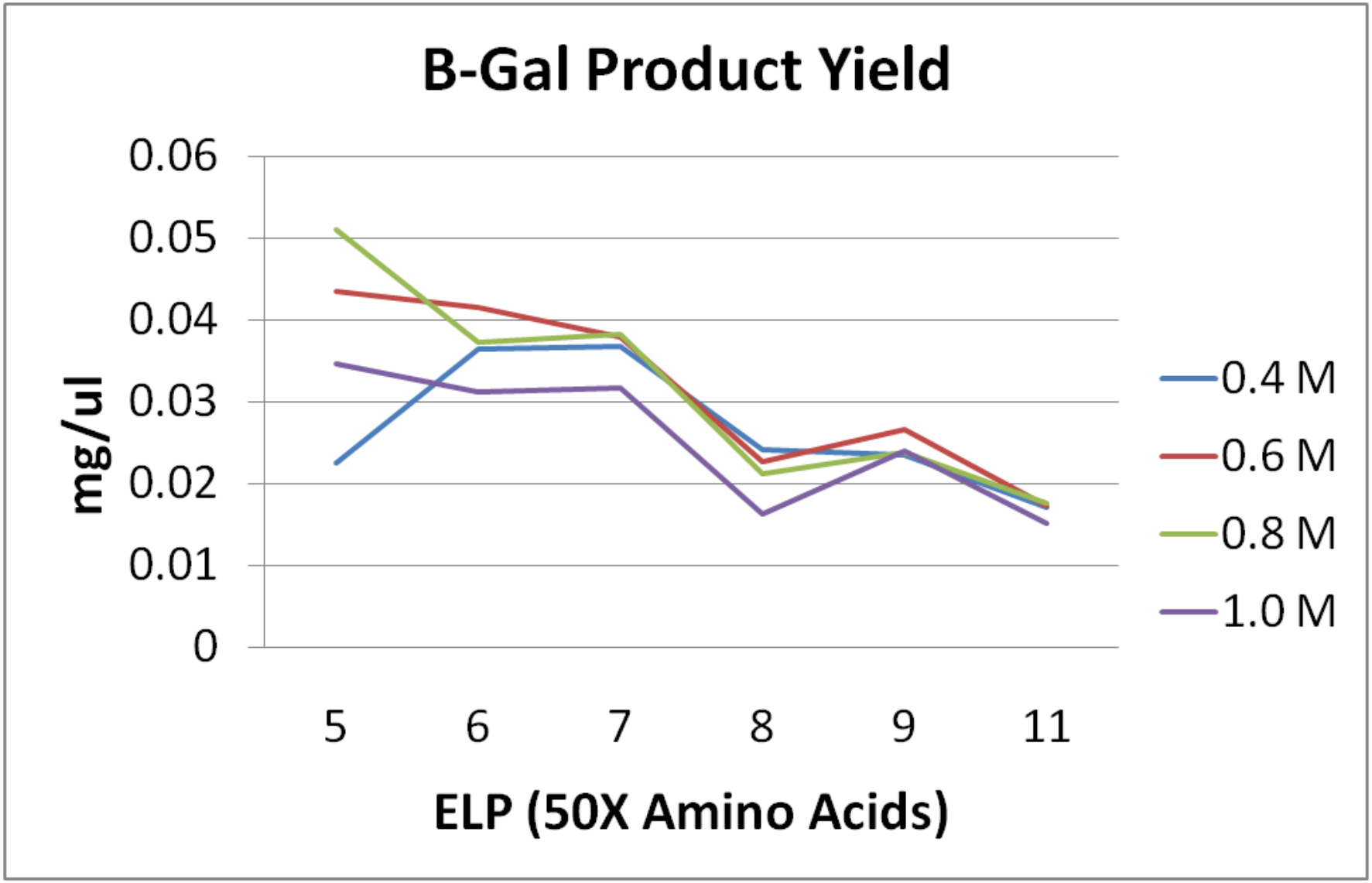
Lane # 1 2 3 4 5 6 7

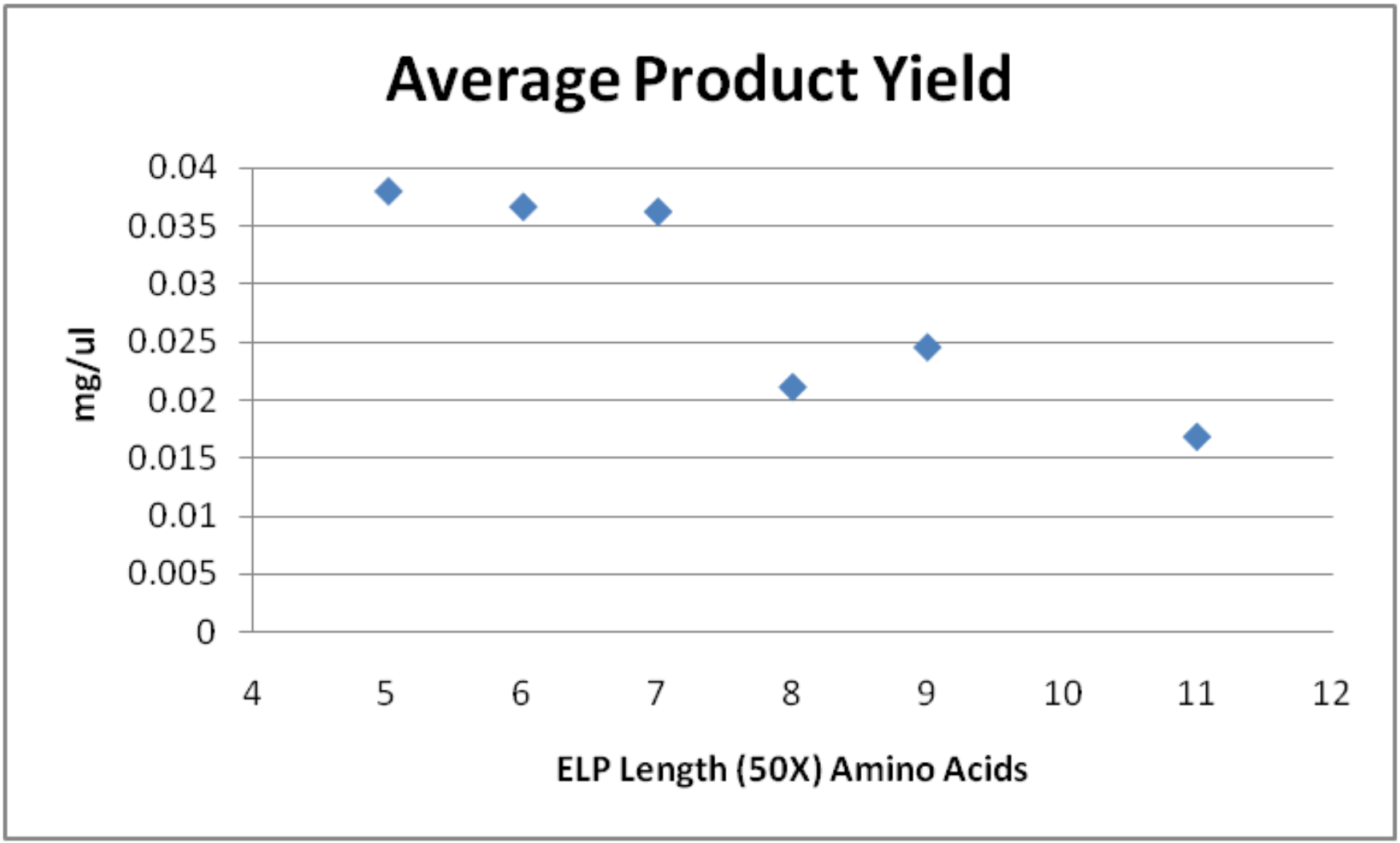


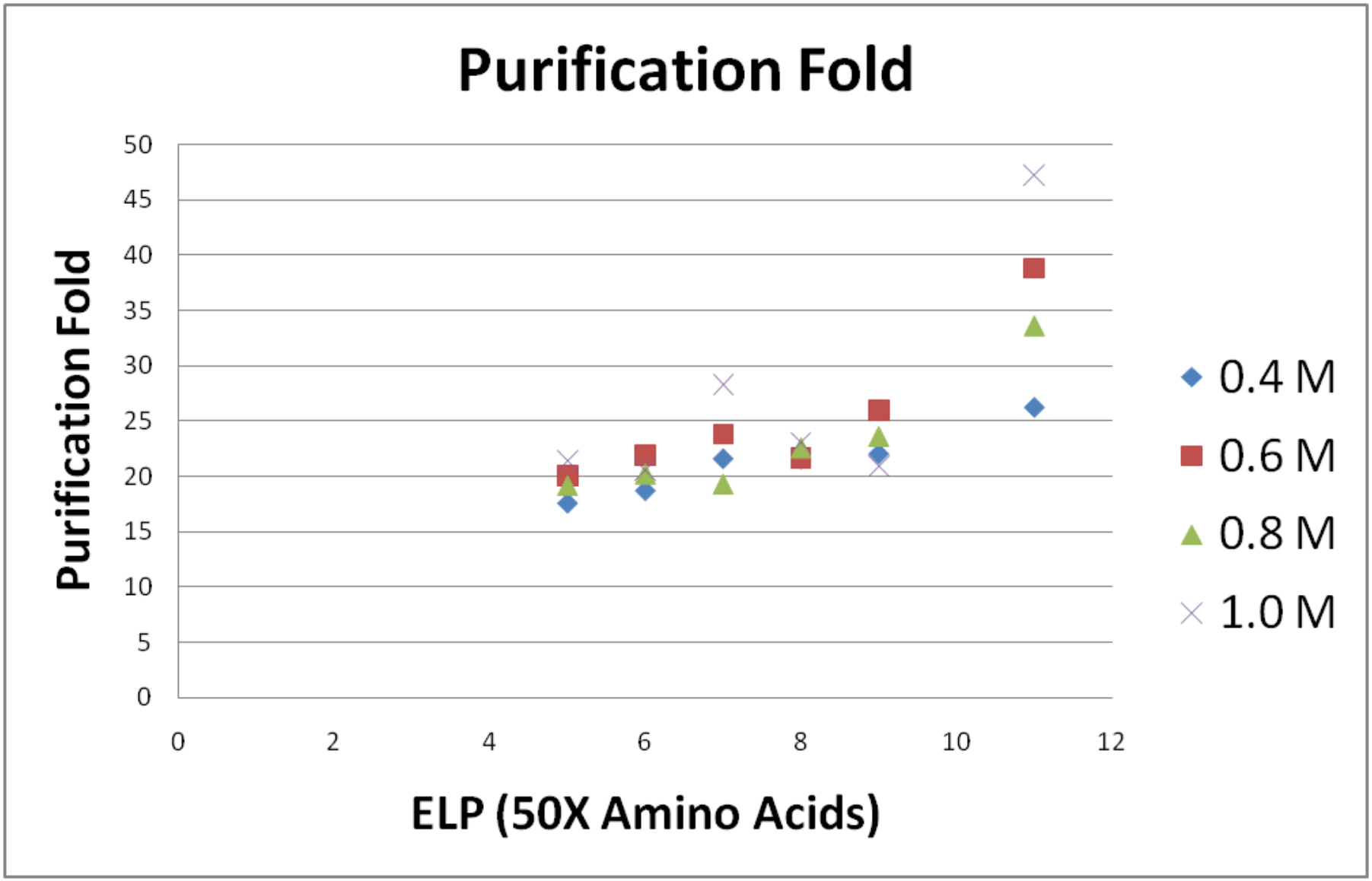
1-Ladder 2-0.4M 3-0.6M 4-0.8M 5-1.0M



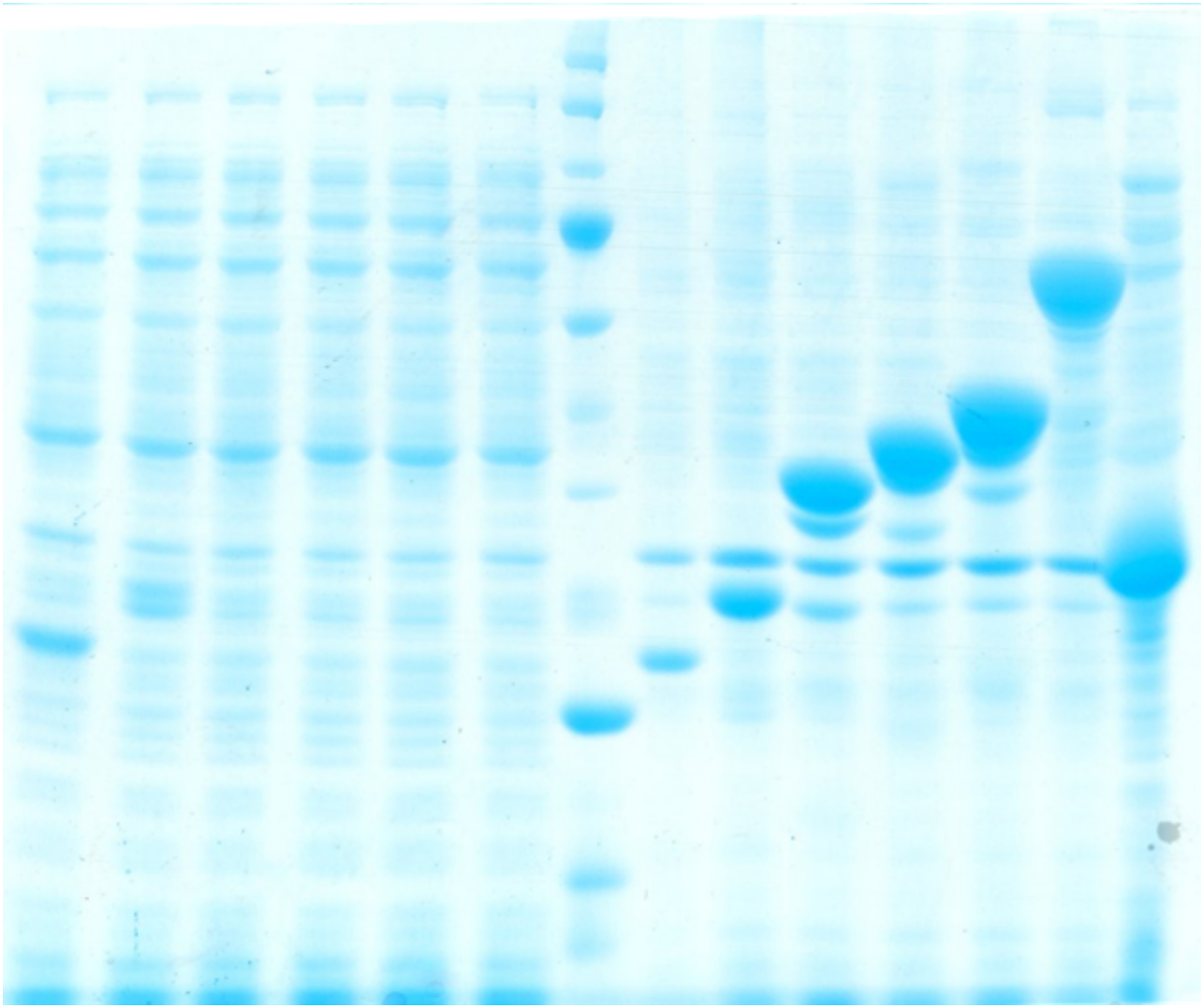
1-Ladder 2-0.4 M 3-0.6 M 4-0.8 M 5-1.0 M


B-Gal Product Yield
0.06
0.05
0.04
0.03
0.02
0.01
0
mg/ul
5
6
7
8
9
11
ELP (50X Amino Acids)
0.4 M
0.6 M
0.8 M
1.0 M


Average Product Yield
mg/ul
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
4
5
6
7
8
9
10
11
12
ELP Length (50X) Amino Acids


Purification Fold
Purification Fold
50
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
ELP (50X Amino Acids)
0.4 M
0.6 M
0.8 M
1.0 M

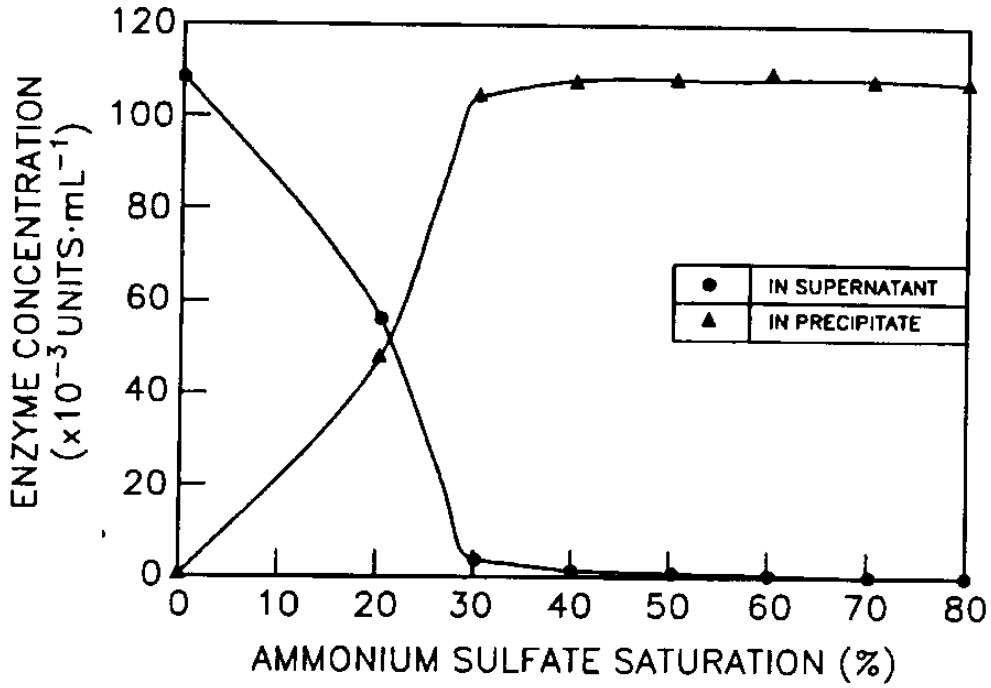
SDS-PAGE Analysis of HP



Lane # 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Discussion and Conclusions

- Sat. ammonium sulfate 5.63 M
- Precipitation without ELP occurs at1.69 M

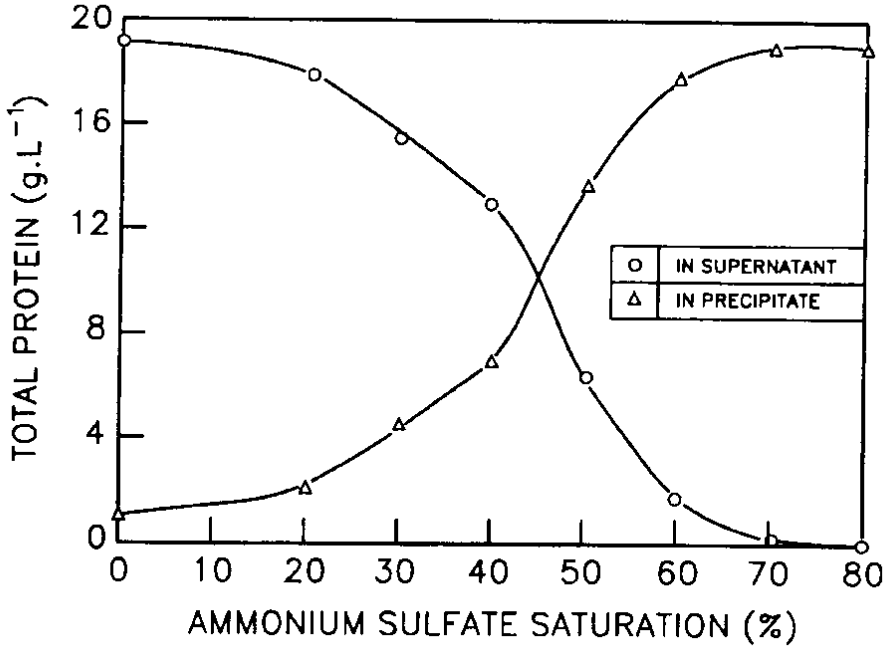


Discussion and Conclusions

- Target protein has an effect on ELP tag length and salt concentration needed
- Can optimize purity and yield by manipulation of the tag length and salt concentration
- Precipitation could be performed at higher temperatures

Discussion and Conclusions

- Salt concentrations up to 1 M appear to have little effect on purity, however past 1 M may introduce significant impurities



Future Work

- Perform purification with E(1,2,4)-I-B-Gal
- Perform purification with E(1,2,4,5,6,11)-I-HP once assay is available
- Determine ammonium sulfate concentration needed for precipitation of free HP and CA

Questions?

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Selection of Intein Variants with Enhanced Cleaving Control using Yeast Surface Display

Michael J. Coolbaugh and David W. Wood

The Ohio State University

October 18, 2011

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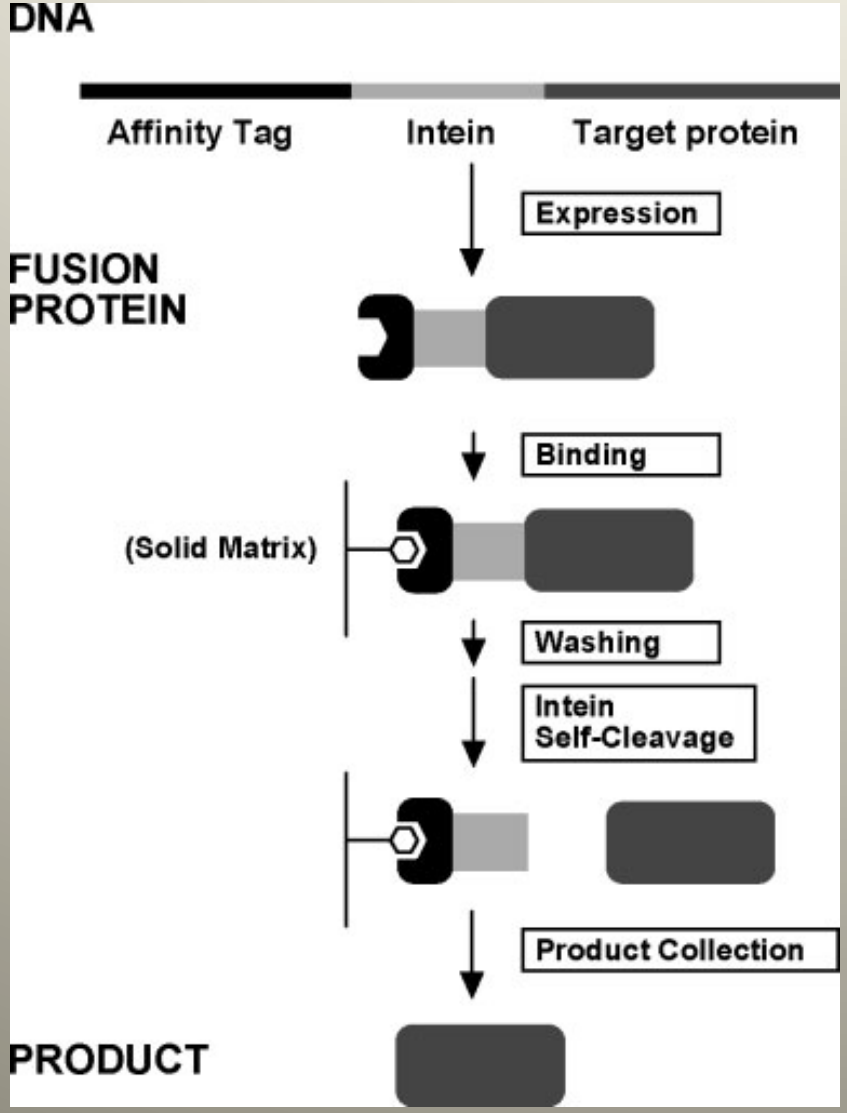
Background/Motivation



Wood (2003)[1]

- Self-cleaving intein based purification tags
- Potential platform technology
- No added protease
- Controlling cleaving reaction
- Project Goals
  - No cleavage at pH 7, 37C
  - Cleavage at pH 5.5, RT
  - CHO Cell Expression

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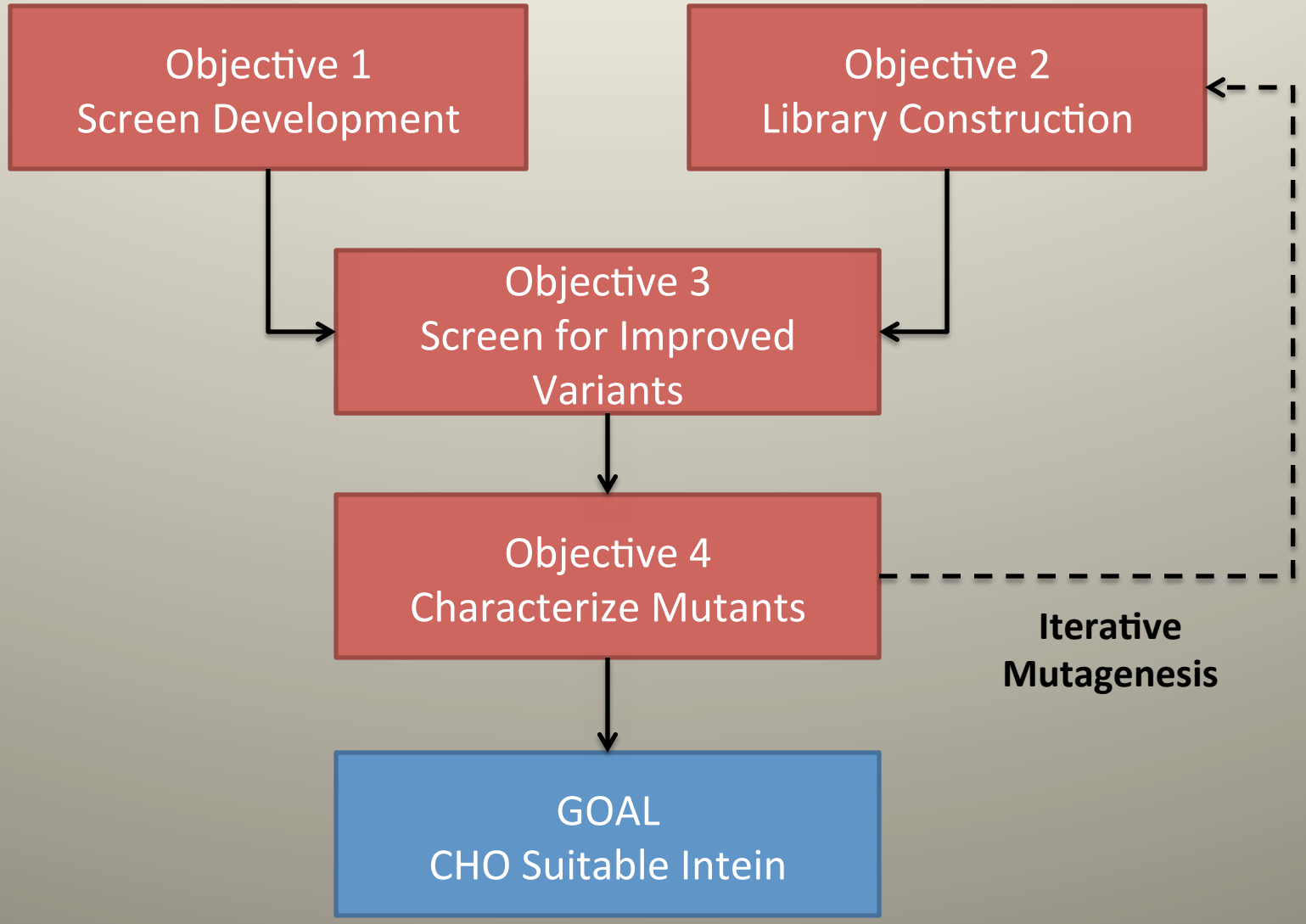
Goals and Objectives



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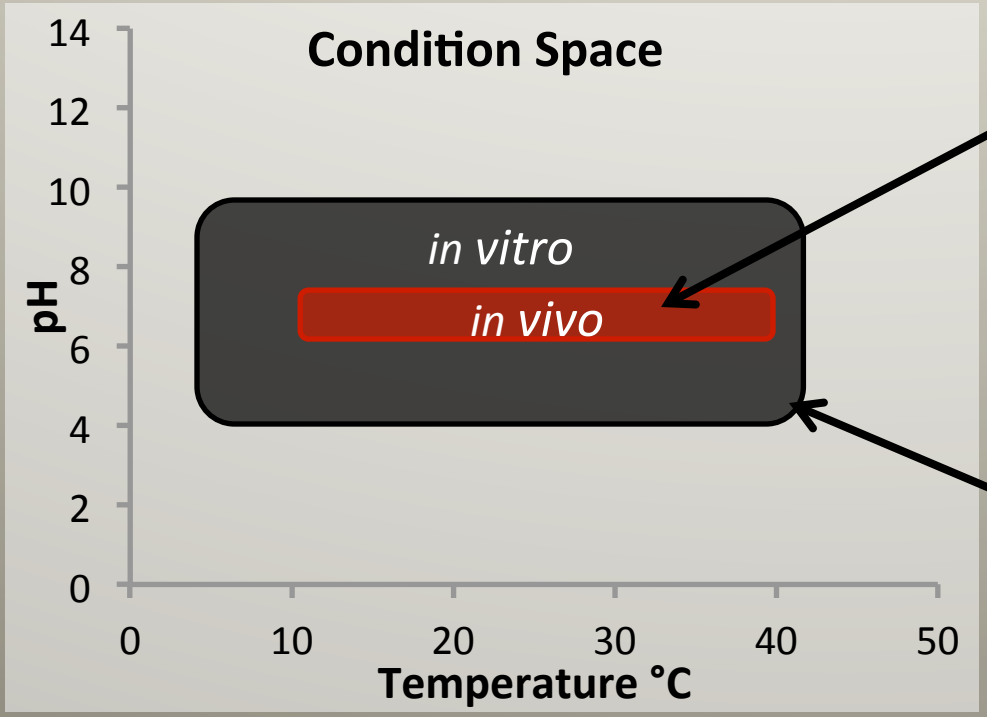
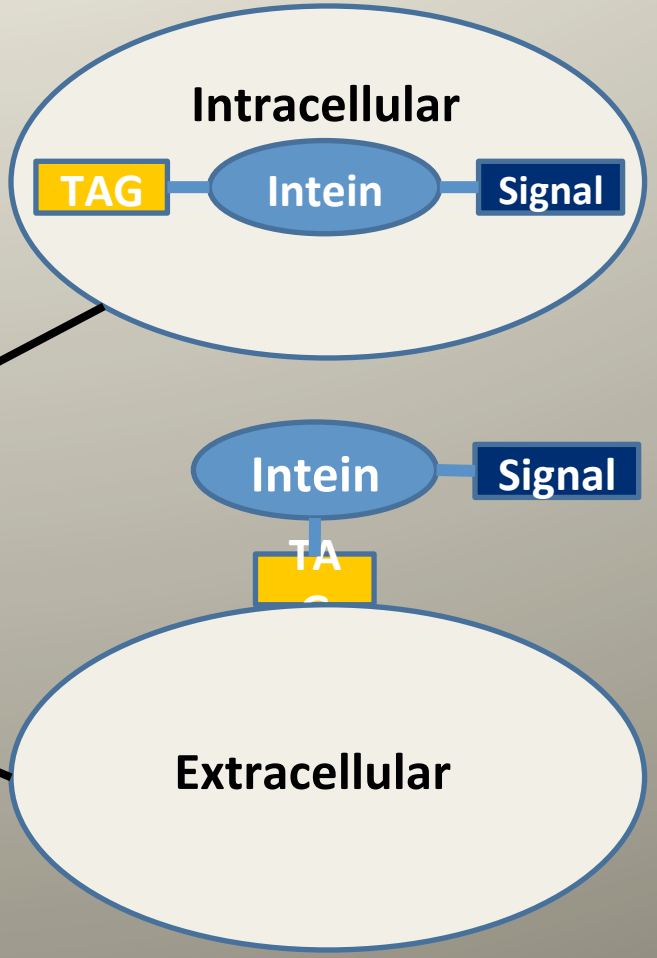
Screen Development: Yeast Surface Display[2,3]

Why Yeast Surface Display?

- Link Phenotype to Genotype
- High Throughput Screening: FACS
- Expanded Condition Space





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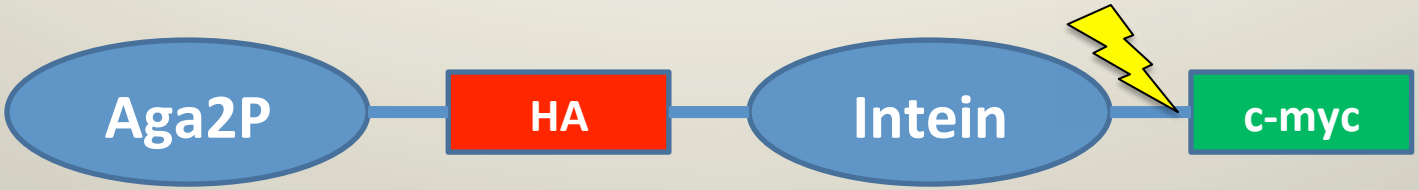
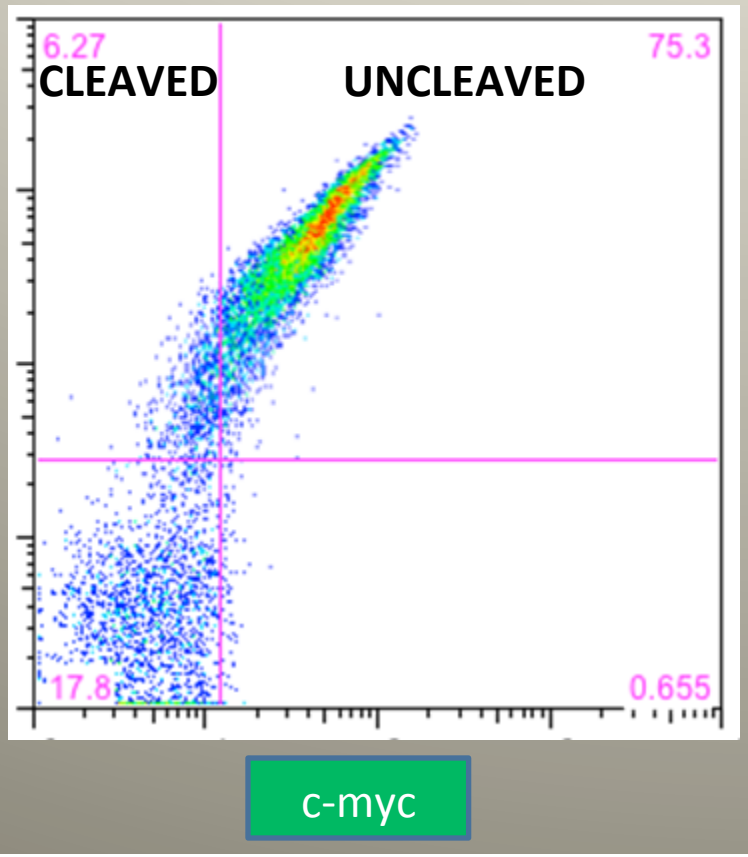
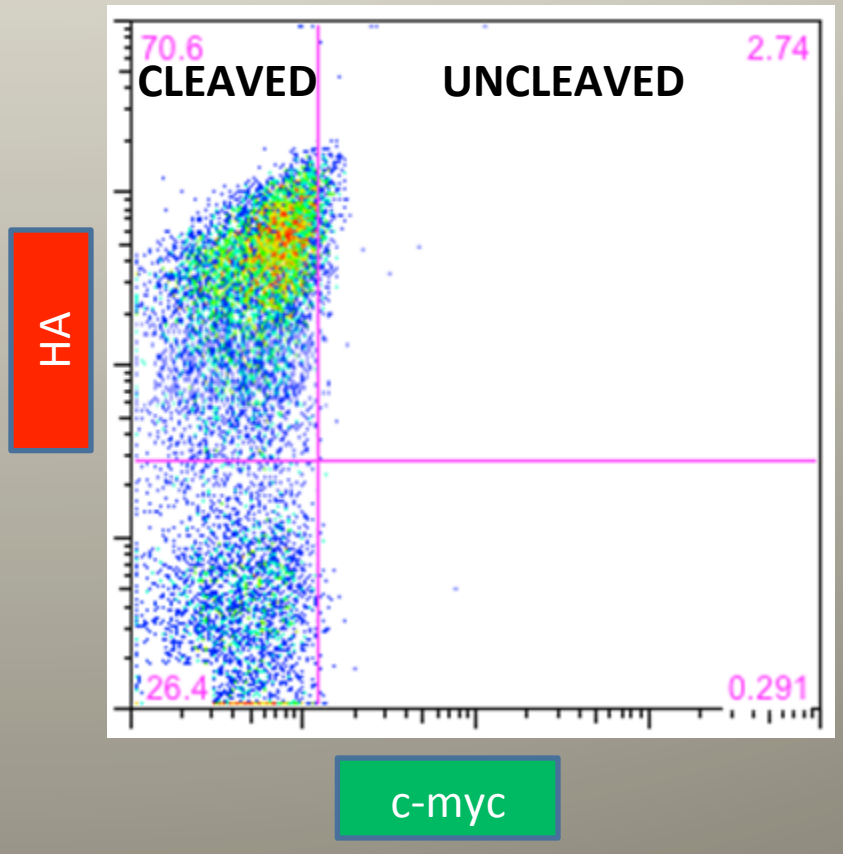
Screen Development: Yeast Surface Display Rationale



Non-Cleaving Intein



Fast Cleaving Intein



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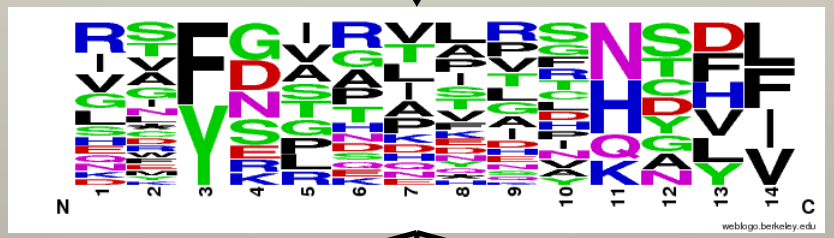
Library Construction

Intein Block F

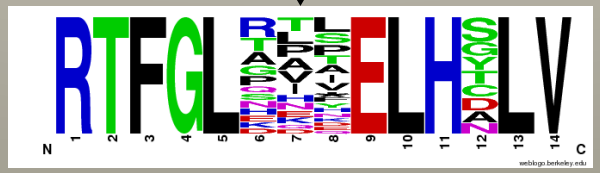
~2x10[18] variants

Natural Diversity[4]
AA Calculator[5]



~1x10[13] variants

Sub-Libraries



Block 1
~2.6x10[4] variants

Block 2
~1.7x10[4] variants

Block 3
~2.2x10[4] variants

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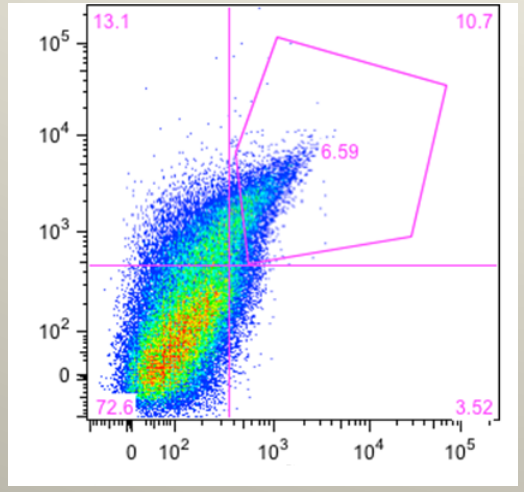
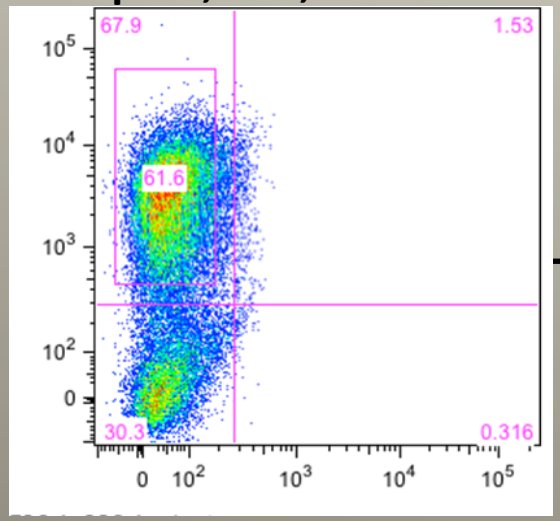
Block 1: Screen and Enrich for Improved Variants

pH 7, 34C, 6 hrs



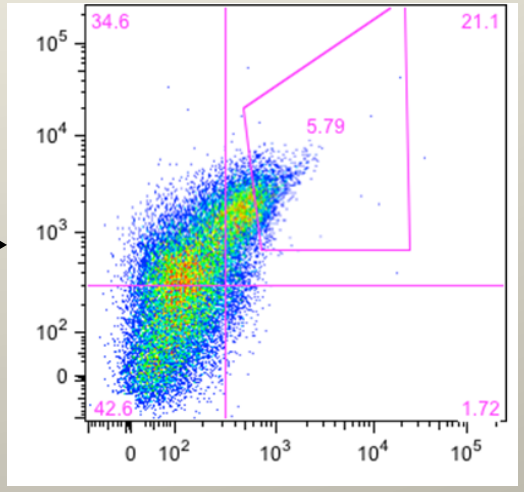
B1-1

pH 6, 25C, 24 hrs



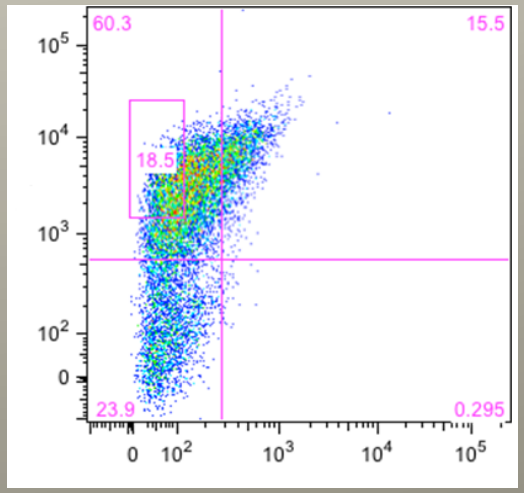
B1-2

pH 7, 37C, 24 hrs



B1-3

pH 5.5, 25C, 6 hrs



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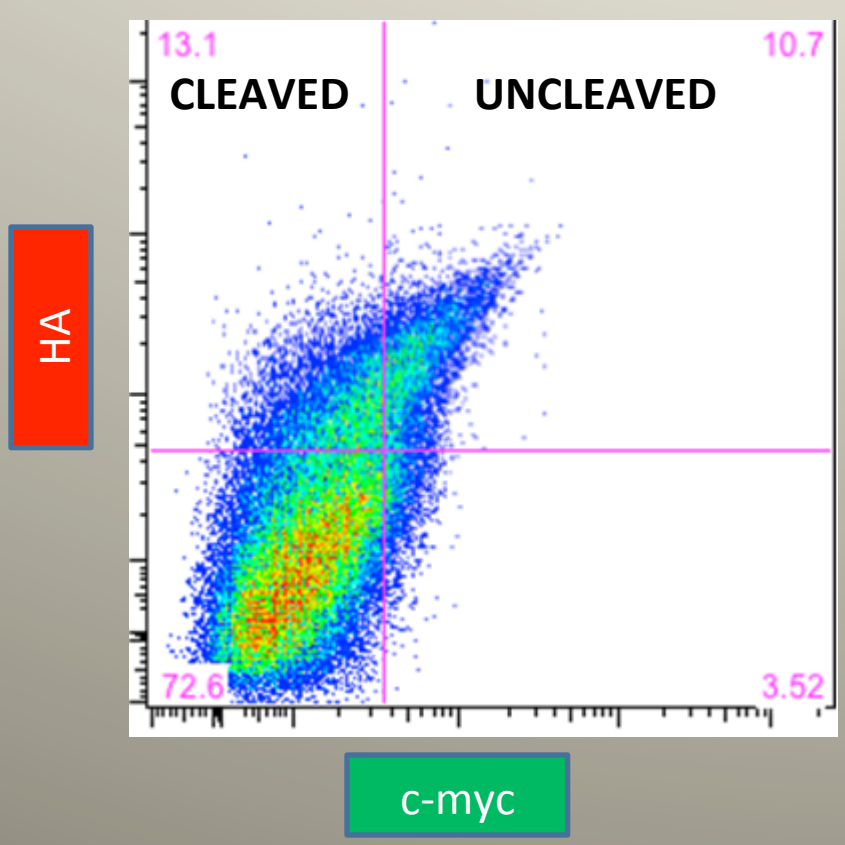
Library Enrichment: Non-Cleaving at pH 7

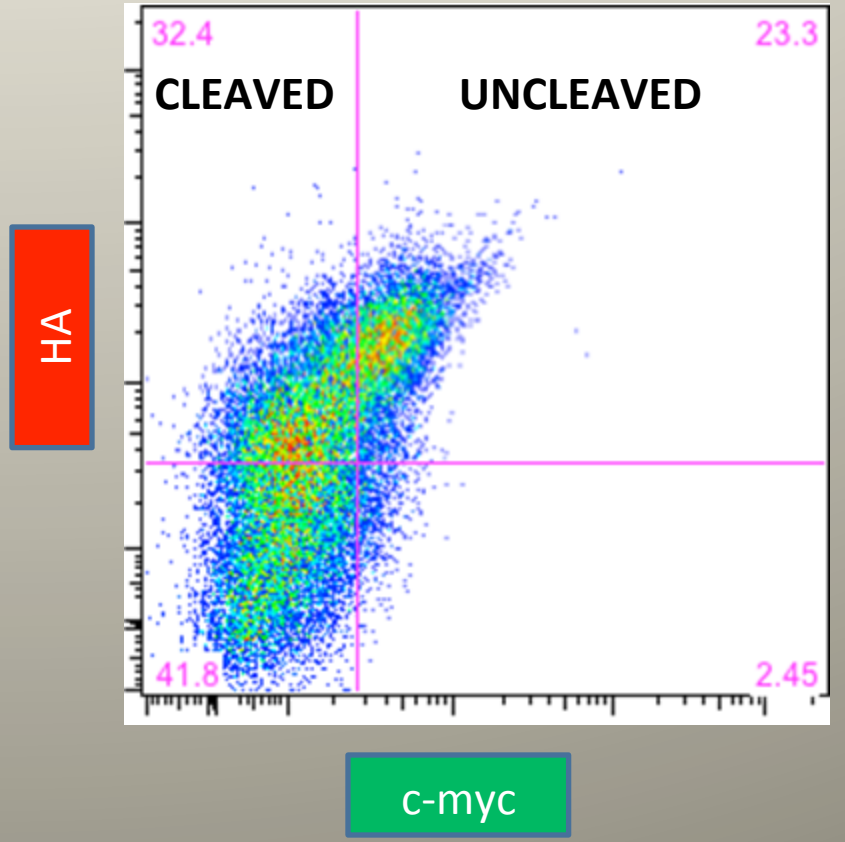
Block 1

pH 7, 34C, 6 hours



Block 1-2

pH 7, 34C, 24 hours



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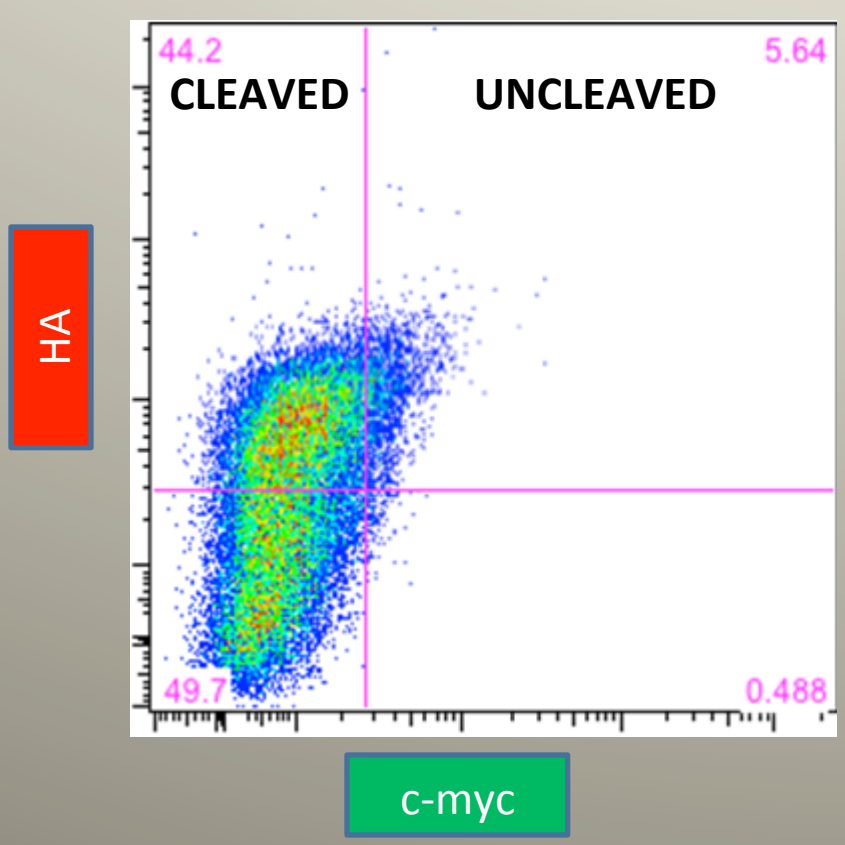
Library Enrichment: Cleaving at pH 5.5

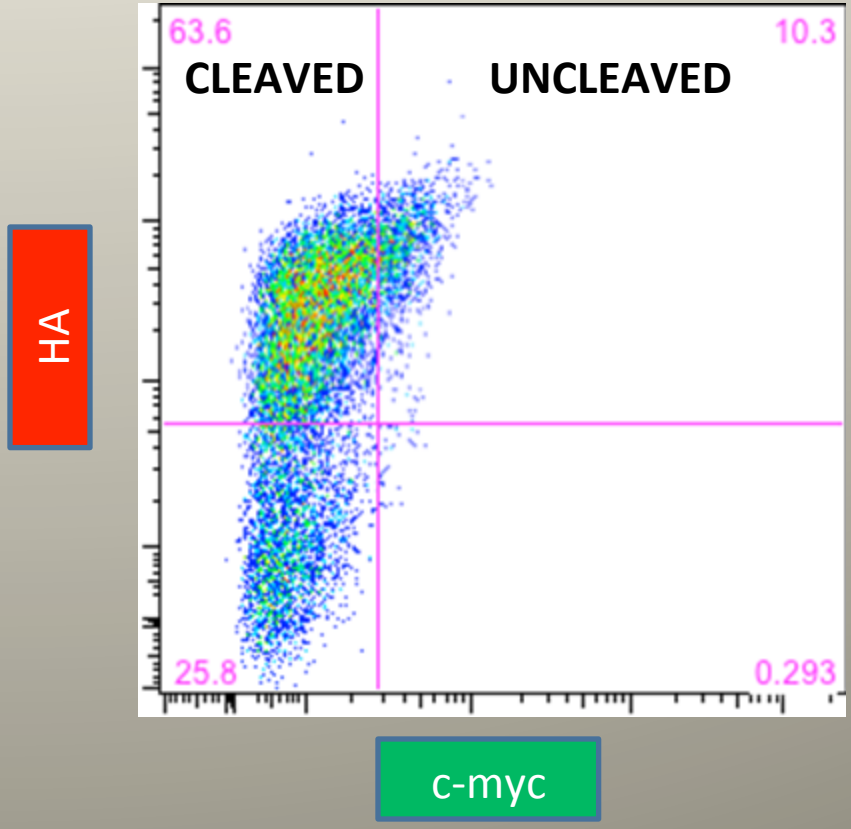
Block 1-1

pH 5.5, 25C, 20 hours



Block 1-3

pH 5.5, 25C, 20 hours



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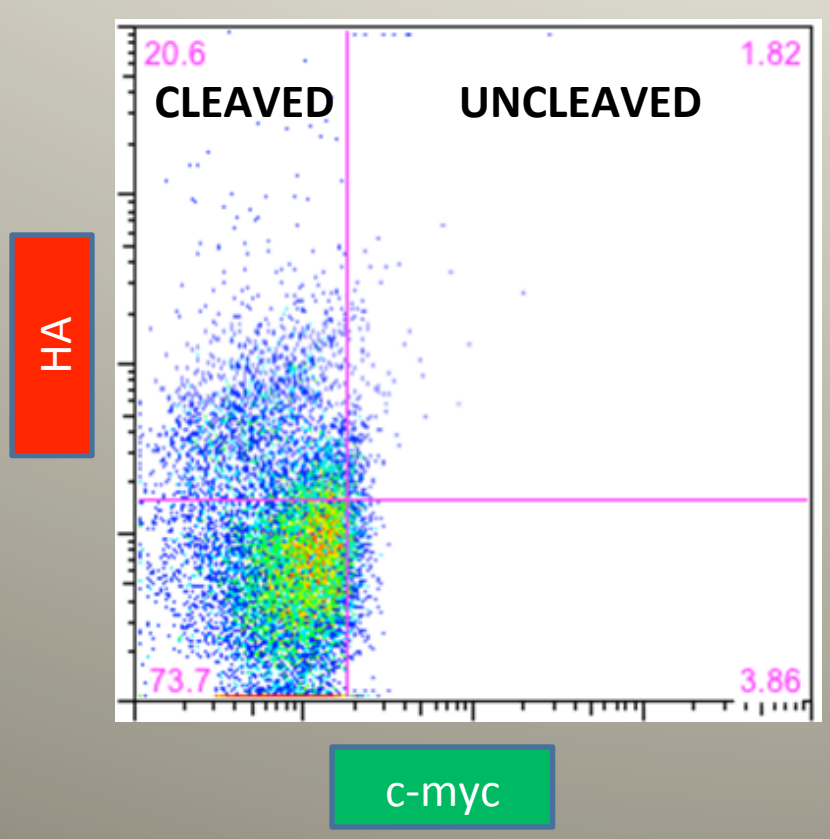
Preliminary Flow Data: Block 1-4

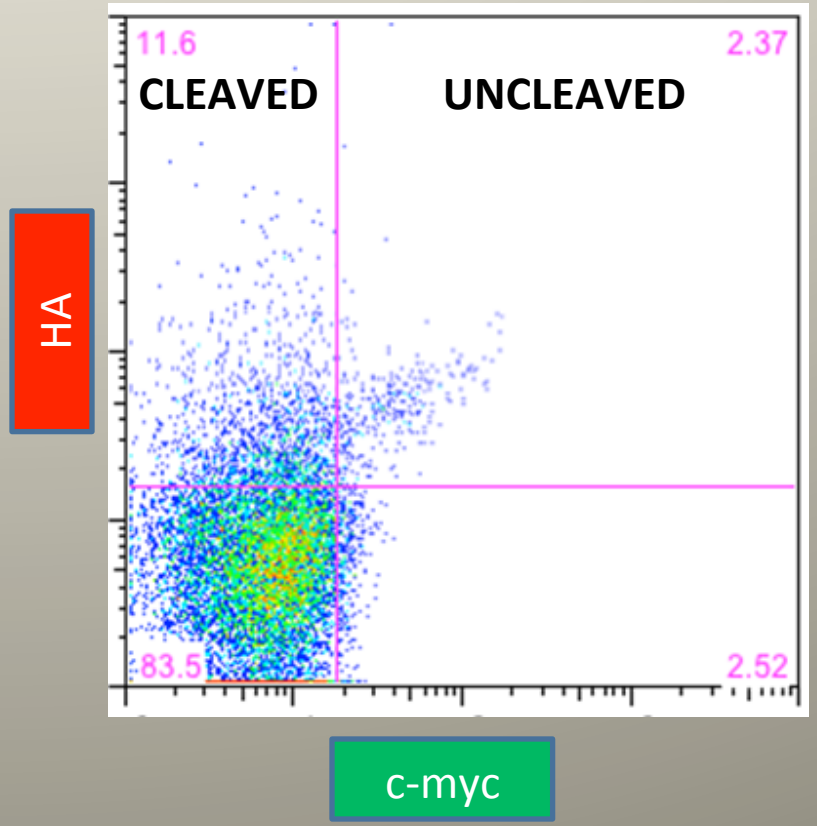
Block 1

pH 7, 37C, 24 hours



Block 1-4

pH 7, 37C, 24 hours



B1-4 Sequence Data

Mutation Name	Sequence	Deviations from WT	Frequency
Mutation 1	****x	4/5	40%
Mutation 2	*xXXx	4/5	30%
Mutation 3	*x*xX	5/5	20%

* Conserved Residue
X Chemically Similar Residue
x Chemically Dissimilar Residue
Note that no sequences were wild type

Summary

- Effective screen developed
  - Distinguish between cleaved and uncleaved intein
- Designed targeted library
  - Complete coverage of sub-libraries
- Enriched library for improved variants
  - Reduced cleaving at pH 7, fast cleaving at pH 5.5

Future Work

- Continue characterizing enriched libraries
  - Flow Data, Sequence Data, SDS-PAGE data
- Continue sorting libraries
  - More stringent negative selection
- Iterative mutagenesis
- Screen error-prone PCR library

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2. Gai, S.A., and Wittrup, K.D. (2007) "Yeast surface display for protein engineering and characterization." **Current Opinion in Structural Biology. 17:**467-73
3. Chao et al.(2006) "Isolating and engineering human antibodies using yeast surface display." **Nature Protocols. 1(2)** 755-68
4. Cochran, J.R. et al (2006) "Improved mutants from directed evolution are biased to orthologous substitutions." **Protein Engineering, Design and Selection. 19(6)** 245-53
5. Firth, A.E., and Patrick, W.M.(2008) "GLUE-IT and PEDEL-AA: new programmes for analyzing protein diversity in randomized libraries." **Nucleic Acids Research. 36**. W281-85

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- OSU Flow Cytometry Core Lab, especially Bryan, Katrina, and Danny
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