Advancements in purifying aggregate proteins through inclusion body separation

2022-2023 ESS SUPER Program

Skills for Undergraduate Participation in Ecological Research

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April 2023

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Research Summary:

The process of recombinant protein expression has unlocked new and more effective ways to obtain proteins for research. Recombinant protein expression is the process of inserting an encoded gene into a microbe so that it produces a target protein. Yet, even with this effectiveness, it is a complicated and multi-step process that has needed troubleshooting at each step and with each unique protein that has been produced. One process that is of interest is the formation of inclusion bodies. Inclusion bodies are amorphous aggregates of proteins that are formed during high level recombinant protein expression and are seen by many researchers as detrimental to the harvesting of said proteins. This study looked at using inclusion bodies from a protein that innately forms them as a way to separate our target protein from other cell materials to then have a more pure final product. We found that roughly isolating our sample using inclusion body separation was effective in producing a purer sample of our target protein. To find these results we used gel electrophoresis, which separates proteins by molecular size. Since we knew what our target proteins' molecular size was we determined the amount of our target protein versus other different sized proteins in our sample.

Introduction

The process of growing and harvesting protein for scientific research has always been labor intensive. In the past, procurement was a destructive process that required several kilograms of biological tissue to obtain even the smallest volume of pure protein (Rosano et al. 2014a). In the early 1970s, however, a procedure was created to produce recombinant proteins in microbial systems (Rosano et al., 2014b). Currently, recombinant expression in Escherichia coli (E. coli) or other bacteria is still the dominant method to procure pure protein for research purposes. This process involves the

insertion of an expression plasmid (which codes for the desired protein) into E. coli so the cultures produce the protein (Rosario et al. 2014a).

Once the target protein is produced it needs to be purified/separated from the rest of the cellular material in order to have a pure sample for study. The addition of histidine tags (His-tags) is a popular tool to enhance the purification process. In this procedure, 6 histidine codons are added to the plasmid next to start or stop codons (Bornhorst et al., 2000). These allow for a smoother purification process later on; the positively charged histidine-tagged proteins easily bind to negatively charged metal ions, separating the proteins from other cellular material (Bornhorst et al., 2000).

With the large number of researchers utilizing this complex protein obtainment procedure, there has been need for trouble shooting at every step (Table 1), especially when growing novel proteins such as aggregate-forming proteins, or prions (Moriyama et al., 2000). A prion is an abnormal form of a protein that is folded incorrectly. Prions have the ability to transmit their incorrect shape to other correctly folded proteins, forming aggregated amyloid structures (Wickner et al., 2004). As seen in Figure 1, prions form an amyloid core due to their aggregation.

One potential method for improving protein purification is the separation of inclusion bodies directly before the His-tag purification steps. Inclusion bodies are amorphous aggregates of proteins found in bacteria and are formed during high level recombinant protein production (Wang 2009). Some proteins innately form inclusion bodies such as our target protein Ure2 (Espargaró 2012). Previous studies have seen the formation of inclusion bodies as detrimental to their experiment but in this study we will explore the unique benefits that inclusion bodies may make possible (Singh 2015).

Greg McKittrick, a graduate student in Ecosystem Science and Sustainability at Colorado State University, has been growing the prion domain of Ure2p, which is an aggregating form of that protein from the yeast species Saccharomyces cerevisiae, without separating inclusion bodies before purification and has been met with suboptimal purity of samples and low yield. It's widely unknown whether separation of inclusion bodies improves purity and/or yield of the final sample. We hope to fill this knowledge gap by comparing the purity of our produced recombinant Ure2 samples with the use of our original protocol that does not separate inclusion bodies against the purity of the updated protocol, which separates the inclusion bodies from other cell material directly before the His-tag purification.

Table 1. Strategies for overcoming common problems during recombinant protein expression in E. coli (from Rosano et al., 2014A)

Problem	Possible explanation	Solutions
No or low expression	Protein may be toxic before induction	Control basal induction: • add glucose when using expression vectors containing *lac*-based promoters • use defined media with glucose as source of carbon • use pLysS/pLysE bearing strains in T7-based systems • use promoters with tighter regulation
		Lower plasmid copy number
	Protein may be toxic after induction	Control level of induction: • Tuneable promoters • Use strains that allow control of induction [Lemo21(DE3) strain] or *lacY*$^{-}$ strains (Tuner^{TM})
		Lower plasmid copy number
		Use strains that are better for the expression of toxic proteins (C41 or C43)
		Direct protein to the periplasm
	Codon bias	Optimize codon frequency in cDNA to better reflect the codon usage of the host
		Use codon bias-adjusted strains
		Increase biomass: • Try new media formulations • Provide good aeration and avoid foaming
Inclusion body formation	Incorrect disulfide bond formation	Direct protein to the periplasm
		Use *E. coli* strains with oxidative cytoplasmic environment
	Incorrect folding	Co-express molecular chaperones
		Supplement media with chemical chaperones and cofactors
		Remove inducer and add fresh media
		Lower production rate: • Lower temperature. If possible, use strains with cold-adapted chaperones • Tune inducer concentration
	Low solubility of the protein	Fuse desired protein to a solubility enhancer (fusion partners)
	An essential post translational modification is needed	Change microorganism
Protein inactivity	Incomplete folding	Lower temperature
		Monitor disulfide bond formation and allow further folding *in vitro*
	Mutations in cDNA	Sequence plasmid before and after induction. If mutations are detected, the protein may be toxic.
		Use a *recA*$^{-}$ strain to ensure plasmid stability
		Transform *E. coli* before each expression round

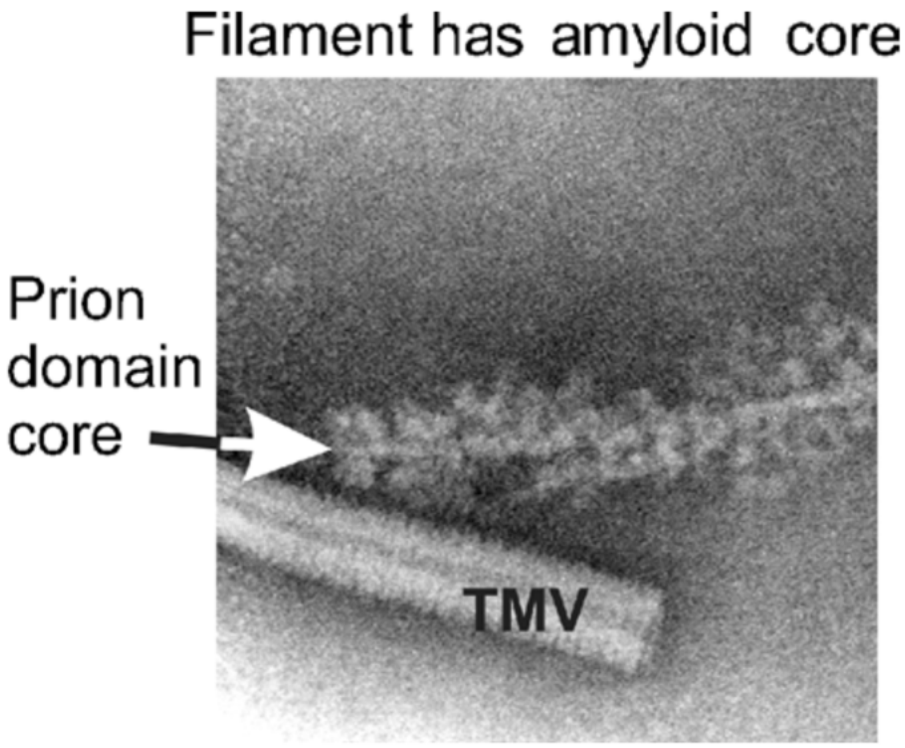

Figure 1. Ure2p amyloid architecture. The Ure2p prion domain forms a central amyloid core (From Wickner et al. 2004).

Research Questions and Hypotheses

Research Question: Can the separation of inclusion bodies increase the purity of Ure2p produced in vitro?

Hypothesis: We expect that the additional step of the inclusion bodies separation will lead to a more pure sample of Ure2p after purification.

Null Hypothesis: We expect the additional step of the inclusion bodies seperation to have no effect on the purity of the final Ure2p sample.

Explanation: Ure2p is naturally insoluble in most conditions; thus, the separation of inclusion bodies is a relatievely simple means of isolating Ure2p from the majority of other cellular materials.

Methods

Transformation was completed on BL-21 (DE3) competent cells. Cells were kept frozen at -80°C and later thawed on ice to avoid heat shock. 20 ***μ***L of cells were aliquoted into a pre-chilled 1.5 mL tube, with 1 ***μ***L of plasmid DNA (PER346) added. After a 30-minute incubation on ice, the cells were heat-shocked at 42°C for 30-45 seconds to increase the rate of plasmid intake. After another two minutes on ice, 180 ***μ***L preheated SOC was [MD10] added to the cells and the mixture was incubated for 1 hour at 37°C.

Once transformation was complete, the colonies were plated. 40 ***μ***L of colonies were applied to each plate, which consisted of nutrient broth, agar, ampicillin, and chloramphenicol. The plates were then incubated for 12-24 hours at 37°C.

After the transformation, the E. Coli cultures were put under the proper conditions to express Ure2p. Individual colonies were placed in 60mL of 2XYT broth containing chloramphenicol and ampicillin and grown overnight. Cells were then harvested through centrifugation, suspended in 2mL of media, and used to inoculate flasks with 500mL of 2XYT containing no antibiotics. These were shaken at 175 rpm at 30°C overnight until they reached an optical density value of 0.6 at 600nm. 500 ***μ***L of 1M IPTG was added to each 500mL flask, inducing the expression of the protein.. Four hours after the addition of IPTG, cells were harvested through multiple rounds of centrifugation. These cells were then stored at -80°C while they awaited purification.

Purification is a crucial step in protein obtainment, as it separates the protein of interest from all other cellular materials. Though the original method of protein purification involved a lysis process that denatured Ure2p's aggregate structure, this newly developed protocol keeps Ure2p under its native pH conditions to preserve its properties as an insoluble protein (Fig. 2). This allows the inclusion bodies (including Ure2p) to separate via centrifugation from the culture's soluble proteins, which were not desired.

After the proteins had been roughly isolated, the remaining insoluble materials were purified. This was accomplished through a series of buffer rinses and sonication for additional lysing. The samples were put in the first buffer, containing imidazole at a pH of 8.0, and sonicated in 30-second intervals for 5 minutes. After the sonication, the samples were placed in a series of urea-based buffers that allowed the Ure2p to bind to a nickel-agarose column, leaving the other cellular materials suspended in the supernatant to be discarded. Once a majority of the Ure2p had binded to the

nickel-agarose, the samples were put in additional buffers that separated them once again, leaving only pure Ure2p in the solution.

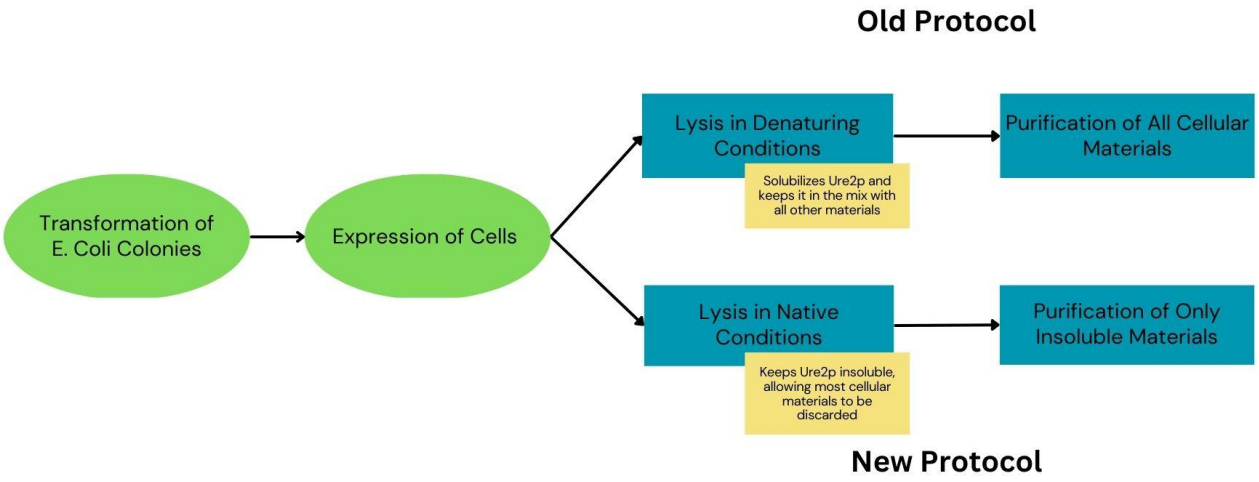

Figure 2. Visual map of proposed changes to purification protocols to account for the aggregant nature of Ure2p.

Results

Lane	Description of Sample
1	Sample before Ni-Agarose purification
2	Blank
3	Supernatant after rinse in first GdHCl buffer
4	Supernatant after rinse in second GdHCl buffer
5	Supernatant after rinse in first Urea buffer
6	Supernatant after rinse in second Urea buffer
7	Supernatant after rinse in third Urea buffer
8	Blank
9	Final elution
10	5 μL protein ladder

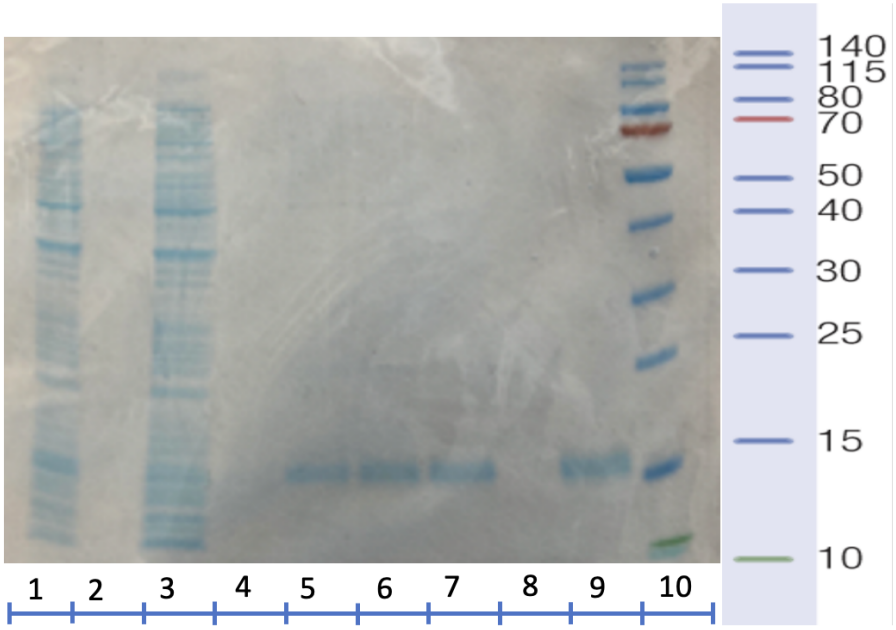

Figure 3. The protein gel electrophoresis (right) and sample load description (left) for a batch of protein purified without inclusion body separation. The blue bands are proteins sorted by size with the figure on the right showing approximate size in kilodaltons.

Lane	Description of Sample
1	Soluble culture material
2	Insoluble material before Ni-Agarose purification
3	Supernatant after rinse in first GdHCl buffer
4	Supernatant after rinse in second GdHCl buffer
5	Supernatant after rinse in first Urea buffer
6	Supernatant after rinse in second Urea buffer
7	Supernatant after rinse in third Urea buffer
8	Final elution
9	Known Ure2p (control)
10	5 μL protein ladder

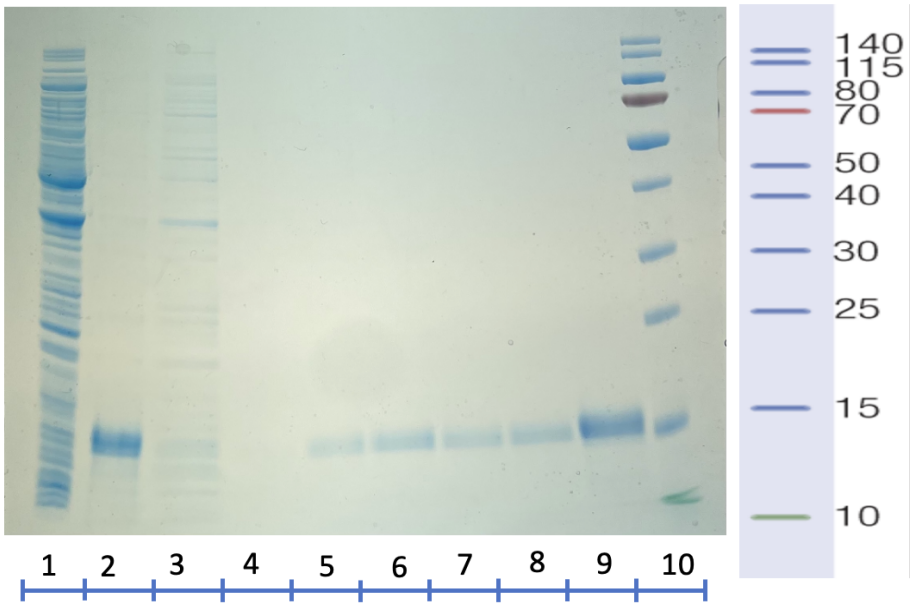

Figure 4. The protein gel electrophoresis (right) and sample load description (left) for protein purified after inclusion body separation. The blue bands are proteins sorted by size with the figure on the right showing approximate size in kilodaltons.

Discussion

The results of the gel electrophoreses indicate that, after the introduction of the new step in the purification process, the samples of Ure2p contained fewer contaminants from other cellular materials. While the original post-lysis sample contained large quantities of material at 40 and 50 kDa, the new post-lysis samples had only faint lines beside the strong line of Ure2p at ~14 kDa. These results are promising, as they indicate that the additional step of inclusion body separation was highly successful in removing a large portion of other cellular materials. These results align relatively closely with our initial hypothesis; though we didn't expect all cellular materials to be completely excluded before nickel-agarose purification, we did expect to see a significant decrease in the presence of non-target proteins in the post-lysis samples. These predictions proved true; the newer protein batches underwent the nickel agarose process with much less excess material, improving the purification process.

Conclusions

The results of this study have highlighted the potential for improvement in existing methods for protein growth and purification. Although the insolubility of aggregate-forming proteins is often seen as a complication in this process, its ability to separate from the majority of soluble cellular proteins provides it with a unique advantage. The separation of inclusion bodies removes a large portion of the irrelevant materials in a sample and makes the following nickel-agarose purification much more effective.

This protocol adjustment is shown to improve sample purity during purification of Ure2p. Thus, inclusion body separation could prove beneficial in any future studies of aggregating proteins with similar properties. Increased purity could improve study results and remove some levels of confounding noise.

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Acknowledgements

This work was made possible by Dr. James Henricksen, Julie Gauthier, and Dr. Richard Conant of the Natural Resource Ecology Laboratory, and was supported by the entirety of the L-Sugars Research Team of Colorado State University's EcoCore Research Facility. This project was completed as part of the Skills for Undergraduate Participation in Ecological Research (SUPER) Program, led by Dr. Stacy Lynn.