Optimizing Recombinant Protein Purification Techniques for Bio-Analytical Applications

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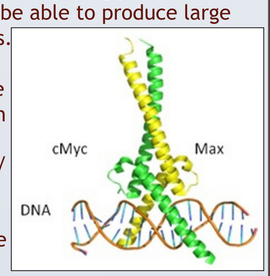
ABSTRACT

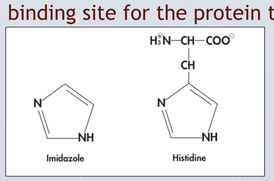
Many modern analytical protein characterization techniques, such as X-ray crystallography, nuclear magnetic resonance, and activity gel shift assays rely on access to large quantities of purified human proteins that have been expressed in bacteria threw a recombinant vector uptake mechanism. In particular, our lab builds electrochemical and optical biosensors to detect proteins involved in cancer that require pure recombinant proteins for validation purposes[1]. However, the process of expressing and purifying functional human proteins in bacteria requires optimization of conditions on an individual protein basis. Here we investigate varied buffer parameters in the process of affinity column chromatographic separation of recombinant Max (myc-associated factor X) transcription factor protein. The gene encoding a version of Max that is fused to a His-tag purification motif was previously introduced into BL21 E. coli bacteria, and this bacteria were used to produce large quantities of cell lysate[2]. However, the process of isolating Max from the thousands of native bacterial proteins requires careful selection of purification procedures and techniques. In particular, the concentration of imidazole, an amino acid mimic used to out-compete Max binding to the Nickel-affinity column, has a significant effect on the final purity of the protein obtained. By varying the concentration of imidazole, we aim to quantitatively determine an effective concentration for optimized purity of Max for testing of our lab's biosensors.

INTRODUCTION

Proteins serve a variety of functions within the body including, but not limited to structural, transport, catalytic and identification. In the process of studying proteins further, it becomes necessary to be able to produce large quantities for analytical purposes. The goal of this research is to gain further understanding of the optimal conditions for production and purification of a particular protein – Max. As a part of a Myc/Max complex[3], this protein is associated with breast and kidney cancers when found in the blood stream in high concentrations, from the lysing of cancer cells. By using biosensors to test blood samples, a screening process would become possible for patients, indicating whether further oncological diagnostic testing would be necessary.



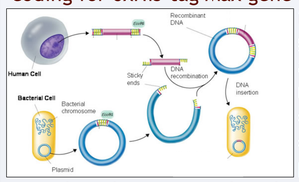
In order to analyze this protein complex, the purification of Max protein must be individualized. The use of a 6xHis-tag (poly Histidine amino acid residue tail), provides a selective binding site for the protein to the Nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices[4]. With a low concentration of imidazole added, low-affinity binding of native proteins is prevented, leaving the 6xHis-tagged Max proteins still bound in the column. The variability is in the concentration necessary to remove background proteins, without affecting Max binding[5].



MATERIALS & METHODS

Expression

BL21 E. Coli bacteria containing recombinant DNA vector coding for 6xHis-tag Max gene and ampicillin resistance inoculated in autoclaved growth media. Ampicillin was also added to Luria Broth media, selecting for competent bacteria containing the vector plasmid. After overnight growth, culture was induced with isopropyl-ß-D-thiogalactopyranoside (IPTG), promoting production of Max gene within bacterial cells. Cultures were centrifuged, and cell pellets extracted while supernatant was discarded.



Separation

The cell pellet was then combined with lysis buffer, Dnase I (degradation of bacterial DNA), AEBSF (protease inhibitor), and lysozyme crystals. The lysing of the bacterial cells was also aided by the application of sonication, which caused cavitation of the bacterial cells, shredding them in solution. As components of bacterial cells are freed, centrifugation was applied to compact cell macromolecules. The supernatant containing soluble Max protein was collected, and new cell pellet removed and discarded.

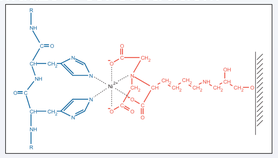
Isolation

Supernatant then separated into equal amounts for Trial (A-F). Each trial was applied to Ni-affinity column chromatography, with the use of Ni-resin slurry. Trial sample mixed 1:1 with equilibration buffer and added to a column, collecting the flow through. Equilibration buffer allows all non-binding molecules to pass through the column. Then the wash buffer is applied to the of column (testing different amounts of imidazole per trial). Wash buffer inhibits binding of dispersed histidine residues in nontagged background proteins, allowing proteins to flow through the column for collection.[2] The elution buffer is the last to be added, containing a high concentration of imidazole that out competes the 6xHis-tagged proteins for Ni-NTA binding sites, releasing Max protein into collection vials.

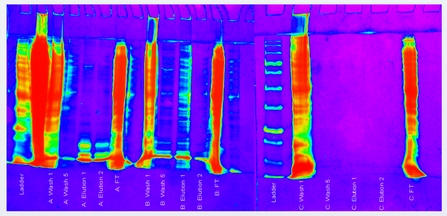
The wash buffers contained imidazole concentrations of 10mM, 75mM, and 150mM,(Trials A-C). The second analysis included concentrations of 37.5mM, 75mM, and 112.5mM, (Trials D-F). These parameters were determined from percentages of elution buffer with a concentration of 250mM imidazole, which is was known for competitive binding with Ni-NTA.

RESULTS

SDS-Page gels were used for analysis of protein purification for each set of parameters of imidazole concentration. Determination of purity confirmed that binding of the 6xHis-tagged Max protein to Ni-NTA was strong enough to withstand low levels imidazole, while higher levels of imidazole out compete for binding sites.



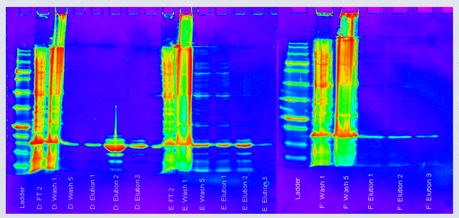
Trial A-C



A: 10mM (Equilibration buffer concentration)
B: 75mM (30% Elution buffer concentration)
C: 150mM (60% Elution buffer concentration)

Trial A: The gel from this trial shows that background proteins were eliminated from the column. Shown in elution 1, Max protein is present in relative purity. Elution 2 exhibits continued Max protein being eliminated from the column.
Trial B: The results from this trial are incongruent with what is expected. From elution 1, it seems to imply that this higher concentration (75mM) of imidazole was not enough to purify Max protein. Possible overflow of gel wells could have lead to this disparity.
Trial C: It is clear from this trial that (150mM) of imidazole will eliminate the desired protein along with background protein during the addition of wash buffer, thus inhibiting purification of Max.

Trial D-F

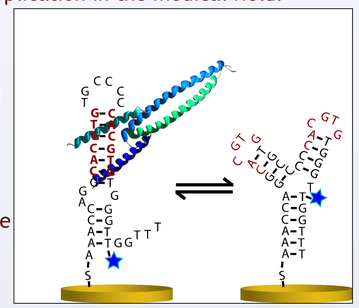


D: 37.5mM (15% Elution buffer concentration)
E: 75mM (30% Elution buffer concentration)
F: 112.5mM (45% Elution buffer concentration)

Trial D: The gel pattern of elution 2 shows the purity Max protein provided by using (37.5mM) concentration of imidazole. These results represent the greatest purity of the trials.
Trial E: The repeat of the concentration (75mM) being used shows a minimal amount of a protein eluted, meaning the majority was lost with the addition of wash buffer.
Trial F: Similar to trial C, the concentration of (112.5mM) was high enough to completely eliminate the protein of interest from the column before elution.

CONCLUSIONS & FUTURE WORK

Once the optimal concentration of imidazole is obtained for use in wash buffer solution, testing for natured Max protein must be determined. Pure samples of natured Max protein are necessary for further study in the use of biosensors and their application in the medical field.



Max protein forms a complex with Myc protein, and together this complex with bind to a specific sequence of DNA. The use of a biosensor attached to this specific DNA sequence allows for the sensor to bind and detect levels of Myc/Max complex. In a laboratory setting, known binding concentrations can be tested and assigned a detectable current read-out level. These experimentally determined values can be set as standards to determined what concentration level of Myc/Max represents a high risk of malignancy. This work with biosensors is significant for future application of diagnostic testing in clinical settings.

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