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Separatome of Escherichia Coli Analysis of DEAE Chromatography as pH of loading changes.

An Undergraduate Honors College Thesis

in the

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College of Engineering
University of Arkansas
Fayetteville, AR

by

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April 30th, 2015

This thesis is approved.

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Thesis Committee:

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Abstract

Recombinant DNA technology is used to produce therapeutics in host organisms and undergoes several purification steps before it can be used by the end user. Escherichia coli which has been widely studied has been the microorganism of choice mainly because of its low production costs. The primary objective of this study was to investigate the optimal conditions to purify a protein of interest with antifungal properties. Varying the loading pH alters the overall charge of both the target protein and host cell proteins (HCPs). Identifying genomic proteins that remain bound regardless of loading pH on DEAE columns will guide future genetic manipulation to enhance purification without sacrificing product yield. The identity of the proteins which bind strongly to the DEAE resin even at low pH are important which if non-essential for the growth of *E.coli* can possibly be deleted to improve column efficiency.

Introduction

In the recent past the demand for biomolecules and therapeutic proteins has seen a tremendous growth with the biopharmaceutical market estimated to be worth $99 billion in 2009 [1, 2]. With protein based treatment gaining popularity and replacing chemical based therapeutics [3], researchers around the world are constantly innovating to facilitate economic biopharmaceutical production. Recombinant DNA technology is used to produce therapeutics in host organisms and undergoes several purification steps before it can be used by the end user. *Escherichia coli*, which has been widely studied, has been the microorganism of choice mainly because of its low production costs. Additionally, the physiological and genetic characterization is already known to researchers which facilitates tailoring the organism to optimally produce the target proteins of choice. Therapeutics such as insulin, human growth hormone, interleukins etc. has already been commercially produced in E.coli. [4, 5] The present project focuses on optimizing downstream purification processes when using E.coli as the host cell as discussed later.

According to an article on pharmaceutical industry published by the World Health Organization, about $8 million is spent just on purifying proteins produced recombinantly. Every extra purification step along with increasing the cost also decreases protein yield with target protein lost during the procedure. According to a recent study, the fermentation facilities required for recombinant production of proteins are believed to be nearing full capacity [6] which makes developing novel techniques to optimize protein purification crucial to meet the demands of a growing pharmaceutical market. Improvement in fermentation techniques are being currently widely studied. The present study focuses on overcoming purification challenges faced during chromatography due to presence of host cell proteins (HCPs).

E.coli produces numerous proteins in addition to the target protein of interest referred to as E.coli proteins (ECPs). Presence of ECPs in the final product can adversely affect humans by stimulating immune responses and should be removed to low levels as detected by a sensitive analytical method during downstream processing of recombinant protein [7]. The industry generally employs multiple orthogonal separation steps to reduce HCPs to acceptable level [8]. These steps also helps in removal of other process related impurities such as residual host cell DNA, and other cell culture components.

The method used for the purification of the E. coli proteins is anion exchange chromatography with fast liquid protein chromatography (FPLC). FPLC is a high-performance chromatography that was developed for proteins, aqueous buffer systems, fast flow rates and availability of stationary phases in most common chromatography modes [10]. This system makes separation possible because it incorporates high level of automation that includes auto samplers, gradient program control and peak collection [10].

Objective

The primary objective of this study was to investigate the optimal conditions to purify a protein of interest with antifungal properties. The studies also aimed to identify the HCPs produced during mass manufacturing of the target protein should the loading pH on DEAE columns be changed. Bioreactors were used to mass-produce the protein of interest by fed-batch techniques. Varying the loading pH alters the overall charge of both the target protein and HCPs, and identifying genomic proteins which remain bound regardless of loading pH on DEAE columns will guide future genetic manipulation to enhance purification without sacrificing product yield.

Materials and methods

Production of extract

Genetically modified cell lines of MG1655 *Escherichia coli* containing a plasmid to produce a fusion protein of antifungal peptide and green fluorescence protein (GFP) was used which does not bind to the DEAE column at lower pH. The cell pellets of *E.Coli* were obtained from Boston Mountain Biotech after fed batch fermentation was performed. Cell pellets were prepared by centrifuging the harvested culture.

Preparation of buffers

The objective of this project was to vary the pH of the loading buffers of the FPLC in order to identify proteins that will remain bound regardless of loading pH. For this purpose, two different pH were chosen. For each pH, two buffer solutions were used for purification in the following concentrations: for buffer A, 25 M Tris buffer and 1 M NaCl and for buffer B, 25 M Tris buffer and 5mM of NaCl. The pH was adjusted by adding diluted HCl until the solutions were pH= 5 and pH=7 for both cases. For the rest of the document, buffers A and B of pH 5 and pH 7 are referred to as buffer A1, B1 and A2, B2 respectively.

In order to prepare the cell clarified lysate for pH 5 that will be loaded to the 1 mL DEAE (diethyl amino ethyl) fast flow column from GE Healthcare, 2.5 g of the cell pellets were first resuspended in 15 mL of buffer A1 and then lysed by sonicating for 15 minutes with a pulse cycle of 20 seconds with 10 seconds rest. The cell lysate was then centrifuged for 90 minutes at 4000 rpm. The supernatant was decanted carefully which comprised the clarified cell lysate. Cell pellets and clarified cell lysates when not being used were always frozen at -20°C between intermediate steps.

Fast Protein Liquid Chromatography

The samples that were loaded into the chromatography column were prepared by mixing 3 mL of the clarified lysate with 12 mL of buffer A1. A step wise gradient was used using buffer A1 and buffer B1. Buffer B1 was increased 20% for every 10 column volumes. This means that the 1 mL DEAE column was subjected to an incremental change of 0.2 M NaCl every 10 mL of flow volume after the step wise elution gradient had started. Peak fractionation was used to collect the eluted proteins with 3 mL samples and were then stored at -20°C. This exact same process was repeated for the protein purification with buffers A2 and B2 for pH 7.

Preparation of sample for analysis

Two of the FPLC runs for each pH, in which similar results were obtained, were selected for analysis and both sample fractions were combined. For pH 5, the peak corresponding to fraction 29 (as seen on figure 1) eluted at 85% buffer B was selected to be analyzed. For pH 7, the peak corresponding to fraction 25 (as seen on figure 2) eluted at 80% buffer B was selected to be analyzed because it was at the highest salt concentration. Amicon Ultra 0.5 mL microcentrifugal filters of 3 kDa MWCO were used to concentrate the samples. First, 400 µL of the selected samples from the FPLC runs with pH 5 and pH 7 were poured into the microcentrifuge tube using a pipette. Following this, the sample was centrifuged for 10 minutes at 10,000 rcf three times until it was concentrated to 50 µL. In order to make the sample more diluted, deionized water was added until it reached a volume of 400 µL. The previous steps were repeated two more times in order to reach the desired concentration (5 µg in 50 µL) to meet the requirements for protein characterization. The filtrate was collected and stored at -20°C.

Protein Identification

The proteins produced by the E. coli were sent for characterization to the Proteomics Core Facility at University of Arkansas for Medical Sciences (UAMS), a facility that provides the service of protein characterization by mass spectrometry. Using the information from the databases Ecogene and Ecocyc, six or seven proteins will be classified as essential or non-essential.

Results and Discussion

The FPLC uses different detectors that are used to follow the progress of the purification. To detect the proteins, multiple wavelength absorbance detectors are used. These chromatograms were obtained using the UNICORN Control Software in the ÄKTA chromatography system. As is evident from the chromatogram shown in Figure 1, there are multiple proteins that bind to

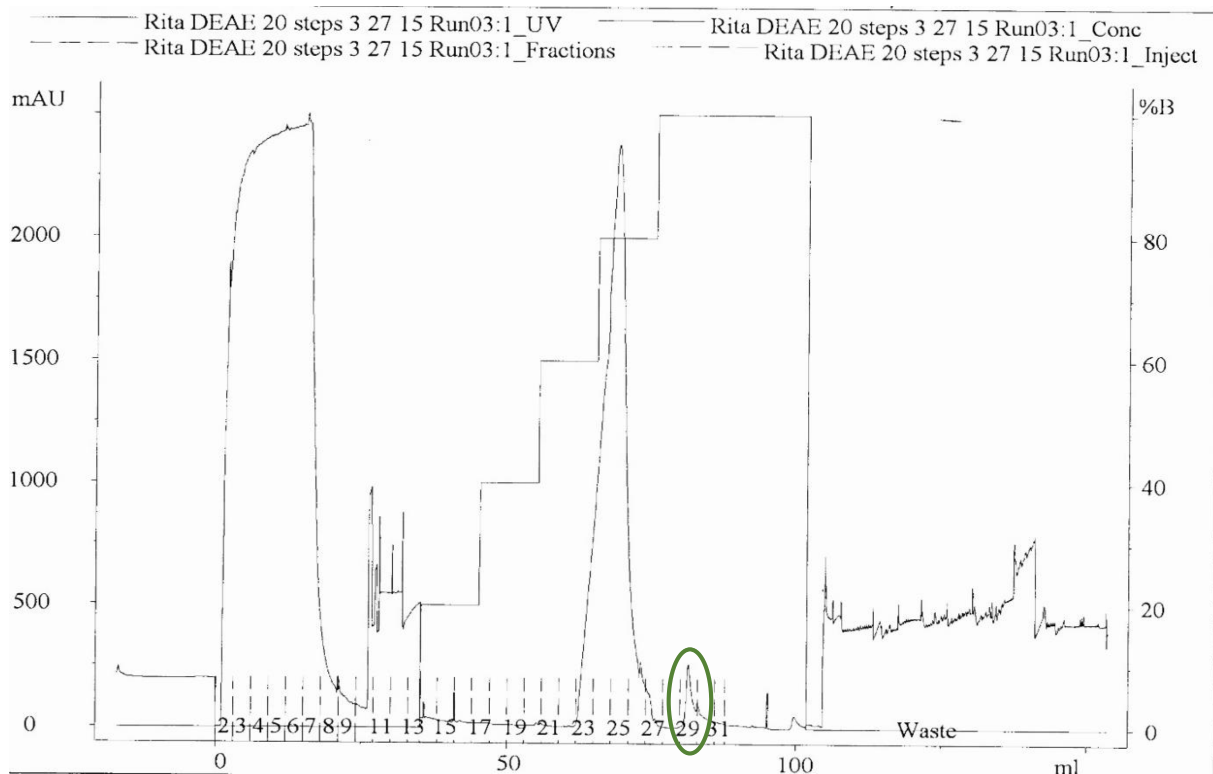

Figure 1: ÄKTA fast protein liquid chromatography chromatogram for pH=5.

the weak anion exchanger DEAE resin even at low pH. Since GFP has a pI of 6.2, it has a net positive charge at a pH of 5 and binds weakly to DEAE. For this project, the identity of the proteins which bind strongly to the DEAE resin even at low pH are important which if non-essential for the growth of *E.coli* can possibly be deleted to improve column efficiency. There is a large peak eluted at 0.8 M NaCl concentration (corresponding to fraction 23-27) and a comparatively smaller peak at 1M NaCl (fraction 29). For purification runs at pH 7, a small peak was eluted at 0.8 M NaCl (fraction 25). Samples from these fractions (number 29 for pH 5, and number 25 for pH 7), were sent for protein identification to University of Arkansas for Medical Sciences Proteomics Core facility. The identity of the proteins shall help to determine if they are essential to the *E. coli*, which will direct further studies.

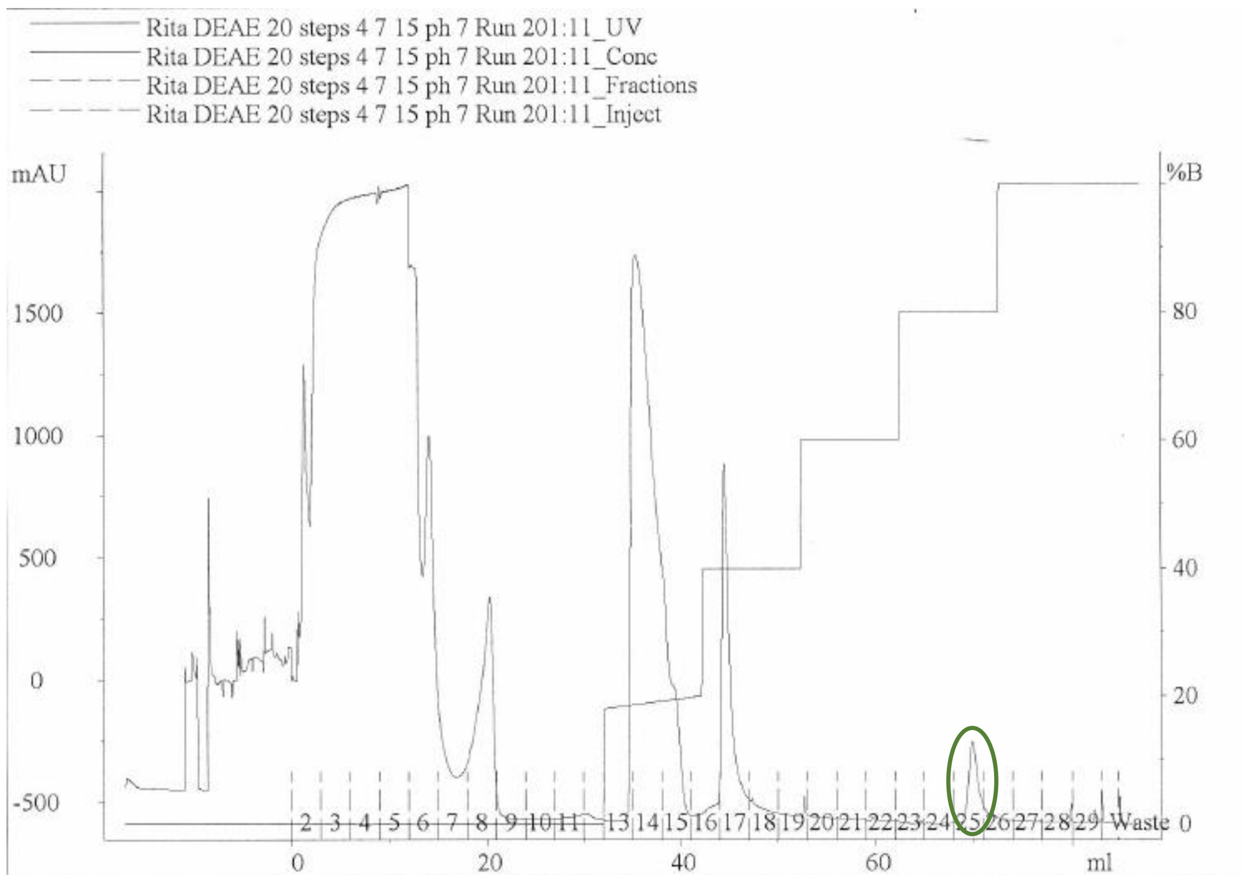

Figure 2: ÄKTA fast protein liquid chromatography chromatogram for pH=7.

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