2005

Development of an upper level undergraduate research based laboratory: The purification, analysis, and dinetic study of cytochrome b_5

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Abstract

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Degree Type

Open Access Senior Honors Thesis

Department

Chemistry

First Advisor

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Keywords

Cytochrome b, Chemistry Research

Subject Categories

Chemistry

DEVELOPMENT OF AN UPPER LEVEL UNDERGRADUATE RESEARCH BASED LABORATORY:

THE PURIFICATION, ANALYSIS, AND KINETIC STUDY OF CYTOCHROME b_5

by

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A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation with Honors in Chemistry

Approved at Ypsilanti, Michigan, on this date _____

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The purpose of this project was to develop an upper level undergraduate biochemistry laboratory (CHEM 453) based upon a novel research project, designed to replace an existing instructional laboratory that employs 'cookbook' exercises. Methods for the expression, purification, spectral analysis of structural integrity, and electron transfer capability of rabbit cyt b_5 were adapted from published work done by Dr. Lucy Waskell of the University of Michigan and Dr. Mansuy of the University of Paris. In our hands, rabbit liver cyt b_5 expressed in *E. coli* was purified to 45% specific content of the theoretical content. Additionally, 37 nmol cyt b_5/mg of protein was found using a Biuret Standard Assay giving a 63% specific content from the theoretical value of 58.8 nmol cyt b_5/ mg protein. The specific content is the amount of cyt b_5 (holo and apo) that is present out of all the protein present. Therefore, the difference of the 18% could be due to cyt b_5 or another protein. In the CHEM 453 lab purification of the cyt b_5 gave a specific content that was 70% of the theoretical value, the same as was obtained previously by Waskell. The rate constant for tetrahydrobiopterin reduction of cyt b_5 was determined to be 0.005 $M^{-1}cm^{-1}$ compared to Mansuy's kinetic rate constant of 0.20 $M^{-1}cm^{-1}$. This discrepancy might be due to differences in concentration of cyt b_5, the reductant, or to the fact that Mansuy used a trypsonized form of cyt b_5 whereas we used the intact rabbit cyt b_5. Reduction was confirmed to be a second order process. The research effort was successful in preparing a research-based experiment for the CHEM 453 course.

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INTRODUCTION

Cytochrome b_5 (cyt $_5$) is a membrane bound protein containing a heme prosthetic group [1]. Cyt b_5 is involved in the desaturation of fatty acids. Fig. 1 provides a representation of cyt b_5's involvement in the cascade reaction that introduces a double bond into fatty acids.

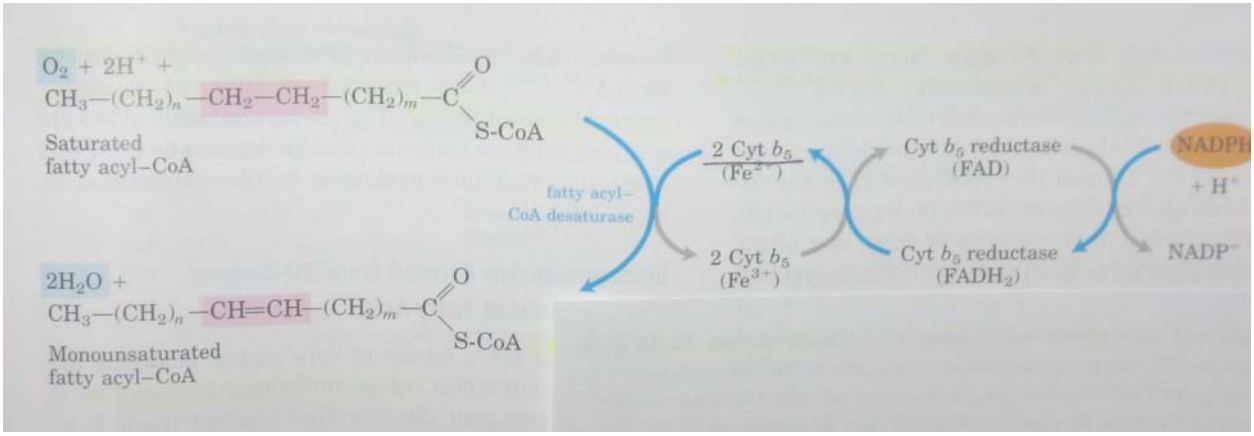


Fig.1 Electron transfer reaction in desaturation of fatty acids [2].

Cholesterol biosynthesis also involves cyt b_5 as an electron carrier with the multi-enzyme complex methyl sterol oxidase [3]. Cyt b_5 serves as an electron carrier in the endoplasmic reticulum in the cyt P450 pathway for the metabolism of exogenous compounds [4], and exists in a soluble form in red blood cells where it functions to maintain hemoglobin in its ferrous oxygen-carrying form. In simple terms, cyt b_5 serves as an electron carrier in several of the body's reactions. The secondary structure of cyt b_5 is highlighted in its three-dimensional representation in Fig. 2.

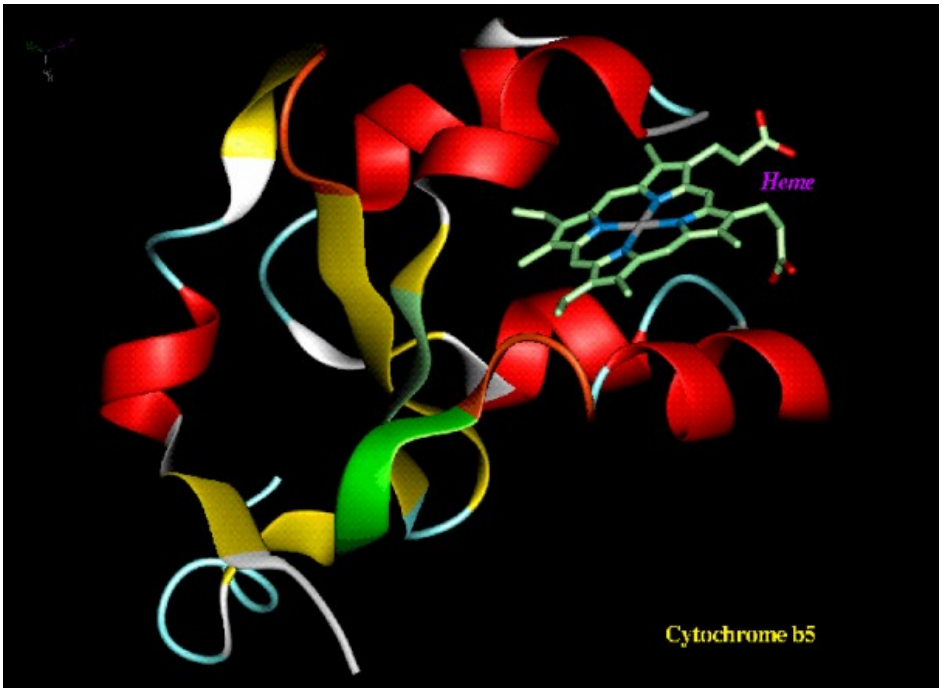


Fig. 2. Cyt b_5 structure (notice heme incorporation and iron in middle of heme).

The Chemistry department along with the Biology department at Eastern Michigan University (EMU) is in the process of transforming upper level undergraduate laboratory courses from verified "cookbook" experiments to incorporating experiments based upon current research taking place at EMU and elsewhere. The purpose of this lab was to develop the CHEM 453 lab (biochemistry) into a research-based class. Whereas not all the research is novel (some of it is verified work), the students are provided with opportunities to conduct novel research experiments. Each CHEM 453 lab section has two parts: a methodology portion and a research-based portion. It is the hope of those involved in the teaching of this course that eventually the entire methodology portion of the laboratory, which is based on cookbook approaches, will be incorporated into the research-based portion. The goal of the laboratory is for students to experience common biochemistry methods as well as become familiar with the research approach.

Published methodology pertaining to research with cyt b_5 was incorporated in the CHEM 453 course for the second half of the winter'06 semester. These experiments are based on Dr. Mansuy's kinetic work at the University of Paris with cyt b_5 and Dr.Waskell's work at University of Michigan involving the purification and analysis of cyt b_5 expressed in *E. coli*. Students repeated the work conducted by the researchers incorporating modifications developed in our research lab, and attempted to achieve the same results as well as some novel results.

BH_4 [(6R)-5,6,7,8-tetrahydropterin] is a cofactor required by several enzymes and is involved in one electron transfer to iron porphyrins (i.e. cyt b_5) with formation of an intermediate cation radical similar to the one detected in the NO synthase. For haemoproteins with reduction potentials more positive than -160 V, such as cyt b_5, there is good correlation between the second order reaction rate constant and the redox potential, E_m (Fe^{3+},Fe^{2+}) [5]. The second order rate constant of cyt b_5 as determined by Mansuy *et al.* [5] is 0.20 $M^{-1}s^{-1}$.

In Waskell's lab, approximately 5% of cyt b_5 expressed in *E. coli* was holoprotein, that is, protein in which the heme was incorporated, with the remaining of the protein existing as apoprotein, which does not contain heme. After heme addition to the solution containing the heme protein and subsequent purification, holoprotein content of cyt b_5 rose to 70% of the theoretical value for specific content[6]. The final cyt b_5 preparation was free of any major detectable protein contaminants when analyzed by SDS-PAGE.

METHODS

The purification and analysis procedure was adopted from research conducted by Dr. Lucy Waskell and her research group at the University of Michigan. The second part of this lab, the kinetic study of cyt b_5 was adopted from research by Dr. Mansuy at the University of Paris.

Both procedures were adopted and modified to meet the needs of EMU undergraduate students in the CHEM 453 instructional lab.

I. Purification and Analysis of cyt b_5

Cyt b_5 was produced in large quantities by over-expressing the membrane-bound form of rabbit cyt b_5 in an *E.Coli* strain C41 donated by Dr.Lucy Waskell. The cyt b_5 gene is carried in a pLW01 plasmid. The C41cells were first transfected with the pLW01 plasmid by heat shock. Each 1.5 ml microcentrifuge tube contained 100 µl of C41 cells and 50 µl of Luria Burtania (LB) broth (no ampicilian added), and allowed to grow overnight. Then 50 µl of the overnight culture was transferred to a 0.5 ml microcentrifuge tube, heat shocked at 42 ^{o}C for 90 seconds in a hot water bath, and then immediately placed on ice for 2 minutes.

The heat-shocked culture was then transferred to new microcentrifuge tubes and incubated at 37 ^{o}C for 15 minutes without stirring. The transformed cells were then plated on LB plates containing 100 µg/ml ampicilian. Bacterial cells that did not contain plasmid were subjected to the same treatment and served as negative controls. The plates were incubated at 37 ^{o}C overnight for 16 hours. The colonies were then picked and grown up in two 500 ml Erlenmeyer flasks containing 100 ml of Terrific Broth (TB) medium and ampicilian at a final concentration of 100 µg/ml. The colonies were then incubated in a shaker for 18 hours at 35 ^{o}C at 180 rpm.

Two 500 ml Erlenmeyer flasks were prepared with 0.2 ml of the transformed cells, 90 ml TB, 10 ml of 10 mM potassium phosphate buffer (KP_i) at pH 7.0, and 1 ml of 10 mg/ml ampicilian. The two flasks were incubated in an Innova shaker for 16 hours at 37 ^{o}C with shaking at 140 rpm. When the optical density had reached 0.6 to 0.8, as monitored in a

UV/Visible spectrophotometer, 1 ml of isopropyl B-D-thiogalactopyranoside (IPTG) was then added. Each flask was incubated an additional 24 hours after the induction (IPTG added) at 37 ^{o}C with shaking at 140 rpm and then placed in the cold room.

The two 500 ml Erlenmeyer flasks containing 100 ml of cells containing expressed cyt b_5 were then combined. The cells were harvested at 8000 rpm for 10 minutes at 4 ^{o}C in a Sorvall RC-5C centrifuge. The supernatant was decanted and the cell pellet was resuspended in 200 ml of ice-cold buffer A. Buffer A contained 10 mM KP_i and 1 mM ethylenediamine tetraacetic acid disodium salt (Na_2EDTA) at pH 7.0. The re-suspended cells were pelleted at 8000 rpm for 10 minutes, the supernatant again decanted, and the cells were re-suspended in 25 ml of ice cold buffer B containing two mini-protease inhibitor tablets. The composition of buffer B is identical that of buffer A except that it also contained 1% tergitol. Four aliquots (1 ml) of the cells containing expressed cyt b_5 were subjected to centrifugation, labeled, and placed in a -20 ^{o}C freezer for future use.

Following the harvest of C41 cells containing cyt b_5, the cell solution was analyzed for apo cyt b_5 and holo cyt b_5 content. 1 ml of the cell solution was spun down for 1 minute at 13,000 rpm, decanted, resuspended in 1 ml of cold buffer B, and vortexed. The detergent sodium deoxycholate was added to a final concentration of 0.4% and the cell solution was then sonicated two times for 30 seconds at medium power at 0 ^{o}C. The cell solution was then diluted 50-fold to a final volume of 1 ml, and heme was titrated in the UV/Visible spectrophotometer at an absorbance of 412 nm. A sonicator used to lyse the cells is shown in Fig 3.

Fig. 3. A Sonicator used to lyse bacterial cells

Fig.4 shows a picture of French Press in the CHEM 453 lab used in lieu of the sonicator to disrupt bacterial cells. The French Press is a considerably better method of lysing cells.

Fig.4. French Press Lysis Cells

In order to complete the heme titration to determine the amount of apo cyt b_5, 2 µl increments of 1.0 mM heme stock solution were added and the absorbance at 412 nm was monitored with time until it reached a steady value (10 to 30 minutes). The calculations needed to quantify the amount of apoprotein is described in the results section. To determine the amount of holo cyt b_5 in the preparation, another sample from the same solution was diluted in the same

manner and a few grains of sodium dithionite were added to reduce the ferric cyt b_5 to ferrous cyt b_5, and the spectrum was monitored between 350 and 500 nm. The calculations used to quantify the amount of holoprotein in the preparation are given in the results section.

After determining the amount of heme that was required to be added so all of the apoprotein would be converted to holoprotein, an appropriate amount of the 1.0 mM heme stock solution was added to the protein preparation. The protein solution was then centrifuged at 100,000 *g* for 1 hour at 4 ^{o}C and the pellet was discarded.

A DE-52 anion exchange column was used to purify cyt b_5. First 120 mg of pre-swollen DE-52 was rehydrated in 500 ml of water. The supernatant was filtered several times (pulled off with a Pasteur pipette) until the gel appeared homogeneous. After hydrating overnight the water was exchanged for buffer C. Buffer C contains 20 mM Tris-HCl, 1 mM Na_2EDTA, and 0.4% sodium deoxycholate, pH 8.0. The gel was then poured in the DE-52 column and allowed to pack for two days. Fig. 6 shows the DE-52 column set-up with two reservoirs that served to contain the elution buffers that were used in a gradient elution mode for resolving holo cyt b_5 and removing it from the column.

Fig. 5. DE-52 column set up for gradient elution of cyt b_5

The column was equilibrated with approximately 500 ml of buffer C until the affluent buffer was at the same pH as the eluent buffer (pH 8.0). The flow rate was set to approximately 2 ml/min and the cyt b_5 preparation that had been treated with heme and submitted to centrifugation was loaded to the top of the column.

The cyt b_5 band, which migrates on the column as a red band, was washed with 400 ml of buffer until it was approximately halfway down the column. Then 200 ml of buffer C containing 0.1 M sodium chloride (NaCl, salt) was used to further wash the column. Finally, a gradient from 0.1 to 0.4 M NaCl of buffer C was used to wash the band. Once the band was near the bottom of the column a fraction collector was set up to collect the purified cyt b_5 overnight.

After the purified cyt b_5 was collected, the peak ratio $A_{418/280}$ was determined using a UV/Visible spectrophotometer. Fractions with a peak ratio from 1.0 to 1.6 were pooled together as well as fractions with a peak ratio greater than 1.6. The fractions were then concentrated with an Amicon concentrator with a YM-3 membrane at a volume of 10 ml. The concentrated fractions C1 (1.0 to 1.6) and C2 (greater than 1.6) were used in the SDS-PAGE gel run along with cyt b_5.

Lastly, the cyt b_5 was analyzed using several methods including a standard biuret assay and denaturing gel electrophoresis (SDS-PAGE) following purification. It is important to note that at this point, cyt b_5 was purified by two *separate* methods, dialysis, and column purification (discussed above). In the research lab half of the cyt b_5 sample was purified by dialysis followed by column purification at a column drop rate of 0.0337 ml/min. However, the second half of the cyt b_5 sample was purified by dialysis only. Each sample went through the same treatments discussed further on. The BSA contained six test tubes with varying concentrations from 1.5, 3.5, 5,7.5, 10, and a blank.

The 15% SDS-PAGE gel was run with a 15% separating gel (pH 8.8) and 4% stacking gel (pH 6.8). The cyt b_5 samples were boiled for three minutes to denature the protein. 5 µl of a suitable protein standard "ladder" was loaded into two wells, 10 µl of column purified cyt b_5 samples in six lanes, two lanes of 10 µl of non-column purified samples of cyt b_5, and 5 µl of myoglobin (another standard) in one lane. The column-purified cyt b_5 samples were mixed at a 50:50 ratio with SDS-PAGE buffer, whereas non-column purified cyt b_5 samples were mixed in a 5:95 SDS-PAGE buffer ratio. The SDS-PAGE gel was then run for 90 minutes at 100 V for the first half hour and then at 200 V for the last hour. The gel was then stained overnight and destained for two days.

II. Kinetic Study of cyt b_5

The second portion of the experiment was adopted from work done by Mansuy et al [5] at the University of Paris.

The kinetic study of cyt b_5 used a kinetic graphing program that EMU acquired in the winter'06 semester. This kinetic program gathers data from 350 to 700 nm from a Shimadzu UV/Visible spectrophotometer. The kinetic study takes approximately 40 minutes for each sample.

BH_4 was used at a concentration of 1.2 mM along with 180 µM cyt b_5, 100 mM KP_i at pH 7.0, for a total volume of 1 ml. Everything was added in solution and used as blank. Then, 6.67 µl of 0.6M BH_4 was added last, the cuvette was quickly inverted, and placed in the spectrophotometer to measure absorption.

Afterwards, the kinetic data was transferred to the graphing programs Microsoft Excel and Graphical Analysis. The absorbance data was corrected by subtracting each absorbance

value from that corresponding to the steady state absorbency and the natural logarithm of the corrected absorbance was taken. Additionally, the reciprocal of the natural logarithm was plotted versus time, to determine the rate constant for reduction and whether or not the reduction process was second order.

RESULTS

The purpose of this experiment was to develop a research-based lab that incorporates several analytical methods that are traditionally taught in the CHEM 453 lab. In order to achieve this purpose, two lab exercises incorporating current research on cyt b_5 were adopted and molded to fit the needs of EMU CHEM 453 students. Table 1 summarizes the data collected from the cyt b_5 assay in the research lab, CHEM 453 lab, and the published data.

Table 1. Summary data for cyt b_5 assay

Cytb_5 assay data						
	Research		**CHEM 453**		**Published Data**	
Purification & Analysis	apo	holo	apo	holo	apo	holo
Cell Culture	97%	3%	95.30%	4.70%	95%	5%
Heme add #1	71.60%	29.40%	N/A*	N/A*	30%	70%
Heme add #2	43%	57%	N/A*	N/A*	N/A*	N/A*
After DE-52 column	N/A*	N/A*	30%	70%	30%	70%
BSA determined	63%**		N/A*	N/A*	N/A*	N/A*
Kinetic rate constant, k	0.005$M^{-1}cm^{-1}$		N/A*	N/A*	0.20 $M^{-1}cm^{-1}$	

* N/A = Not available

** BSA Determined is 37 nmol cyt b_5/ mg protein as compared to theoretical value specific content 58.8 nmol cyt b_5/mg protein (63%)

The following paragraphs describe the results from both the research lab and the CHEM 453 lab.

I. Research Lab

A difference spectrum for the cyt b_5 preparation obtained in the research lab produced an absorbance difference of 0.010 absorbance units. This number was found by taking the difference of the peak absorbance at 413 nm and the absorbance minimum at 409 nm for cyt b_5 in the reduced state (Fe^{2+}). The concentration of the holo cyt b_5 was found using Beer's Law. Calculation 1, as shown below, determined the ratio of the holo cyt b_5 to the apo cyt b_5. The total amount of cyt b_5 prepared was 131.5 mg. In calculation 1, the amount of apo and holo cyt b_5 is calculated.

Calculation 1.Difference Spectrum

Purpose: To find the ratio of holo:apo cytb_5

$A = Elc$

$.010 = (185\ mM^{-1}cm^{-1})(1\ cm)(c)$

$c = 5.4 \times 10^{-5}$ x 20-fold dilution $= .00108$ mM

1.08 μM

(0.200 L)(1.08 μmol/1L)(17,600 μg cytb_5/1 μmol cytb_5) = 3802 μg

3.8 mg holo cytb_5 in culture

(3.8 mg/131.5mg) (100%) = 3% holo cytb_5

97% apo cytb_5

Another analysis to determine the ratio of holo:apo cyt b_5 was used during the EMU research lab. Heme titration was performed on a 1 ml sample of cyt b_5 and 2 μl increments were added to the 1ml cyt b_5 sample. Table 2 provides the raw data produced from the heme titration.

Table 2. EMU Research Lab Heme Titration Data

heme (μl)	Abs at 413 nm
2	0.034
4	0.068
6	0.1
8	0.125
10	0.15
12	0.177
14	0.196
16	0.216

From the data in Table 2, the graph shown in Fig. 6 was obtained.

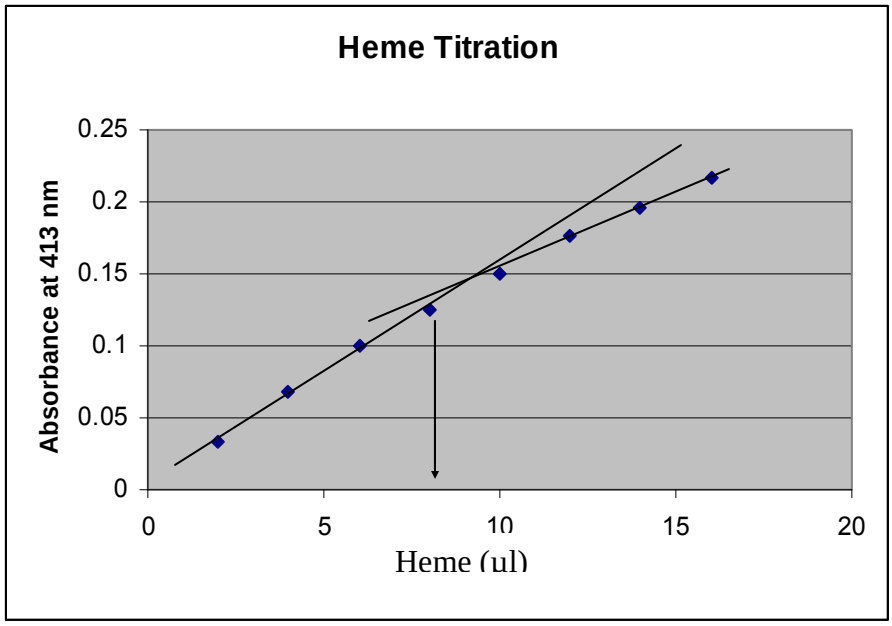


Fig. 6. EMU Research Lab Heme Titration

By adding best fit lines to Fig. 6, it is possible to find the point at which a break in the slope of the lines occur, the break point. Using the break point derived from the graph of 8 μl, further calculations were performed. The amount of holo cyt b_5 calculated is shown previously in calculation 1. Calculation 2 shows the amount of apo cyt b_5 calculated.

Calculation 2. Heme Titration

Purpose: To find apo:holo ratio of cyt b_5

- 8µL was the volume of heme corresponding to the break point.
- Concentration of heme used in titration was 0.1 mM

$(8x10^{-6}L \text{ heme})(0.1mM \text{ heme}/1L) = 8x10^{-7} \text{ mmol heme}/1 \text{ ml in cuvette}$

1:1 heme to cytb_5

$8x10^{-7}$ mmol cyt b_5/1ml x 50 (fold dilution) = $4x10^{-5}$ mmol cyt b_5/ml

$(4x10^{-5}$ mmol cyt b_5/1 ml)(200 ml)(17,600 mg cyt b_5/ mmol cyt b_5) =

140.8 mg cyt b_5 apo

4.4 mg cyt b_5 holo

145.2mg cyt b_5 total

After the heme titration, calculation 3 was performed to determine the amount of heme needed to achieve the maximum expression levels of holo cyt b_5. This was the second purpose of performing a heme titration.

3. Calculation to Determine Amount of Heme Solution Needed to Convert Apo- to Holoprotein

Purpose: Determine how much heme to add to achieve maximum holo cyt b_5

(132mg cytb_5) (1mmol cytb_5/ 17,600mg cytb_5)(1mMol heme/ 1mMol cytb_5) (1L/ 4.5mMol heme)

= 1.83 ml of heme solution to add to 132 mg (16 m1) cyt b_5 sample

= 0.915 ml of heme solution to add to 66 mg cyt b_5 sample

Instead, three increments of 250 µl of the 4.5 mM heme solution were mixed in with 66 mg of the cyt b_5 sample. After the first addition of the 4.5 mM heme solution, the absorbance of the cyt b_5 was found to be 0.317 absorbance units at 413 nm. Again, using Beer's law, it was possible to find out the amount of holo cyt b_5 present. Calculation 4 shows the steps in detail.

Calculation 4. Holo cyt b_5 Increase I

Purpose: To determine the amount of holo cyt b_5 present after addition of 250 µl 4.5 mM

- Use Beer's law C = A/eb

C = (0.317)/((117 mM^{-1})(1 cm))

C = (0.00271 mM)* 51(dilution factor)

= (0.13821 mM/L in cuvette) * (0.016 L)

=2.2 µmol cyt b_5 in stock solution

(2.2 µmol cyt b_5) * ((17,600 µg cyt b_5)/µmol)) = 38720 µg cyt b_5

38.7 mg cyt b_5 holo

Another 250 µl of the 4.5 mM heme solution was mixed to the same 8 ml of the cyt b_5 sample. The cyt b_5 sample produced an absorbance of 0.611 absorbance units at 413 nm. Calculation 5 shows the steps used to find the amount of holo cyt b_5 produced in the heme titration.

Calculation 5. Holo cyt b_5 Increase II

Purpose: To determine the amount of holo cyt b_5 present after additional addition of 250 ul 4.5 mM

- Use Beer's law C = A/eb

C = (0.611)/((117 mM^{-1})(1 cm))

C = (.00522/mM)* 51 (dilution factor)

(0.266 mM/L in cuvette) * (0.016 L)

= 4.3 µmol cyt b_5 in stock solution

(4.3 µmol cyt b_5) * ((17,600µg cyt b_5)/µmol)) = 75680 µg cyt b_5

75.68 mg cyt b_5 holo

Finally, a third addition of 250 μl of 4.5 mM heme was mixed with the same 8 ml cyt b_5 sample. The cyt b_5 produced an absorbance at 413 nm of 0.658 absorbance units. Calculation 6 shows the steps used to find the increased amount of holo cyt b_5.

Calculation 6. Holo cyt *b_5* Increase III

Purpose: To determine the amount of holo cyt b_5 present after additional addition of 250 μl 4.5 mM

- Use Beer's law C = A/eb

C = (0.658)/((117 mM^{-1})(1 cm))

(0.2868 mM/L in cuvette) * (0.016 L)

= 4.6 μmol cytb_5 in stock solution

(4.6 μmol cyt b_5) * ((17,600 μg cyt b_5)/μmol)) = 80960 μg cyt b_5

80.96 mg cyt b_5 holo

Afterwards, a Biuret Standard Assay (BSA) was used as another method to aid in the determination of the holo cyt b_5 concentration after heme addition. Shown in Table 3 are the results of the protein assay with the absorbance at 550 nm given for each of the bovine serum albumin standards. Included in Table 3 is the absorbance each tube produced at 550 nm.

Table 3. Biuret Standard Assay Data for Protein

Biuret Assay		
Standard tubes containing bovine serum albumin	Conc, mg/ml	Abs at 550 nm
1	1.5	0.096
2	3.5	0.246
3	5	0.348
4	7.5	0.526
5	10	0.687

The data in Table 3 were used to create the standard curve, shown in Fig.7.

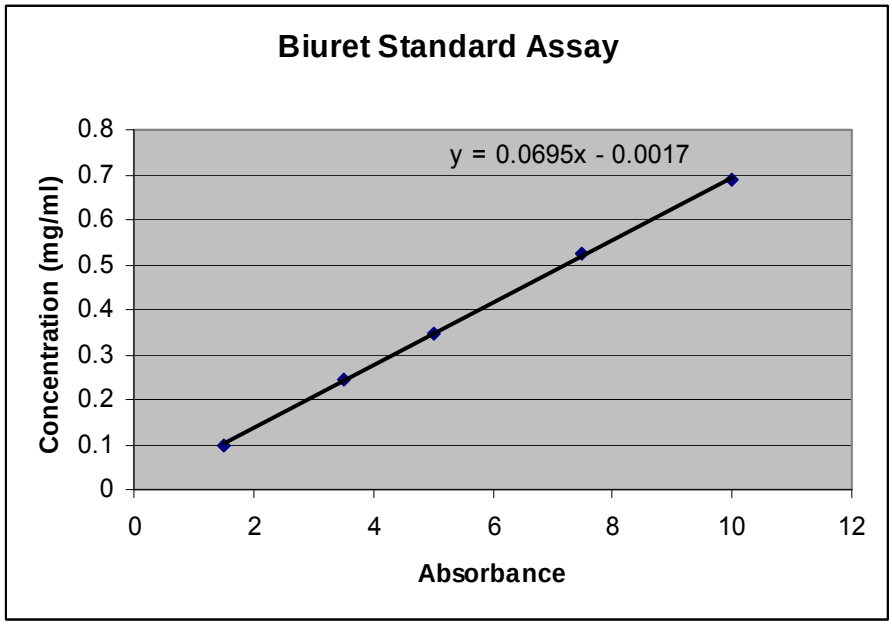


Fig.7. Biuret Standard Assay (BSA) using Bovine Serum Albumin

A BSA tube containing cyt b_5 produced an absorbance of 0.134 absorbance units. By setting the absorbance of cyt b_5 to the best-fit line of Fig. 7 the specific content of cyt b_5 was found. Calculation 7 shows the details involved.

Calculation 7. BSA Determined cyt b_5 specific content

Purpose: To determine specific content

- 0.134 absorbance units for cyt b_5
- Best Fit line y = 0.0695x – 0.0017
- Theoretical Value is 58.8 nmol cyt b_5/mg

$0.134 = 0.0695x - 0.0017$

$0.134 + 0.0017 = 0.0695x$

$0.1357 / 0.0695 = 0.0695x / 0.0695$

(1.95 mg/ml) * (4 dilution factor)

= 37 nmol holo cyt b_5/ mg protein

= 37/58.8 = 63% specific content

Another BSA with varying concentrations of heme was performed because there was suspicion that the additional heme present in the cyt b_5 sample was contributing to the absorbance reading.. Shown in Table 4 are the results obtained in terms of heme contribution to the assay.

Table 4. Biuret Standard Assay: effect of Heme

Heme contribution		
Tubes	Abs	Concentration of heme (mM)
0	0.483	0
1	0.626	0.025
2	0.76	0.05
3	1.023	0.1

The graph shown in Fig. 8 was obtained using this data.

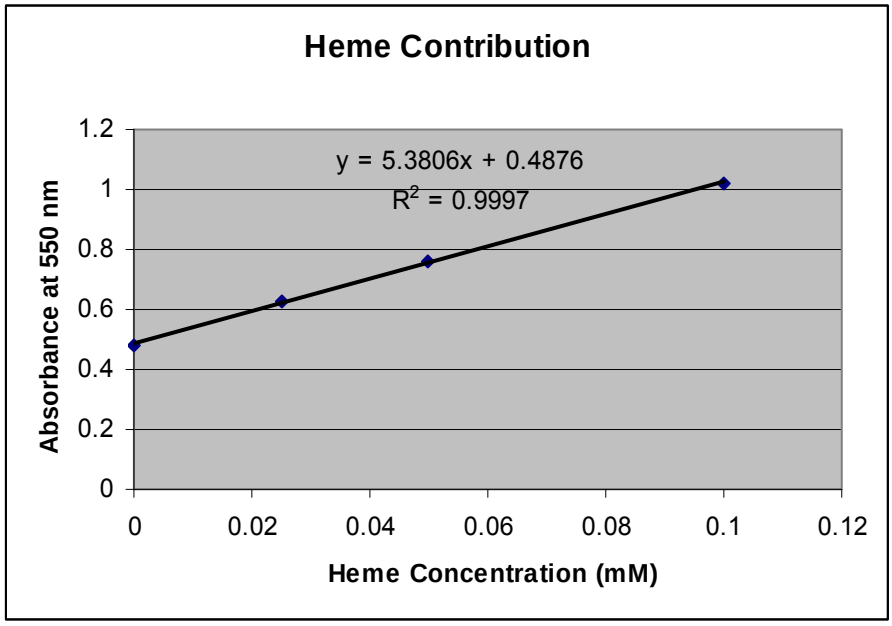


Fig. 8. BSA Heme contribution: effect of Heme concentration

The heme was found to contribute to a 19% overestimation of the amount of protein in the holoprotein sample 19%. Therefore, the BSA yielded 44% holo cyt b_5 before purification.

Before attempting to purify the cyt b_5 sample on a prepared DE-52 column, the cyt b_5 sample was dialyzed to remove detergent and salts. A spectrum of the buffer produced an absorbance of 0.018 at 413 nm. The sample, once loaded onto the column, did produce one fraction (C1) of purified cyt b_5. However, the remainder of the sample was not able to be eluted from the column and would have given fraction C2 if it was possible.

The other half of the cyt b_5 (8 ml) that had not undergone previous heme addition nor the BSA had three aliquots of 250 µl of 4.5 mM heme solution added to it. Each addition produced an absorbance of 0.294 and 0.375 at 413 nm. Calculation 8 shows the treatment of the data.

Calculation 8. Holo cyt b_5 Increase I (2nd half)

Purpose: To determine the amount of holo cyt b_5 present after additional addition of 250 µl 4.5mM

- Use Beer's law C = A/eb

$C = (0.294)/((117\ mM^{-1})(1\ cm))$

(0.128 mM/L in cuvette) * (0.016 L)

= 2.05 µmol cyt b_5 in stock solution

(2.05 µmol cyt b_5) * ((17,600 µg cyt b_5)/µmol)) = 36,080 µg cyt b_5

36.08 mg cyt b_5 holo

Calculation 9 shows the treatment for the second addition of heme.

Calculation 9. Holo cyt b_5 Increase II (2nd half)

Purpose: To determine the amount of holo cyt b_5 present after additional addition of 250 μl 4.5 mM

- Use Beer's law C = A/eb

Concentration of cyt b_5 converted from apo to holo protein

= (0.375)/((117 $mM^{-1}cm^{-1}$)(1cm))

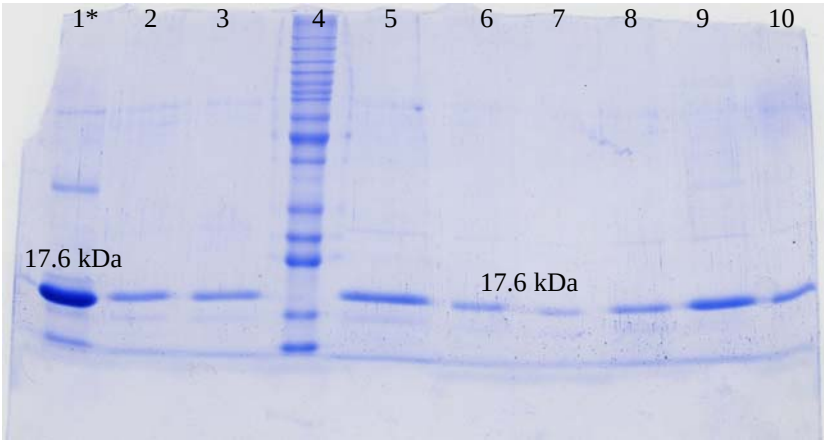
(.163 mM/L in cuvette) * (.016 L)

= 2.62 μmol cyt b_5 in stock solution

(2.62 μmol cyt b_5) * ((17,600 μg cyt b_5)/μmol)) = 46,112 μg cyt b_5

46.11 mg cyt b_5 holo

After the heme additions to the sample of cyt b_5, the sample was dialyzed similar to that of the first sample. Following dialysis, a SDS-PAGE gel was run. Shown in Fig.9 is a picture of the gel run.



***Lanes 2, 3, 5, 6, 7, 8 were purified on column DE-52. Lanes 9 and 10 are unpurified cyt b5 lysate. Lane 1 is Myoglobin and Lane 4 is 100kDa Protein ladder.**

Fig. 9. SDS-PAGE of the cyt b_5 preparation

Concluding the research lab experiments was the kinetic assay on cyt b_5, which used the purified cyt b_5 samples from the CHEM. 453 lab. Shown in Fig.10 is a picture of the kinetic assay performed on cyt b_5.

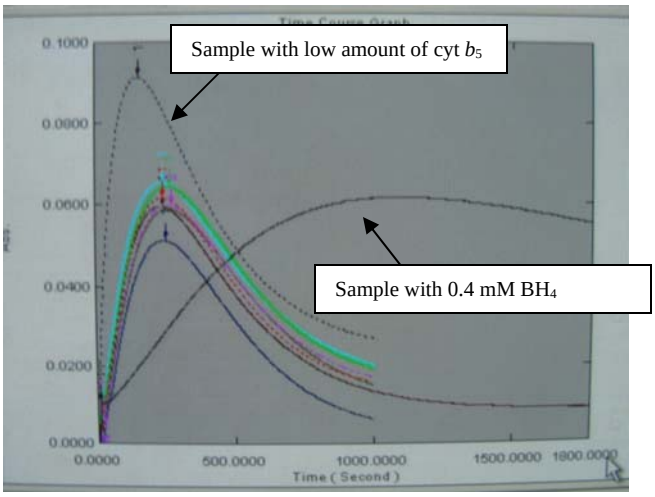


Figure 10: Kinetic Assay

Shown below is an enlarged view of the kinetic assay performed on cyt b_5.

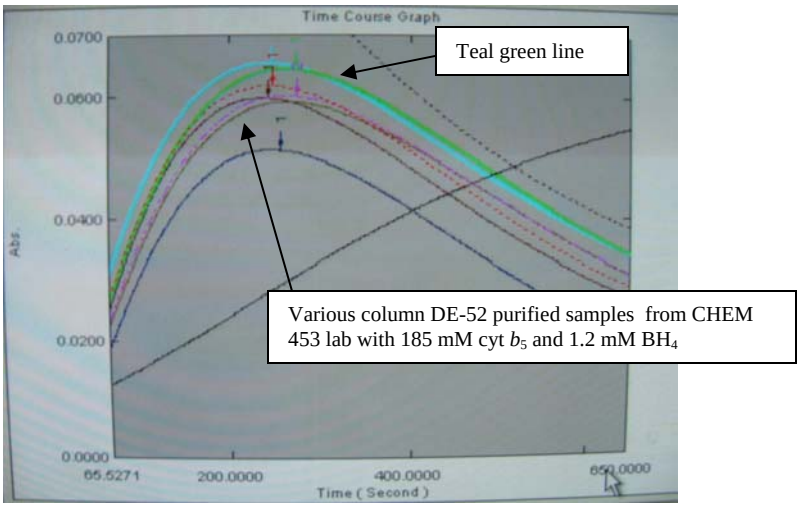


Fig. 11. Kinetic Assay Close-up of data in Fig.10

From the kinetic assay of the teal green line is the rate reaction graph below.

Figure 12: Kinetic Rate Reaction of cyt b_5

The slope from the graph is the rate constant, k of cyt b_5 at a concentration of 185 mM and 1.4 mM BH_4.

II. Results Obtained in the CHEM 453 Lab

The CHEM 453 lab for the Winter '06 semester repeated the exact same procedure that the EMU research lab had prepared. There were a few differences between the labs that will be discussed further. The CHEM 453 class however did not have the chance to repeat the kinetic assay on cyt b_5.

The CHEM 453 lab measured the apo cyt b_5 by heme titration the same way as the EMU research lab. Calculation 10 below was performed in order to find the heme concentration.

Calculation 10. Heme Concentration

Abs of heme =0.106

C = A/ eb

0.106/((56 mM^{-1}/cm)(1 cm)) = 0.001893 M heme concentration

Shown below in Table 5 is the data produced from the heme titration.

Table 5: Heme Titration (CHEM 453)

Heme titration	
heme added (μl)	Abs (413)
2	1.035
4	1.391
6	1.734
8	2.005
10	1.86

From Table 5 the following graph was obtained.

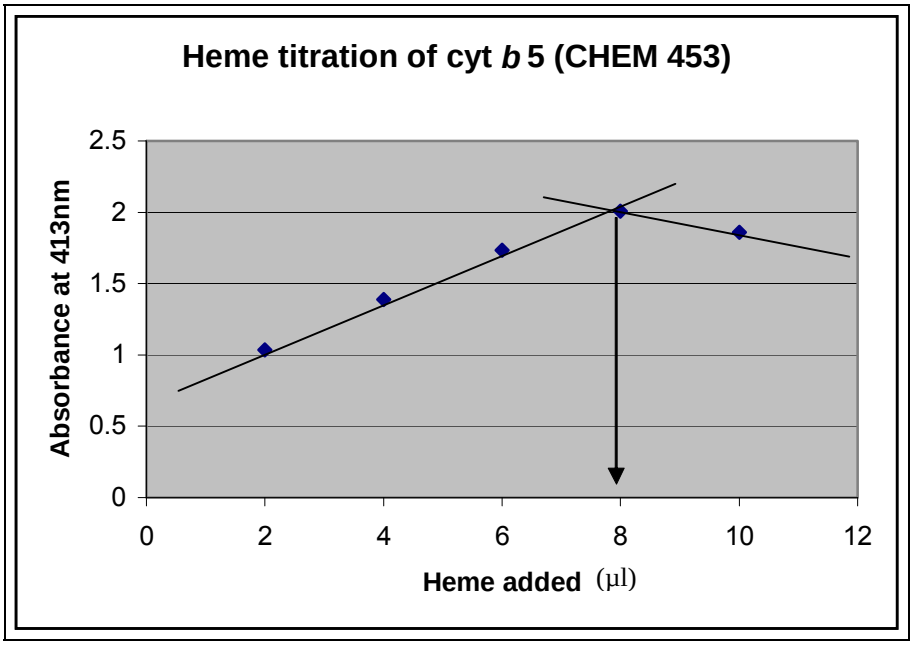


Fig. 13. CHEM 453 Heme Titration of cyt b_5

Like in the research lab, the break point in the CHEM 453 lab was 8 µl. Show in calculation 11 the amount of apo cyt b_5 present is determined.

Calculation 11. Apo cyt b_5

Purpose: To calculate the amount of apo cyt b_5 present before heme addition

$(8x10^{-6}$ L heme)(1.893 mmol heme/1 L) = $1.5144x10^{-5}$ mmol heme/1ml in cuvette

1:1 heme to cyt b_5

(1.514x10^{-5} mmol cyt b_5/1 ml) x 51(fold dilution) = $7.723x10^{-4}$mmol cyt b_5/ml

(7.72344x10-4 mmol cyt b_5/1 ml)(1000 ml)(17,600 mg cyt b_5/ mmol cyt b_5)

= 13593.3 mg apo cyt b_5

=13.5 g apo cyt b_5

An absorbance spectrum was run on a 1 ml sample of cyt b_5 to find the amount of holo cyt *b*5 present. The sample had a characteristic absorbance spectrum of the oxidized cyt b_5 solution (Fe^{3+}) from 550 to 350 nm. Then, like the difference spectrum in the research lab the absorbance difference between 424 nm and 409 nm was used with the cyt b_5 extinction coefficient 185 $mM^{-1}cm^{-1}$. Calculation 12 provides the detailed steps.

Calculation 12. Holo cyt b_5

Purpose: To calculate the amount of holo cyt b_5 present pre-heme addition

C = (0.014)/ (185 mm^{-1})(cm^{-1})

C=7.5675x10-5 mM

$7.5675x10^{-5}$ mM x 51 (fold dilution) = 0.00385 µM = 3.85 µM

(1 L) (3.85 µM/1 L) (17600 µg/µM cyt b_5) = 67926 µg cyt b_5

679.26 mg holo cyt b_5

Together, these two numbers are used to calculate the apo:holo ratio, shown in calculation 13.

Calculation 13. Apo: Holo Cytb_5 ratio

Purpose: To calculate the apo:holo ratio of cytb_5
14272.5 mg total cytb_5
13593.3 mg apo cyt b_5/14272.5 mg total cyt b_5 x 100 = 95% apo cyt b_5
679.26 mg holo cyt b_5/14272.51926mg total cyt b_5 x 100 = 5% holo cyt b_5

Shown in Fig. 14 is the absorbance spectrum of ferric (solid line) and ferrous (dotted line) cyt b_5 after DE-52 purification.

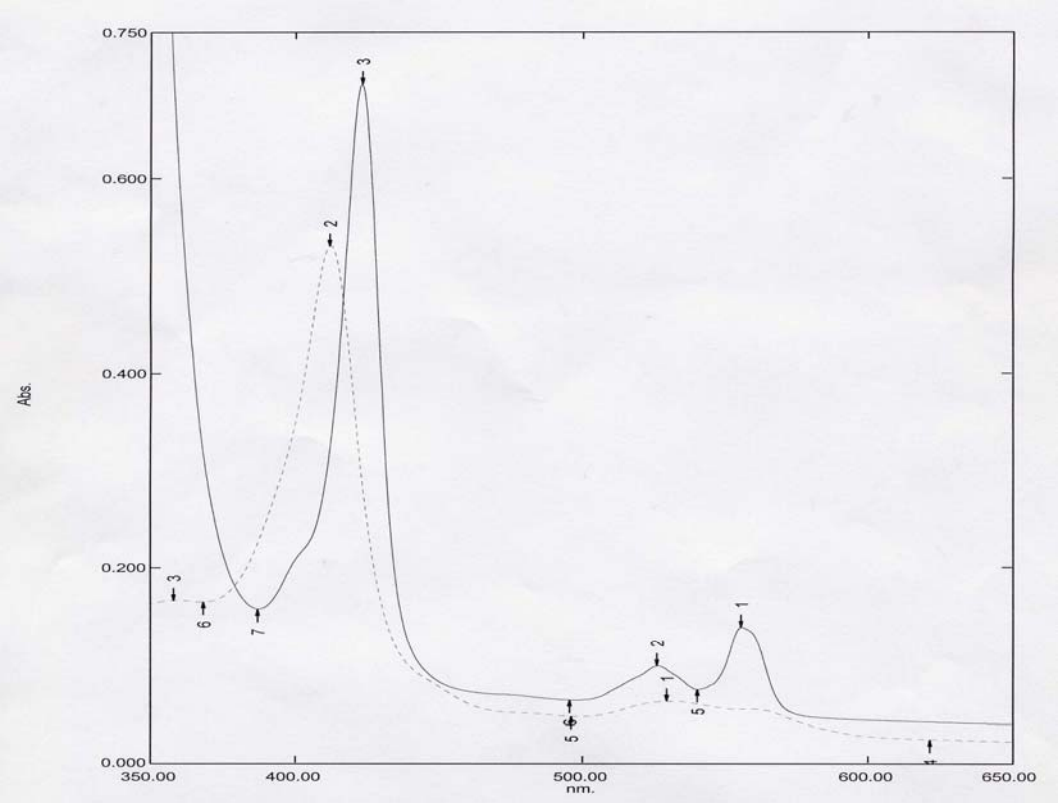


Fig. 14. Absorbance Spectrum of Ferrous (dotted line) and Ferric (solid line) cyt b_5 following DE-52 Purification

Shown in Fig. 15 is a difference spectrum run on the cyt b_5 sample.

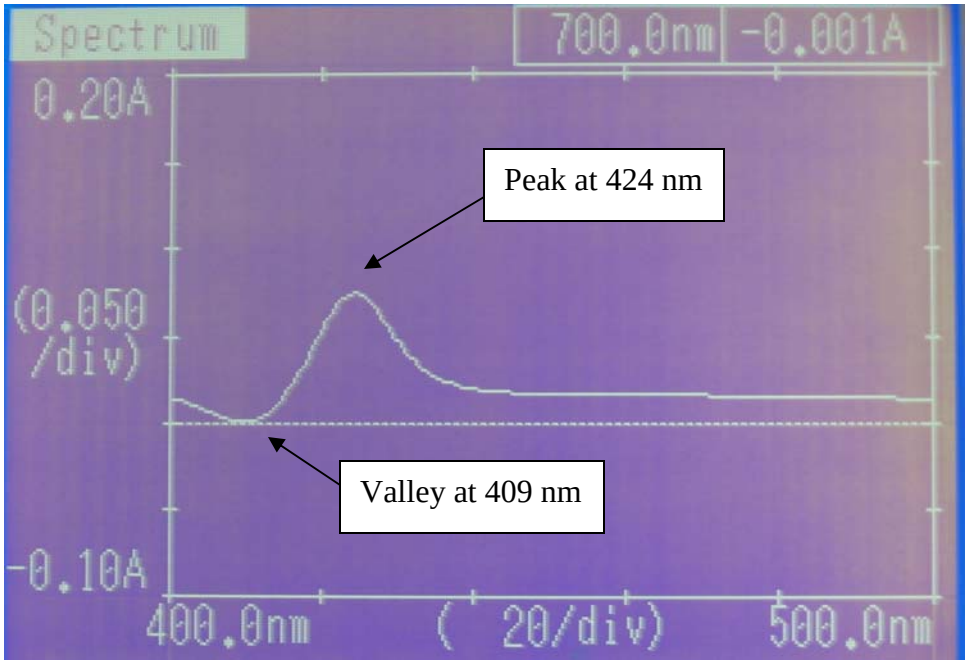


Fig. 15. Difference Spectrum (ferrous cyt b_5 minus ferric cytb_5)

Calculation 14 shows the steps below for the determination of the Difference Spectrum:

Calculation 14. Difference Spectrum

C1 (cyt b_5 that came through column)

- All absorbencies below from Difference Spectrum on C1

@409 Abs is −0.010

@424 Abs is +0.059

difference is Abs of +0.069

- Use absorbance difference between 409 nm and 424 nm to find concentration

$C = 0.069/185\ mM^{-1}cm^{-1}$

$C = 3.72972973 \times 10^{-4}$ mM x 20 (fold dilution) = 0.00746 mM cyt b_5

7.459 µM

(7.4594595 µM/1000 ml)(5 ml)(17600 µg/cyt b_5) = 656.432432 µg holo cyt b_5

6.56 mg holo cyt b_5

150.88 mg cyt b_5 total

C1 came from the concentrated fractions from 1.0 to 1.6

Calculation 14 continued

C2 (batch process)

- All absorbencies below from Difference Spectrum on C2

@409 Abs is +0.001

@424 Abs is +0.074

difference is Abs of +0.073

- Use absorbance difference between 409 nm and 424 nm to find concentration

C = 0.073/185 mM-1cm-1

C= 3.945945946 x 10^{-4} mM x 20 (fold dilution) = 0.0078918919 mM cyt b_5

7.89 µM

(7.89 µM/1000 ml)(5 ml)(17600 µg/cyt b_5) = 656.432432 µg holo cyt b_5

6.94 mg holo cyt b_5

Calculations 13 and 14 represent two different fractions, one that came through the column (C1) and the other that was pooled by the batch process (C2).

From the calculated holo and the apo calculated by the $A_{260/280}$ the specific activity was found as shown in calculation 15.

Calculation 15. Recovery of Purified Cyt b_5 after DE-52 column

(6.94 mg holo C1cyt b_5 + 6.56 mg C2 holo cyt b_5)/19.29 mg pre column = 70% holo

The calculated amount of heme was added to the cyt b_5 solution. Shown in Fig. 16 is the red band of cyt b_5 on the column.

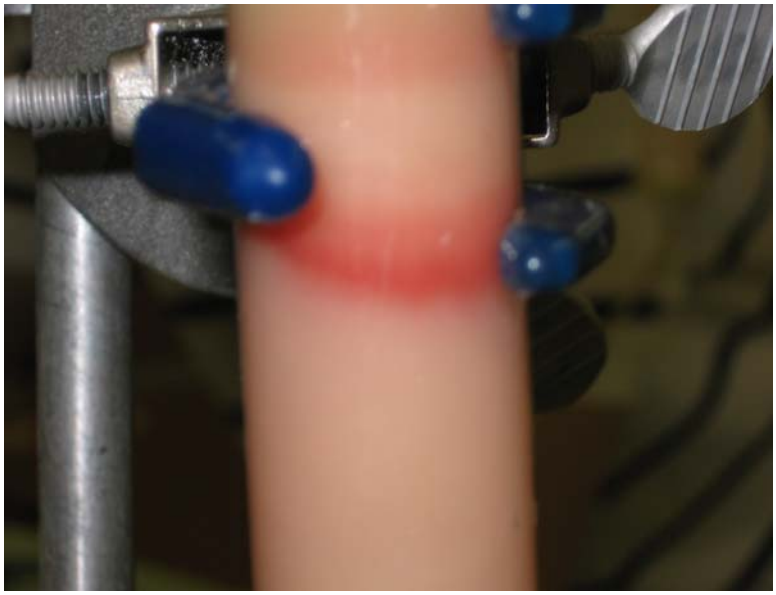


Fig. 16. Cyt b_5 on column initially

As the cyt b_5 moved down the column, the band broadened significantly as expected. Shown in Fig.17 is a picture of cyt b_5 on the column when it started to come off the column.

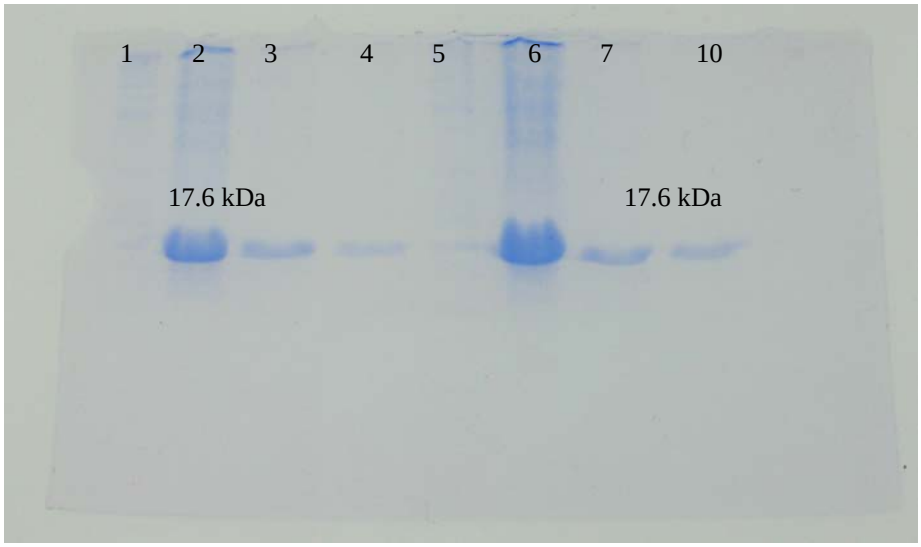
Fig. 17. Cyt b_5 on column during elution

The DE-52 column produced two purified cyt b_5 fractions (C1 and C2). Table 6 shows the absorbance readings of the pooled fractions.

Table 6. Fraction Pooled Data

Pooled Fractions	
Fraction #	Abs at 413
8	0.385
9	0.487
10	0.421
11	0.368
12	0.305

Lastly, C1 and C2 fractions, centrifuged samples prior to chromatography, and a 100 kDa ladder were run on a SDS-PAGE gel as in the prep lab.



*Lanes 1, 2, and 6 are 100kDa protein ladder. Lanes 3 and 7 are C1 fraction and C2 fraction subsequently. Lanes 4 and 8 are cyt b_5 lysate.

Fig. 18. SDS-PAGE Gel of cyt b_5 purified in the CHEM 453 course

Lastly shown in Fig. 19 is a picture of the C1, C2, and UC samples.

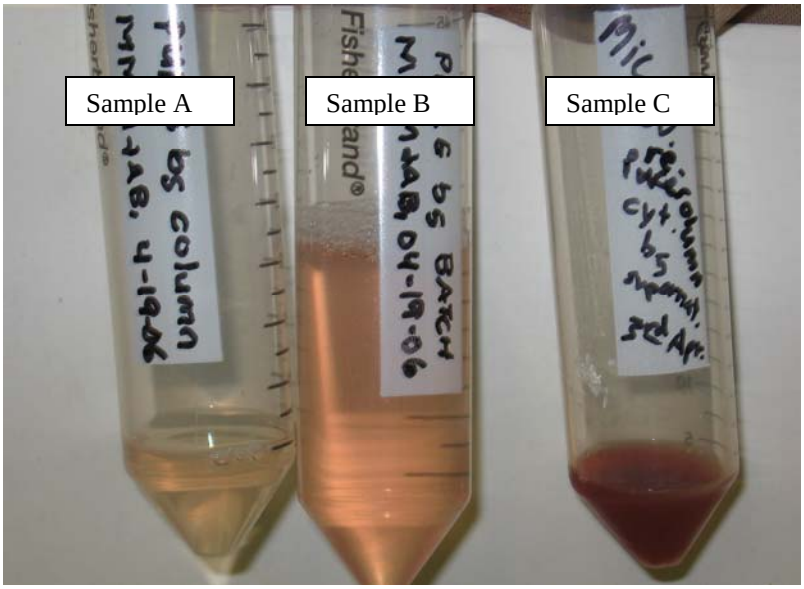


Figure 19: Visual comparison of cyt b_5 samples

DISCUSSION

The protein cyt b_5 is found in many organisms including rabbits, pigs and humans. Cyt b_5 is a robust protein with a red color due to a heme containing an iron atom, which cycles between the ferrous and ferric state as it passes electrons to organic and protein acceptors. The rabbit protein was expressed at levels so high that the bacterial cells became red during the course of the expression. Whereas much of the cyt b_5 was expressed as apoprotein, heme could be easily reconstituted to give fully active holoprotein. Batch or gradient elution of the protein after DE-52 column chromatography gave preparations of cyt b_5 that were electrophoretically pure and had specific contents that were not substantially different from published values.

The largest problem with the preparations isolated in the research lab was the additional non-incorporated heme present in the cyt b_5 solution. Some of the extra heme did dialyze out of the cyt b_5 solution; although so did a very small amount of cyt b_5. However, since the dialysis tubing selects for molecules of a molecular weight less than cyt b_5 is 17.6 kda, there is no need to

worry. The small amount of cyt b_5 that dialyzed out is most likely due to cyt b_5 with the tail cleaved by proteases. This occurred despite the fact that protease inhibitor tablets were included in the assay.

Another error that may have been caused by the additional heme was that the second fraction of cyt b_5 was not able to be eluted from the column. The second fraction of cyt b_5 was never recovered, though thankfully, half of the cyt b_5 solution was retained for further studies. Besides the previous errors, the additional heme also contributed over expected results to the Biuret Standard Assay which from subsequent analysis was provided a correction factor.

Two more errors present in the research lab were 1.) the addition of 5% sodium deoxycholate (detergent) to the cyt b_5 solution to lyse the cell and 2.) the constant freezing/thawing of the cyt b_5 solution. Sodium deoxycholate is a detergent that is negatively charged and is therefore an appropriate detergent to be used on a positively charged column. Interestingly, cyt b_5 was retained on the column despite the presence of a small amount of this detergent. The constant freezing/thawing over a long period of time (greater than one year) can cause the protein to lose its integrity.

Other than the errors mentioned above, the research lab minimized possible random errors. This was achieved by keeping a strict, sterile space with 70% ethanol, autoclaving, gloves, and all other sterile protocol. Additionally, the research lab always kept the samples on ice or in the freezer and was aware of the lab's surroundings.

In the CHEM 453 lab there was no extra non-incorporated heme to serve as a problem and tergitol (a non-charged detergent) was used instead of sodium deoxycholate. In addition, a much better method involving the French press purchased by EMU was used to lyse the C41 cells versus sonication as was done in the research lab and in the Waskell laboratory.

Another difference in the CHEM 453 lab was that the protein assay was never employed and in neither the research lab nor the CHEM 452 was a second column used in purification as was done in the Waskell lab. Alternatively, the CHEM 453 lab used an $A_{412/280}$ spectrum to determine the content of the preparation. In addition, the CHEM 453 lab prepared *E.coli* cells, whereas the research lab did not. The CHEM 453 lab was not able to run the kinetic experiment because of the lack of time.

Some errors present in the CHEM 453 lab were that 1.) the students did not always practice sterile technique with their cyt b_5 solutions 2.) there were times during which the cyt b_5 was not on ice .Thankfully, the robust nature of cyt b_5 aided in maintaining its integrity. A third problem that occurred was that, of the four columns that were used in CHEM 453 lab, two apparently did not elute the second fraction of cyt b_5. Fortunately, the batch process, a method not thought of previously, successfully extracted purified cyt b_5. Additionally, the increase in holo cyt b_5 was not tested for, after heme addition and French Press lysis.

Upon examination of the data in Figs. 9, 18, and 19, it is possible to see that column chromatography of cyt b_5 resulted in it purification. The results from the purification and analysis portion of the research lab show 44% for specific content of cyt b_5 and 70% for the CHEM 453 lab. When compared to Waskell's specific activity of 70%, purification of cyt b_5 occurred.

The kinetic assay on cyt b_5 was explored in the research lab only because the lack of time prohibited the CHEM 453 lab from taking these measurements. The rate constant k, was found to be $0.005\ M^{-1}cm^{-1}$, not close to the rate constant reported by Mansuy as $k = 0.020M^{-1}cm^{-1}$. This could be due to two reasons. One reason is that it is most likely that Mansuy used a different concentration of BH_4 as well as cyt b_5. In addition, it is important to note that Mansuy's lab used

trypsin recombinant bovine liver cyt b_5 versus the rabbit membrane cyt b_5 used in our research lab.

Ways to improve this experiment would be to run another heme titration after purification to test for holo cyt b_5 increase; and use either a Sonicator with a probe that is dispensed within the bacterial solution (as opposed to a cup-horn that surrounds the solution) or a French Press Lysis system (as the CHEM 453 course did).

I. Student Participation

One of the most important successes of this project was student involvement and training. Undergraduate students in CHEM 453 learned methods taught traditionally in a verified "cookbook" course in a new research based course and presented their work in detailed lab reports on the "research" conducted.Not only did the undergraduate students in CHEM 453 produce better specific contents than the EMU research lab and the same results as the Waskell lab; but also aided in refining the CHEM 453 lab for future use. Shown in Figure 20 is a picture of a student taking a difference spectrum.

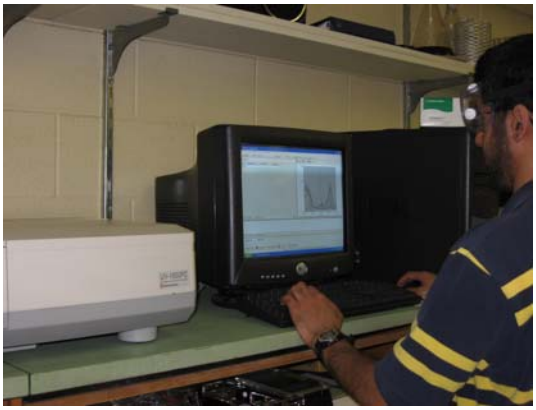


Figure 20: Student taking Difference Spectrum

Shown in Fig. 21 is a picture of a student examining the red band of cyt b_5 on the column.

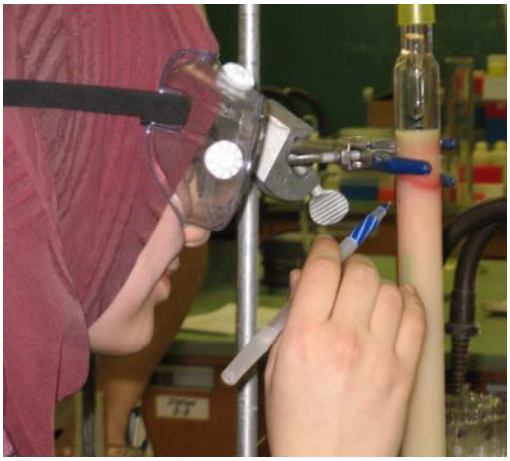


Fig. 21. Student examining DE-52 Column During cyt b_5 Chromatography

II. Future research and current presentations

It is the hope of those involved that others interested at other universities will be able to adopt the CHEM 453 protocol developed during the past year. Already, two presentations of current work in the EMU research lab were presented at the 2006 Undergraduate Symposium at EMU and the American Society for Biochemistry and Molecular Biology (ASBMB) conference 2006 in San Francisco, California. Shown in Fig. 22 is a picture of the 2006 UG Symposium presentation at EMU.

Fig. 22. Student Presentation at EMU

Shown in Fig. 23 is a picture of the 2006 ASBMB Conference presentation.

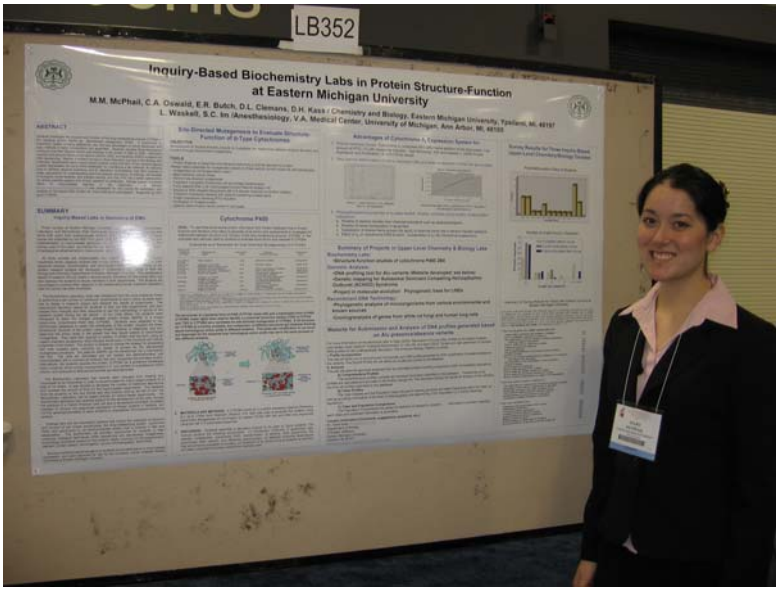


Fig. 23. Student presentation at ASBMB

Eventually, as the years progress the cyt b_5 experiment will evolve and branch out into other experiments In conclusion, this project produced a laboratory experiment that involved research themes and techniques currently emphasized in the CHEM 453 course. Prospects in the near future include an enzymatic study to study cyt b_5 protein with other enzymes, more kinetic studies on cyt b_5 variants, and a web site to accompany the CHEM 453 course. In this way, the CHEM 453 lab will hopefully remain as a research-based lab reflecting current research, or perhaps, even delving further into novel research.

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