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Expression and Purification of Recombinant Saposin B for Coenzyme Q10 Purification

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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Honors Capstone Project in Biochemistry

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Abstract

Coenzyme Q10 (CoQ10) plays a critical role in energy production in humans. Low levels of CoQ10 have been linked to diseases such as Parkinson's and Huntington's disease. CoQ10 levels decrease as we age, and use of statin drugs also lowers CoQ10 levels. Oral supplementation however increases CoQ10 levels. However, the lack of industrial sources of CoQ10 and the difficulty in CoQ10 purification has resulted the need to address these problems. *Sporidiobolus johnsonii* (*S. johnsonii*) has been reported as a natural producer of CoQ10. This work is aim to increase the production of CoQ10 in *S. johnsonii* through genetic engineering. Current purification methods for CoQ10 are difficult and expensive. A protein based purification method may alleviate the current problems associated with typical LC purification. Saposin B (Sap B) has been shown to bind with CoQ10 at selective pH's. We hypothesized that utilizing a Sap B coated support resin that an affinity purification method for CoQ10 could be produced. To this end, the recombinant Sap B was expressed and bound via a HIS-Tag to a sepharose IMAC bead. This work demonstrates that CoQ10 can be bound and released by a Sap B based affinity resin by manipulating the pH.
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Chapter 1. Introduction

1.1 Coenzyme Q10 (CoQ10)

1.1.1 Introduction

Coenzyme Q10 (CoQ10) is produced naturally by our body and is necessary for basic functioning cells. Professor Frederick Crane from the University of Wisconsin-Madison was the first to isolate it in 1957, and showed that CoQ10 is vital in the electron transport chain [1]. In 1958, the structure of CoQ10 was determined by Dr. Karl Folkers from Merck, and it was designated as 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-diquinone. The structure is composed of two major parts- a quinone ring and a long carbon chain (Figure 1). CoQ10 is also known as Q10 or ubiquinone-10 [2]. Ubiquinones are compounds that are being produced by all organisms, however the number of isoprene units is species specific. Human produce CoQ10, which contains a decaprenyl group, while *E. coli* synthesizes CoQ8, which contains an octaprenyl group [3] for example.

![Figure 1. Structure of CoQ10](image)

1.1.2 CoQ10 is an electron carrier in the mitochondrial electron transport chain

CoQ10 plays a vital role in energy production in humans. CoQ10 is fat-soluble and is located in the inner membrane of the mitochondria. It plays a critical role in the electron transport chain, which acts as an electron carrier [4]. In the inner membrane of the mitochondria, electrons from NADH and succinate pass through the electron transport chain to oxygen and subsequently reduced it to water. As
electrons are being transferred, H\(^+\) ions are pumped across the membrane such that a proton gradient is created (Figure 2). The proton gradient is used by ATP synthase to generate ATP. Specifically, CoQ10 serves as an electron carrier from enzyme complex I (NADH-ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) to complex III (cytochrome bc\(_1\)) in the electron transport chain [5].

Figure 2. CoQ10 plays a vital role in the mitochondrial electron transport chain.

1.1.3 CoQ10 act as lipid antioxidant

CoQ10 acts as an antioxidant, which can counteract the damaging effects caused by the physiological process of oxidation. CoQ10 is a perfect antioxidant because as it changes from the ubiquione to ubiquinol under the redox cycle (See Fig 3), it easily gives up electrons, such that it serves as a free radical neutralizer [5, 6].

Figure 3. Sequential single electron reduction of quinone to quino
1.1.4 Diseases associated with CoQ10 deficiency

Organs requiring a great deal of energy, such as the heart and kidneys, are very sensitive to CoQ10 levels [6]. CoQ10 has great clinical potential for treating diseases such as Parkinson’s disease, Huntington’s disease, chronic heart failure, and Meniere’s disease [7]. Studies have also shown that CoQ10 has the potential to protect against renal tissue injury and renal dysfunction induced by cisplatin, which is commonly used for cancer treatment [8]. To manage their cholesterol levels, many people take statin drugs on a daily basis, which are now among the most commonly used drugs in the world [9]. The wide consumption of statin draws attention to their adverse effects, including the lowering of energy associated reduced with CoQ10 levels. Consequently, doctors encourage patients to take CoQ10 along with statin drugs. It is recommended that one take 30-60mg/day to prevent CoQ10 deficiency. Studies have shown that CoQ10 is non-toxic; we can take up to 1.2 g kg\(^{-1}\) day\(^{-1}\) [10]. No absolute contraindications are known for CoQ10.

1.1.5 Biosynthetic pathway of CoQ10

CoQ10 naturally present in small amounts in a wide variety of foods, which include organ meats such as heart, liver and kidney. Beef, soy oil, sardines, mackerel and peanuts are good sources of CoQ10. Approximately 30mg of CoQ10 intake is equivalent to 1 lb. of sardines, 2 lbs of beef, or 2.5 lb. of peanuts [11]. The biosynthetic pathway of CoQ10 consists of three major steps: 1) production of aromatic quinone group, 2) production of the isoprene tail, and 3)
covalent attachment with subsequent modification. [12,13, 14].

Figure 4. Biosynthetic pathway of CoQ10

1.1.6. Chemical synthesis of CoQ10

CoQ10 can be synthesized chemically using trimethoxy toluene and solanesol as starting materials (Fig. 5) [15]. The benzyl chloride intermediate is generated after five steps from the starting material, trimethoxy toluene. The solanesol is converted into vinyl alane by two synthetic steps [16]. CoQ10 is produced by
coupling the benzyl chloride and vinyl alane intermediates, which is only a 50% yield from the two starting materials. In addition, the by-product, non-natural Z-isomer is produced on a large-scale production of CoQ10, which is very difficulty to purify [17]. Although as to today, this is the best-known organic synthesis method for CoQ10, it has not proved viable on a commercial scale. As a consequence, microbial fermentation is the dominant large-scale route for large-scale CoQ10 production [18].

![Diagram of CoQ10 synthesis](image)

Figure 5. The organic synthesis procedure of CoQ10 from solanisol and trimethoxy toluene. A overall 50% yield is achieved from these two starting materials with multiple steps, which is not viable in a commercial scale.

1.1.7 Microbial production of CoQ10

Genetic engineering in *E. coli* has been attempted multiple times to increase the production of CoQ10. However, ubiquinone is species specific, *E. coli* naturally produces CoQ8 [3]. In other words, CoQ8 needs to be purified out from the desired CoQ10, and the available isoprene precursor will be used for the synthesis of both CoQ8 and CoQ10.
Increasing the flux through the non-mevalonate isoprenoid pathway is the major focus on genetically engineering *E. coli* for CoQ10 production [19]. In addition, a non-native *dpps* gene is needed to insert into *E. coli* to increase the CoQ10 production. Many other genetic engineering strategies have been attempted with limited success. These studies indicate that a more efficient route to production of CoQ10 will involve identifying a new organism, which naturally produces CoQ10.

### 1.1.8 Sporidiobolus

*Sporidiobolus* is one of the genuses under Basidiomycete. The Basidiomycete phylum is within the fungi kingdom, with fungi classified as eukaryotic microorganisms with chitin cell walls. *Rhodosporidium, Sporidiobolus, Rhodotorula* and *Sporobolomyces* make up the Sporidiobolus family [20]. All *Sporidiobolus* species are red pigmented due to carotenoid production. Carotenoids act as antioxidants, and have been used for food coloring and flavoring [21].

![Figure 6. The relationship among members of the *Sporidiobolus* genus.](image)
S. johnsonii is very closely related to S. salmonicolor [22]. Both S. johnsonii and S. salmonicolor produce the carotenoids β-carotene, torulene, and torularhodin. R. sphaerocarpum is another member of the Sporidiobolus genus, also known as the yeast with the highest native production of CoQ10 [23].

1.2 Saposins

1.2.1 Introduction

Saposin A, B, C and D are highly conserved non-enzymatic, heat and protease stable proteins. The saposin family consist approximately 80 amino acids with six highly conserved cysteine residues and a conserved glycosylation site (Fig 7) [24, 25]. There are four alpha helices and three disulfide bonds in the protein, which creates a V-shaped monomer (Fig. 8). Two monomers form a dimer and create a hydrophobic pocket for lipid binding. The N-terminal alpha helix, helix 1, spans approximately 17 amino acids followed by the conserved N-glycosylation site. The helix 1 is followed by helix 2, which also consists about 17 aminos acids. After a hairpin turn, a 21 amino acid sequence comprises alpha helix 3, followed by the C-terminal alpha helix (helix 4), spanning about 12 amino acids. The two intramolecular disulfide bonds hold helix 1 and 4 together, and the hairpin turn is held together by the third disulfide bond [26].
Figure 7. ClustalW alignment using SDSC clustal program using human Saposin A, B, C, D. Disulfide linkages are shown by solid lines. Conserved glycosylation site, N, is outlined. Identical amino acids are in blue and labeled with (*). Similar amino acids are in green labeled with (: ).


All four saposins (A, B, C, D) are generated from the same prosaposin gene [27]. First, a 54 kDa protein is expressed, and glycosylated to 78 kDa, then protein is proteolytically processed in the lysosomes and result the mature saposins A-D, each of them weigh between 8-14 kDa.

1.2.2 Prosaposin gene produce Saposin A, B, C and D genes are measured in kb (kilobases)

The prosaposin consists approximately 20 kDa gene with 15 exons and codes for saposins A, B, C and D [28] (Figure 9). The coding strand that produces the 524 amino acid polypeptide is obtained from splicing the prosaposin RNA. The major splice variant takes place at exon 8 in the SapB coding region [29], which will be the main focus for this project.
Figure 9. Prosaposin gene. Exons are showed in the shaded area. Coding regions for Saposin A, B, C and D are indicated by the bars above.

1.2.3 Mechanism of saposins

The exact mechanism of the saposins is still a matter of controversy. It has been shown that all four saposins interact and modify lipid membranes in a certain manner. Saposins are non-enzymatic, however saposins aid in the hydrolysis of sphingolipids. Currently, it has been shown that saposins can increase the reaction rate by binding to the hydrolytic enzyme and subsequently induce a conformational change [30]. Other studies report that saposins can perturb the lipid membrane sufficiently to present the substrate to the enzyme. In specific, saposin B is believed to be responsible for lipid solubilization [31].

1.2.4. Saposin B binds with CoQ10

Saposin B is an 81 amino acid protein [32], which also have shown to bind and transfer lipids [33]. Studies have shown that saposin B binds to CoQ10 with natural glycosylated human saposin B used in these CoQ10 binding studies. In addition, binding studies between CoQ10 and gamma-tocopherol and other isoprene lipid were successfully demonstrated [34]. More importantly, scientists
have shown that the non-glycosylated saposins A, C and D, which was expressed using *E. coli*, still stimulate lipid hydrolysis[35].
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Chapter 2 Expression and Purification of Recombinant Saposin B for Coenzyme Q10 (CoQ10) Purification

2.1 Introduction

CoQ10 plays a key role in energy production in humans [1]. Organs requiring a great deal of energy, such as the heart and kidney, are very sensitive to CoQ10 levels [2]. CoQ10 has great clinical potential for treating diseases such as Parkinson’s disease, Huntington’s disease, chronic heart failure, and Meniere’s disease [2]. Studies have also shown that CoQ10 has the potential to protect against renal tissue injury and renal dysfunction induced by cisplatin, which is commonly used for cancer treatment [3]. To manage their cholesterol levels, many people, on a daily basis, take statin drugs, which are now among the most commonly used drugs in the world [4]. The wide consumption of statin draws attention to their adverse effects, including the lowering of energy associated reduced with CoQ10 levels. Consequently, doctors encourage patients to take CoQ10 along with statin drugs. It is recommended that one take 30-60mg/day to prevent CoQ10 deficiency [5,6]. No absolute contraindications are known for CoQ10, and adverse affects are rare.

While CoQ10 is in great demand, producing it on an industrial scale presents some challenges. Currently, commercial production focuses on bacterial and yeast fermentation routes. Currently, high-performance liquid chromatography (HPLC) is the most common purification method, but it requires toxic and dangerous chemicals such as chloroform, a known carciogen [7]. Because people consume
CoQ10, any toxic residue from purification is unacceptable. In addition, co-elution of fatty acid triacylglycerols makes the purification more difficult and expensive [8]. Our aim for this work is to develop a protein binding purification method for CoQ10 by taking advantage of the body’s natural CoQ10 binding protein (Saposin B) that binds CoQ10 in a pH-dependent manner.

Saposin B (SapB) is a lipid-binding protein that humans produce naturally, which can bind and transfer CoQ10 and α-tocopherol. It has also been shown that native Sap B stimulates degradation of cerebroside sulfates by arylsulfatase A [9]. SapB is 9kDa human non-catalytic glycoprotein consisting of 81 amino acids. SapB also has three disulfide bonds and folds into a V-shape monomer (Figure 1A). Two monomers form a dimer with a hydrophobic pocket (Figure 1B). Other characteristics of SapB include, heat, protease, and pH stability [10, 11].

Figure 1. Crystal structure of SapB. A: single chain of the homodimer. B: SapB crystallized as a homodimer revealing a hydrophobic pocket at the interface of the dimer permitting lipid binding. The bound molecule is a phosphatidylethanolamine from E. coli that was copurified with the protein.
Literatures have shown that native SapB binds CoQ10 and the recombinantly repressed SapB from *E. coli* stimulates the hydrolysis of cerebroside sulfates. This findings suggest that glycosylation is not necessary for lipid binding and solubilization, but rather is likely required for maturation of each individual saposins from the prosaposin [12]. With these findings, the hypothesis of this work is CoQ10 can be purified from a microbial extract via construction of a recombinant expressed SapB based affinity resin.

In addition, this work reports the first complete characterization of recombinant expressed SapB. There is no depth analytical investigation of recombinant SapB folding, disulfide bond formation, or thermal stability. There is no CD spectra and melting temperature of recombinant SapB is reported up to date. The natural formation of disulfide bonds in the recombinant SapB has not yet explored.

Moreover, studies toward increasing the expression of SapB through bioreactor have only been done in *P. pastoris*. It has been reported that 160mg SapB/ L media is produced by fermentating *P. pastoris* for four days, which also produces an equal mixture of glycosylated and non-glycosylated SapB. This mixture complicates the process of purification and future studies of the protein [13]. Here, we reported a yield of great than 100mg SapB/ L media in 24 hours fermentation using *E. coli* [14].
This work demonstrates a more thorough investigation on recombinant SapB folding, and addresses the issue of yield. Additional studies such as proof of three disulfide bonds, alpha-helicity and thermal stability are also presented. Melting CD was also conducted to investigate the effect of disulfides. Finally, the affinity between SapB and CoQ10 on a solid support was also studied and shown to be pH dependent [13].

2.2 Saposin B

2.2.1 Construction of DNA Vector

The human SapB was first cloned with PCR primers to create \textit{NcoI} and \textit{XhoI} restriction sites. The SapB gene was purified from the agarose gel (Figure 2A). SapB gene was isolated from the agarose and digested with both \textit{NcoI} and \textit{XhoI}. The \textit{pET27b+} expression vector with a pelB leader sequence and a His-tag, was also digested with \textit{NcoI} and \textit{XhoI} along with calf intestinal phophatase (Figure 2B). Digestion products were purified on an agarose gel. Digested gene and vector were then ligated to construct the \textit{pET27b+_Sap B} plasmid. The \textit{pET27b+_Sap B} plasmid was transformed into \textit{E. coli} BL21 GOLD DE3 for Sap B expression.
The transformation mixture was plated out on LB Kan agar plates and incubated at 37°C overnight. Single colonies grew on plates and were subsequently selected and grown in 5ml LB Kan liquid culture overnight. Plasmid was isolated, digested with NcoI and XhoI and run on an agarose gel for confirmation of gene insertion (Figure 3). The diagnostic agarose gel showed the Sap B and pET27b+ bands, suggesting the correct insertion. The plasmid was also submitted for sequencing for final confirmation. The sequence (Figure 4 & Figure 5) confirmed the ligation was successful.
Figure 3. Diagnostic agarose gel of the pET27b+_SapB plasmid digestion. Digestion product showed the 254bp SapB band and the 5414bp pET27b+ band, suggesting successful gene insertion.

ACCTGCTGCCGACCAGCTGCTGCTGCTGCTGCTGCTCCGTGCTGGCCAGCC
GGCGATGGCCATGGATGGCGATGTTTGCCAGGATTGTATTTTCTGATGATGGT
GACCGATATCCAGACGCGGTTCGTACCAACAGCACGTTTGTGCAGGC
CCTGGTGGAACATGTAAAGAAAGAATGCGATCGCCTGGGCCCGGGTAT
GGCAGATATCTGTAAACTACATCAGCCAGTACTCTGAAATTGCGAT
CCAGATGATGATGCACATGCAGCCGAAAGAAATTTGCGCGCTGGTTGG
CTTCTGTGATGAACTCGAGATCAAACGGGCTAGCCAGCCAGAACTCGC
CCCGGAAGACCCCGAGGATGTCGAGCACCACCACCACCACCACCACCAG
ATCCGGCTGCTAAACAAAGCCCGAAGGAGCTGAGTTGGCTGCTGCTCC
ACCGCTGAGCAATACTAGCATCAACCCCTTGGGCCT

Figure 4. DNA sequence of pET27b+_SapB plasmid showing the correct gene insertion.
Figure 5. Amino acid sequence translated from the DNA sequence from Figure 4. The correct SapB amino acid sequence and the His Tag showed the successful vector construction. Bold AA represent the normal human sapB. Green (italics) AA represent the HSV tag. Blue (underlined) AA represent the his-tag used for sapB purification.

2.2.2 Recombinant expression of SapB in *E. coli*

Upon confirmation of successful ligation, SapB was expressed. Single colonies were picked and growing in 5ml LB Kan culture overnight at 37°C, with 0.5ml of this overnight culture used to inoculate a 50ml LB Kan broth. Once an OD$_{600}$ of 0.4 was reached, the cultures were induced with either 0.01 mM or 0.1 mM isopropylthiogalactoside (IPTG). Culture samples were taken prior to induction, and after induction at 1 hr, 4 hr, and overnight. The samples were centrifuged, and the media was decanted into a falcon tube containing immobilized metal affinity column (IMAC) resin. After 1 hr binding time, the resin was washed with PBS buffer (20mM PBS pH=7.4), and then with PBS buffer containing imidazole (250mM) to elute the SapB protein. All eluted samples were run on a 15% SDS-PAGE protein gel and coomassie stained (Figure 6).
From figure 6, it shows that SapB protein is not present in the media until 18 hours. Therefore, subsequent SapB expressions are in the following conditions: 0.01 mM IPTG induction at 0.4 OD with overnight expression at 30°C.

Based on the data from the SDS-PAGE gel, and the MALDI-MS data (vide infra), SapB was successfully expressed. Fermentation was then performed using a bioreactor to increase the yield of SapB. A single colony was picked and growing in 5ml LB Kan culture overnight at 30°C. 0.5ml of the overnight culture was
transferred to 100ml LB Kan media and grown overnight at 30°C. The total 100ml overnight culture was inoculated in a 1L bioreactor. The system was maintained at pH 7 and 35% dissolved oxygen. After about eight hours, 25g of glucose was added, and IPTG was added to a final concentration of 0.1mM to induce protein production. Fermentation conditions are shown in Figure 7. Media was harvested after 17 hours for purification. The cell density at the media collection time was 6.9± 0.4g DCW/L.

Figure 7A. Bioreactor during the *E. coli* fermentation.
Figure 7B. Fermentation of E. coli for SapB production. Red (A): agitation, Green (B): temperature (°C), Blue (C): dissolved oxygen (DO).

2.2.3. Purification by immobilized metal ion affinity chromatography

Fermentation media was collected, clarified by centrifugation and run on fast protein liquid chromatography using immobilized metal affinity chromatography. The binding buffer for the purification was 20mM PBS, pH 7.4, 0.5M NaCl and the elution buffer was 20mM PBS, pH 7.4, 0.5M NaCl, 250mM imidazole. The five ml IMAC column was equilibrated with binding buffer and 150ml of filtered media was loaded into a super loop. The whole binding experiment was run at 2ml/min, then elution buffer was changed from zero percent to ten percent (25mM imidazole) to elute nonspecific bindings, subsequently 100% elution buffer was used to elute Sap B (Fig. 8). Eluted Sap B fraction was run on 15%
SDS PAGE gel (Fig. 9). A dark band around 12kDa was seen on the gel, suggestive of SapB-His$_6$. Eluted SapB-His$_6$ fractions were quantified using Bradford assay. The yield of SapB through fermentation was 105 ± 15 mg/L.

Figure 8. FPLC trace showing purification of SapB from the fermentation media. Binding and elution was at a flow rate of 2ml/min. The two flow through peaks (3+4) represent 150 ml and 50ml loading respectively. Elution started with a 10% elution buffer (25mM imidazole) to eluted non-specific binding species in peak 5 and finished with 100% elution buffer (250mM imidazole) to elute SapB in peak 6.
The gel in Figure 9 suggests that SapB has successfully been purified by a single step IMAC purification. The sample that weight approximately 12kDa, which bound to the IMAC column and eluted with imidazole, and it showed great than 95% pure. Based on the calculation, the constructed gene that encodes 107AA SapB-His$_6$ should weight 12.2 kDa.

### 2.3.3 SapB characterization and quantification

The 12kDa band showed up in the SDS PAGE gel was consistent with the mass of SapB. However, for more conclusive confirmation, MALDI was used with saturated sinapic acid (SA). The SA matrix was in a 7:5 mixture of acetonitrile:
water with 0.1% TFA. The MALDI-MS data of SapB after IMAC purification and dialysis is shown in Figure 10. Internal angiotensin standard was added and produced a peak at 1,294.3 amu, which is 3.2 amu less than the anticipated peak at 1,297.5 amu. The calculated molecular mass of the 107 AA of the recombinantly expressed SapB is 12,191.8 amu. The peak detected at 12,182.2 amu in Figure 10 is determined to be SapB. The smaller mass as compared to the calculated mass due to the presence of three disulfide bonds (6 units) and the 3.2 mass units off from the MALDI-MS. As a result the expected peak 12,191.8 amu should appear as 12,182.6, representing the [SapB+ H]^+. The peak detected at 12,182.2 was determined to be the singly charged Saposin B monomer. The peak at 6091.8 amu is the doubly protonated [SapB+ 2H]^{2+}, and the singly charged dimer [(SapB)_2 +H]^+ was detected at 24383.7 amu.

Figure 10. MALDI-MS data for non-trypsin digested SapB in SA matrix showing the singly charged SapB monomer at 12,181Da, the doubly charged monomer 6090Da, and the singly charged dimer at 24,365 Da.
In addition, a coomassie stained band was cut from an SDS-PAGE gel and sent to Texas A&M University Protein Identification Laboratory for independent confirmation. Verification was done by post digestion MALDI-MS. Figure 11 showed the peaks detected after tryptic digestion, reduction, and alkylation, provided MS peaks supporting the identification of the band as SapB (Table 1).

![Figure 11. MALDI-MS data from trypsin digested protein bands. See table 1 for identification of the mass peaks.](image)

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Table 1. MALDI-MS data of the trypsin digested eluted protein compared to predicted MS peaks for SapB. Complete coverage of the protein is obtained by MS peaks corresponding to amino acid 1 to 107.

After the confirmation of SapB production, the Bradford assay was used to quantify the yield of SapB. A calibration curve was made using bovine serum albumin (BSA), which is shown in Figure 12. Based on the Bradford analysis, the yield was reached at 105 ±15 mg SapB per liter, with error representing standard deviation from expressions conducted in triplicate. The higher cell density possible during fermentation provides more cells for protein production. In addition, the extracellular localization resulting from the pelB leader sequence allows a larger amount of protein per cell to be made by reducing the toxic accumulation of protein in the cell, and reducing protein exposure to cellular proteases.

![Figure 12. Calibration curve from the Bradford assay used to quantify SapB production. BSA was used as the standard.](image)
With the confirmation of the successful SapB expression, and the significant increased yield, the next necessary step is to demonstrate the proper folding of SapB. One of the major characteristics for SapB is the saposin fold form by the three disulfide bonds. In order to investigate the number of disulfides present, a sample of pure and dialyzed SapB was prepared for MALDI-MS exactly the same as for Figure 10. This sample of pure SapB was then divided into two separate falcon tubes, one with dithiothreitol (DTT) added (1mM), which would reduce the disulfide bonds and the other one has no DTT added. Both MALDI-MS samples were run side by side. There was a six mass unit increase in the DTT treated sample as compare to the non-DTT treated sample (Figure 13). This data suggested there are three disulfide bonds in SapB, which consistent with literatures.

Figure 13. MALDI-MS data for DTT (1mM) treated and non-DTT treated SapB
sample. The increase of six mass units in the DTT treated sample suggested there are three disulfide bonds prior DTT treatment.

In addition, SapB was also react with CNBr in formic acid for 18 h and then lyophilized and dissolved in CHCA matrix solution to obtain the MALDI–MS data shown in Figure 14 (see also Figure 15). The observed connection of amino acids near the C and N termini in the absence of the interrupting amino acids supports that the C and N termini amino acids are connected by a disulfide bond.

Figure 14. MALDI–MS data of the cleaved SapB showing fragments at 5832 and 5963 Da. The amino acids corresponding to these two mass fragments are shown in Figure 15.
Figure 15. Amino acids making up the mass fragments shown in Figure 14. The indicated amino acids remain connected through a disulfide bond.

Additional evidence for disulfide formation is the elution profile with and without 1mM DTT, was shown in Figure 16. The elution profile using the same binding (20 mM PBS 7.4, 0.5 M NaCl) and elution buffers (20 mM PBS 7.4, 0.5M NaCl, 250 mM imidazole) and use of a linear gradient elution provided SapB elution at about 40% elution buffer (or 100 mM imidazole). After reaching the 100% elution buffer (250 mM imidazole), a different elution buffer was used containing 1 M imidazole. No additional protein was eluted from the IMAC column. However, when 1 mM DTT was used in both buffers and added to the crude media, no SapB was detected in the eluted peak near 40% elution buffer as had been the case for SapB with no DTT. After reaching the 100% elution buffer (250 mM imidazole), 1 M imidazole buffer (also with 1 mM DTT) was used to elute a significant amount of SapB.
Figure 16. FPLC elution profile of 150 mL SapB containing media (left) without DTT and (right) with 1mM DTT. Gradient from 0 to 100% elution buffer (250 mM imidazole), followed by elution with 1 M imidazole. Note the eluted protein at 1 M imidazole in the profile contained DTT. Numbers correspond to lanes in SDS-PAGE gels in Figure 17.
Figure 17. Coomassie stained SDS-PAGE gel of the FPLC fractions from Figure 14 (A, left) without DTT and (B, right) with 1 mM DTT. The normal purification (left) gave samples in lane 1) crude media, 2) flow through (FT) 1, 3) FT 2, 4) FT3 5) FT 4, 6) first part of peak indicated by ‘6’ in Figure 14A, 7) tailing part of the peak indicated with ‘7’, 8) fraction corresponding to 1 M imidazole labeled ‘8’. The DTT purification (right) gave samples in lane 1) FT 1, 2) FT 2, 3) FT 3, 4) first peak indicated with a ‘4’ in Figure 14B, 5) eluted peak at 1 M imidazole labeled ‘5’, 6) fraction at end labeled ‘6’, and 7) marker.

The binding affinity of the his-tag for the IMAC resin is significantly increased when DTT is added to the purification. This result is consistent with the DTT reducing disulfide bonds to unfold protein which makes the His-tag more accessible for the binding to the IMAC resin. The non-DTT elusion showed that SapB was released at about 100mM imidazole (Lane 7, Figure 17A), and no
elution at higher imidazole concentration (Lane 8, Figure 17A). The present of the eluted peak at 1 M imidazole (Lane 5, Figure 17B) and no SapB was eluted earlier suggests that protein is unfolded by DTT and such that the his-tag is more available and binds to the IMAC column stronger.

In addition to the three disulfides characteristic, literatures also state that SapB has a high content of alpha helixes. Circular dichroism (CD) was used to investigate the presence of alpha-helices of SapB. CD spectra were recorded in 20mM PBS 7.4 in a quartz cell with 0.1 cm path-length (Figure 18). The spectrum indicates the presence of alpha-helical folds (as expected for SapB) by the peak detection at 208 nm and 222 nm. Further more, melting CD was also performed to study the natural folding and thermo stability of SapB. The absorbance at 222 nm was monitored up to 90°C and the melting curve is shown in Figure 19.

Figure 18. Wavelength scanning CD spectrum of SapB [170µg/ml =1.4x10^{-5}M] in 20mM PBS 7.4 in a 0.1 cm path length cell.
Figure 19. Melting CD of recombination SapB up to 90°C using the sample as in Figure 20. A) no DTT. B) 1mM DTT.

The two different CD spectra in Figure 18 shows the expected unfolding and folding given its helical content. However, in Figure 19, the melting CD spectra is shown for SapB without DTT and with DTT. In Figure 19A, it shows the proper folding of SapB, which is stable and maintains its folded structure up to 90°C. The cooling CD spectra flows the exact same path back to the beginning. Figure 19B shows the CD spectra with the addition of 1mM DTT. The CD spectra does not follow the same path during cooling, suggesting a loss of folding.
3.4 Summary

SapB was successfully expressed in *E. coli* and purified using IMAC. Eluted SapB was shown to be greater than 90% pure based on a coomassie stained SDS-PAGE gel. MALDI-MS confirmed the production of the his-tagged SapB protein. Both CD and melting CD were performed to characterize recombinant SapB. The presences of three disulfide bonds are deduced by MALDI-MS and the CNBr reaction. The expression of SapB reached 105mg/L by fermentation [13].
References:


Chapter 3 The Use of Recombinant Expressed His-tagged Saposin B for CoQ10 Affinity Binding Resin

3.1 Introduction

SapB binds CoQ10 in a pH dependent manner [1]. Taking advantage of this binding specificity, a SapB affinity binding resin can be constructed for CoQ10 binding. Following confirmation that recombinant SapB is properly folded, the final step is to prepare the CoQ10 solution for resin binding. However, since CoQ10 is very hydrophobic, the aqueous solubility of CoQ10 is problematic. This problem can be overcome by dissolving CoQ10 in isopropanol (IPA) and then adding the IPA to PBS to a final concentration of 10% IPA [2].

3.2. Saposin B folds correctly in 10% isopropanol

Prior to binding studies between CoQ10 and SapB, CD spectra of SapB in 10% IPA must be analyzed to investigate the folding of SapB in this solvent mixture. The CD spectra of SapB in 10% IPA (Figure 20A) still exhibits a significant α-helical content. A melting CD experiment was performed with the same sample under the same condition as previously used (Figure 2.19) with the exception that the sample was diluted 10% with IPA. The Figure 1 shows that SapB is folded similarly in the 10% IPA solution as it is in the PBS without IPA (Figure 2.18& 19).
Figure 1. CD spectra showing folded SapB in 10% IPA/PBS. A) wavelength scan CD and B) melting CD showing stability up to 90°C and refolding upon cooling.

As our proposed purification approach exploits the pH dependent binding of CoQ10 by SapB, we also utilized CD to probe SapB folding in 10% IPA/ PBS at pH 7, it is critical to perform CD in 10% IPA/PBS at pH 5, 7 and 9 (Figure 2).
Figure 2. CD spectra of SapB at equal concentration in 10% IPA/PBS pH = 5, 7, and 9. The SapB at pH 5 shows less alpha-helical content.

The CD spectra in Figure 21 shows the difference in the folding of SapB at pH 5, 7 and 9 in 10% IPA. The degree of changes in alpha-helicity between pH 7 and 9 is relative small, suggesting no significant change in the confirmation between pH 7 and 9. However, there is a drastic change in alpha-helicity content at pH 5. The reduced alpha-helicity content corresponds to a less tightly folded structure. This more flexible structure presumably allows SapB bind lipids such as CoQ10 with higher affinity.

3.3 The interaction between CoQ10 and the SapB resin

3.3.1 CoQ10 calibration curve

A method of detection and quantification is needed in order to quantify the potential interaction between CoQ10 and SapB. In this work, HPLC method was used to quantify CoQ10. Three different calibration curves were made for the three solutions at different pH values (Figure 3).
Figure 3. CoQ10 calibration curves determined by peak area detected at 210 nm. Three different calibration curves were made for pH=5, 7 and 9. Each showed a linear range over an order of magnitude from 0.002 to 0.04 mg/ml with an $R^2$
3.3.2 SapB resin binds with CoQ10

Two different types of IMAC resin, silica and sepharose based, were tested for the non-specific interaction of CoQ10. 100µl of solid resin was incubated with 1ml of CoQ10 solution (0.1mg/ml in 10% IPA, PBS pH 5) for one hour, followed by 2 x 1ml washings. HPLC was used to quantify the combined washings of CoQ10. The silica based IMAC resin bound 39 ± 3 % of the CoQ10 at a pH of 5 and 20 ± 2 % CoQ10 was bound at a pH of 7. The sepharose based IMAC resin bound 25 ± 6 % at a pH of 5 and 25 ± 14 % of the CoQ10 at a pH of 7. At pH 7, the non-specific binding between silica and sepharose IMAC resin is very similar, however at pH 5, the silica based resin had a 50% higher background binding. This data suggests that sepharose based IMAC is preferable for construction of SapB for CoQ10 purification at pH5.

Following CoQ10 background binding studies, we investigated the ability of SapB coated resin to bind CoQ10 in a pH dependent manner. Again, the non-protein control experiments were also conducted. When the SapB coated resin was generated, CoQ10 solution was added and rotated at 4°C for 30 minutes. Centrifugation was followed, and supernatant was removed, subsequently, 1ml of 10% IPA/PBS was added to wash any non-bound CoQ10. The combined washings were analyzed by HPLC (Figure 23). The data shows that the SapB coated resin provides no significant binding of CoQ10 over the control at a pH of 7 or 9. However, the binding of CoQ10 to the SapB coated resin at a pH of 5 was greatly higher (93% bound CoQ10 at a pH of 5 vs 32% bound CoQ10 at a pH of
7) than that of the nonSapB control, binding almost all of the CoQ10 as detected by HPLC. The binding at pH 7 was not statistically different from the non-protein control resin.

Figure 4. SapB coated sepharose IMAC resin binding of CoQ10 at pH of 5, 7 and 9. Data was obtained from three different preparations of SapB with each preparation having all three pH experiments in triplicate.

The data from Figure 4 shows that SapB based resin can bind with CoQ10 in a pH dependent manner. To quantify the binding efficiency, a molar ratio of bound CoQ10 to SapB was calculated, taking into account background CoQ10 non-specific binding to the resin. For the binding at pH 5, a molar ratio of 0.44 was calculated, which suggests a dimer binding mechanism.
With the demonstration of pH dependent binding of CoQ10 to the SapB coated resin, the next critical step is to demonstrate pH-induced release CoQ10 from SapB. The pH dependence studies of SapB binding to reveal there was relatively no affinity binding for CoQ10 that at pH 9, suggesting that a change to pH 9 will elute the CoQ10 from the SapB resin. A 55% recovery of CoQ10 was obtained as a result of changing the pH over several elutions (15x1.5ml) over 24 hours. This data demonstrates that SapB binds CoQ10 on a solid surface and release CoQ10 in a pH dependent manner.

### 3.3.3 SapB resin is reusable

As the ability to reuse an affinity resin is very important for economical use, we investigated SapB resin. Each binding experiment was conducted as previously described, with the exception that the resin was equilibrated back to a pH of 5 after the final elution of CoQ10 at pH of 9. The efficiency was determined by comparison of the molar ratio of CoQ10 binding to SapB, and data is shown in Figure 5.
Figure 5. Recyclability of SapB affinity resin. 1st and 2nd use experiments were conducted with at least six samples. 3rd use experiments were conducted in triplicate. Data for 1st use was obtained using a background correction of CoQ10 binding to resin. Data for 2nd use and 3rd use did not include a background binding correction.

Figure 6. CoQ10 recovery efficiency for resins used for multiple subsequent
purification trials. There is little loss in fraction CoQ10 recovered for (up to three) subsequent uses of SapB resin.

The graph in Figure 5 shows that SapB resin will rebind CoQ10 for subsequent uses with a slightly decreased efficiency after each use. The first use of the resin provided a ratio of bound CoQ10 to SapB of 0.44 ± 0.03, as expected based upon the dimer binding of SapB. The second use only bound CoQ10 with a ratio of 0.38 ± 0.02, a decrease by 14%. The third use of the resin resulted in a binding ratio of 0.27 ± 0.001 mol CoQ10 to mol SapB, a decrease of 29% from the previous use of the resin. This decreased efficiency is likely a result of reduced protein activity.

3.4 Summary and conclusions

A solid state SapB affinity resin binds 93% of 0.1mg CoQ10 at pH of 5 at a molar ratio of 0.44 mol CoQ10/mol SapB, which is consistent with a SapB dimer binding mechanism. By change the pH to 9, CoQ10 was able to release from the SapB affinity resin. In addition, this work also demonstrates that SapB affinity binding resin is reusable [2].
References:


Chapter 4 The Complete Genome Sequence and Analysis of *Sporidiobolus johnsonii*

4.1 Introduction

*Sporidiobolus johnsonii* (*S. johnsonii*) is a relatively uncharacterized organism. Based on morphological and physiological similarities, DNA-DNA hybridization (93.1%), electrophoretic pattern of enzymes and nucleotide sequence analysis, *Sporidiobolus johnsonii* is a synonym of *Sporobolomyces holsaticus* [1].

*Sporidiobolus johnsonii* is a pigmented filamentous [2] and ballistospre-forming yeast species [3], which also closely related to *Rhodosporidium sphaerocarpum*, a yeast with high (1.8 mg Coenzyme Q10 /g DCW) coenzyme Q10 (CoQ10) production [2]. It was shown that the yeast, *S. johnsonii* is a high natural CoQ10 producer (10mg CoQ10 per gram dry cell weight), making it the highest known yeast producing CoQ10. *S. johnsonii* is classified as a basidiomycetous yeast, and pigmented basidiomycetous yeasts are promising producers of carotenoids and coenzyme Q10 [4]. With the complete genomic sequence and analysis, genetic engineering studies can be conducted, which has a great potential to improve medical practices.

![Figure 1](image1.png)

Figure 1. *S. johnsonii* is a pigmented filamentous yeast. Images of *S. johnsonii* from left to right, 1) single colonies on an agar plate 2) in liquid culture during
early log phase showing healthy rounded cells and 3) in liquid culture in stationary phase showing the filamentous nature when nutrients are low.

4.2 Genomic DNA isolation

A single colony of *S. johnsonii* was picked from an agar plate and incubated in 5 ml enriched media (malt extract 5g/L, yeast extract 5g/L, potassium phosphate monobasic 1g/L, 4-hydroxybenzoic acid 0.5g/L, pH=5.65) overnight at 28°C at 250 RPM. This 5 ml overnight culture was then inoculated into a 50 ml fresh of enriched media and incubated overnight at 28°C at 250 RPM. This culture was harvested the next morning, and genomic DNA was isolated using the Epicentre Complete DNA and RNA Purification Kit. The isolation process was conducted as describe in manufacture’s protocol. The isolated genomic DNA was electrophorased on 0.5% agarose gel for secondary purification (Figure 1). The genomic band was exercised and DNA was isolated using the QIAEX II Gel Extraction Kit (500). DNA was then precipitated, with purify reflected by a $A_{260}/A_{280}$ ratio of 1.8. The genomic DNA sample was also run on the 0.5% agarose gel to confirm the purity, in which a single band was detected (Figure 1).
Figure 1. Genomic DNA on a 0.5% agarose gel. Lane 1: Marker; 2: DNA product after first isolation; 3: Genomic band was excised and purified using the QIAEX II Gel Extraction Kit, followed by DNA precipitation.
References:

[1] The yeasts, a taxonomic study By C. P. Kurtzman, Jack W. Fell p694


Chapter 5 Experimental

5.1 Materials and methods

Antibiotics, malt extract, and CaCl$_2$·2H$_2$O were purchased from EMD. NH$_4$OH (30%), NaOH, KH$_2$PO$_4$ and hexanes and acetone were purchased from BDH. Sucrose, and angiotensin I standard were purchased from Sigma. NH$_4$Cl was purchased from Fisher. MgCl$_2$·6H$_2$O was purchased from Acros. Yeast extract was purchased from BP/Bacto. Antifoam C was purchased from JT Baker. CoQ10 standard was provided by PharmaBase (Switzerland). Water used was distilled and deionized with a Barnstead Diamond ultrapurification system. All yeast cells were incubated in a MaxQ 4000 Barnstead Lab-Line shaker with digital temperature control. All *E. coli* cells were incubated in a MaxQ 5000 Barnstead Lab-Line shaker with digital temperature control. The pET-27b(+) vector was purchased from Novagen. The SapB gene in pUC57 vector was purchased from GenScript (see appendix 5). All enzymes were purchased from New England Biolabs. PCR was performed using a Techne Tc-312 thermocycler. Media was sterilized at 121 °C for 20 minutes. Metal salts were aseptically added after sterilization. Fermentations were conducted using a BioFlo110 1L bioreactor (New Brunswick Scientific) with an initial volume of 800 mL. 1 mL aliquots were taken for optical density (OD) measurements at 600 nm on a Varian Cary 50 Bio UV-Vis spectrophotometer. The pH was measured on a Mettler Toledo SG2 pH meter and adjusted as needed with NH$_4$OH. Genome DNA isolate kit, Epicentre Biotechnologies, MasterPure™ Complete DNA and RNA Purification Kit was purchased from Epicentre.
5.2 Saposin B

5.2.1 Chemically Competent *E. coli* BL21 (DE3)

A single colony of BL21 Gold (DE3) was picked and grew in LB media (25g/L) at 37 °C at 250 rpm overnight. 0.5 mL of overnight culture was transferred to 50 mL fresh LB broth and incubated at 37 °C and 250 rpm until the OD reached 0.4. The culture was then sit on ice for 10 min then pelleted by centrifugation at 4000 rpm and 4 °C for 5 min. The pellet was resuspended in a 1:4 culture volume ratio with 100 mM magnesium chloride and iced for 5 min. The solution was pelleted by centrifugation at 4000 rpm and 4 °C for 5 min and resuspended in a 1:4 culture volume with 100 mM calcium chloride and iced for 20 min. The solution was pelleted by centrifugation at 4000 rpm and 4 °C for 5 min, resuspended in a 1:40 culture volume ratio with 100 mM calcium chloride in 15% glycerol, and stored at -80 °C.

5.2.2 pET27b+_SapB vector construction

A PCR reaction mixture contains 117 µL H₂O, 21 µL Thermopol buffer, 10.5 µL forward primer (5’ GACTGGATCCATGGATGGCGATG 3’), 10.5 µL reverse primer (5’ CGTCCTCGAGTTCATCACAGAAGCC 3’), 5 µL dNTPs, 2.1 µL Deep vent polymerase, and 5 µL DMSO was prepared and labeled Mix 1. A second combination of 9 µL H₂O, 1 µL pUC57SapB, and 40 µL Mix 1 was prepared and placed in a Techne TC PCR machine. Conditions were as follows: 95 °C for 5
min followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 74 °C for 30 seconds, then held at 16°C. The PCR product was run on an agarose gel. The band corresponding to the full length SapB PCR product was extracted. 14 µL of the SapB sample was then digested with 1 µL XhoI, 1 µL NcoI, 2 µL BSA, and 2 µL NEB Buffer 3 for 1 hr at 37 °C. The pET27b+ vector was digested using the same conditions. Agarose gel electrophoresis was ran on both digestion mixtures and the bands corresponding to the linearized SapB gene and pET27b+ vector were extracted from the gel. The SapB gene and the pET27b+ vector were ligated by combining 5 µL H2O, 10 µL SapB gene, 2 µL pET27b+, 1 µL T4 DNA ligase, 2 µL T4 DNA ligase buffer, and incubating at 16 °C overnight. 4 µL ligation mixture was added to 50 µL chemically competent BL21 Gold (DE3) E. coli, iced for 30min, incubated at 42 °C for 45 sec, and iced for 5 min. 400 µL SOC media was added, incubated at 37 °C for 60 min, streaked onto LB-kan plates, and incubated at 37 °C overnight. Single colonies of the transformed E. coli were picked, placed in 5 mL LB broth, 5 µL kanamycin (35 mg/mL), and incubated at 37 °C overnight. Freezer stocks of the transformed E. coli were made by combining 0.5 mL media and 0.5 mL cold 20% glycerol and storing at -80 °C. The plasmids from the overnight cultures were isolated and digested with NcoI and XhoI and analyzed by gel electrophoresis. The plasmid sample corresponding to the clearest band at 250 bp on the gel was sent for DNA sequencing and confirmed to be the correct construct.
5.2.3 SapB expression and purification

Single colonies *E. coli* with pET27b+_SapB were picked and put in 5 mL LB broth, 5 µL kan (35 mg/mL), and incubated at 37 °C on a incubator at 250 rpm overnight. Control colonies of non-transformed *E. coli* were picked and placed under similar conditions as those containing the desired vector. After overnight incubation, 5 mL of each broth (one for the non-vector sample and one for the vector sample) was transferred to a 500 mL LB broth, 500 µL kanamycin (35 mg/mL except for the non-vector control which contained no antibiotic), and incubated at 37 °C until the OD_{600} reached 0.4. At this time, both samples were induced with 5 µL 1M IPTG (final concentration 0.01 mM). Following induction, the incubation temperature was reduced to 30 °C. After overnight incubation, the cells were pelleted by centrifugation at 8500 RPM and the media decanted into a large vessel. Fermentative protein expression was performed in a BioFlow110. Initially, a single colony was grown in 5 mL LB-kan for 24 hours at 30 °C. 0.5 mL of this culture was used as inoculum for a 100 mL LB-kan culture, which was then incubated for 24 hr at 30 °C and 250 rpm. All 100 mL of the culture was used as an inoculum for the bioreactor. The fermentation media contained KH_{2}PO_{4} (7 g), K_{2}HPO_{4} (3 g), NH_{4}Cl (4g), yeast extract (5 g), and 800 mL H_{2}O, and 0.3 mL antifoam. A glucose solution (50 g in 150 mL) was prepared and autoclaved separately from the fermentation media. After putting the fermenter through a 20 min autoclave cycle at 121 °C, half of the glucose solution was aseptically added as well as filter sterilized MgSO_{4}·7H_{2}O (0.25 g) and thiamin pyrophosphate (1
mg). 5 mL of a trace metals solution was also added. Trace metals solution was made by mixing NaCl (0.25 g), ZnSO₄ (0.03 g), MnCl₂•4H₂O (0.2 g), FeCl₃ (0.2 g), CuCl₂ (0.015 g), H₃BO₃ (0.03 g), and (NH₄)₆Mo₇O₂₄ (0.13 g) and dissolved in 50 mL 6 N H₂SO₄. Kanamycin (1 mL of 35 mg / mL) was added. The dissolved oxygen was set to 35%, airflow at 1.5 L / min, and agitation was permitted to range from 150 – 500 rpm. The pH was set to 7.0 and controlled by 30% NH₄OH. After about seven hours fermentation, the remainder of the glucose was added and the culture was induced with IPTG to a final concentration of 0.1 mM. After 24 hours total fermentation time, the culture was collected and cells centrifuged. The protein was purified by immobilized metal affinity chromatography (IMAC) using a His column, Buffer A (0.5 M NaCl, 20 mM Tris base, pH 7.4), and Buffer B (0.5 M NaCl, 20 mM Tris base, 250 mM imidazole, pH 7.4). Binding conditions were as follows: 2 mL/min, 0.5 mPa, and 100% Buffer A. Elution conditions were as follows: 2mL/min, 0.5 mPa, 90% Buffer A, and 10% Buffer B, followed by elution with 100% buffer B.

5.2.4 Protein characterization

Bound fractions were analyzed by 15% SDS-PAGE gel electrophoresis by combining 30µL protein sample with 20 µL 2x SDS-DTT loading buffer and boiling for 2 minutes. The protein gel was run for 50 minutes at 45 mA. The commassie stained band corresponding to 12 kDa was cut out and sent to Texas A&M University Protein Analysis Lab. Trypsin digestion followed by MALDI-MS provided ion peaks 1794.64 (AA 71-85), 1837.82 (AA 71-86), 1951.28 (AA
71-86 + IAA), 2261.52 (AA 22-40), 2342.59 (AA 1-21), 2457.93 (AA 1-21 + IAA), 2509.62 (AA 51-70), 2603.36 (AA 86-107). IAA = iodo acetamide modification. Non-digested MALDI-MS was performed at Syracuse University. A saturated solution of sinapinic acid (SA) matrix was made in a 7:5 MeCN:H\textsubscript{2}O solution with 0.1% TFA. This was added in a 1:1 ratio with pure SapB (300 mg / L) dialyzed against 20 mM PBS 7.4 over 24 hours with four buffer changes. Scan range was 3 – 30 kDa. The MALDI was operated in linear positive mode. Angiotensin I was included as an internal standard. For the disulfide study, DTT was added from a 100 mM stock to a final concentration of 1 mM and allowed to react for five minutes. Circular dichroism of SapB was performed on a Jasco J-715 spectrophotometer with the following parameters: 0.5 nm Bandwidth, 50 nm / sec scan rate, 0.5 sec time constant, room temperature (23 °C), scan range 200 – 400 nm. Melting CD data was collected with the same sample as used for regular CD scan measurements. Protein concentrations were quantified by a Bradford assay immediately following the CD experiment. Melting CD parameters were the same as for regular CD in addition to a temperature ramp rate of 1 °C / min, 0.2 °C data interval, temperature range of 25 – 90 °C, monitored at 222 nm, with a 3 sec delay between measurements.

5.2.5 CoQ10 calibration curve

A CoQ10 calibration curve was developed by integration of HPLC peak area detected at 210 nm. To generate the calibration curve, a solution of CoQ10 in isopropanol (IPA) at 1 mg/mL was prepared. This solution was then diluted with
IPA to make several different concentrations (0.01 – 0.5 mg / mL) of CoQ10 in IPA. These samples (1 mL) were then added to a phosphate buffer (9 mL, 20 mM, pH = 5, 7, or 9) to a final IPA concentration of 10% and CoQ10 concentration of 0.001 – 0.05 mg/mL. These solutions (20ml injection volume) were analyzed by HPLC using C18 column. Mobile phase A was pure methanol. Mobile phase B was pure ethanol. Flow 1.5 mL/min. 0 min 50% B, 2 min 50% B, 5 min 95% B, 8 min 95% B.

### 5.2.6 SapB and CoQ10 binding studies

Binding studies were carried out with purified SapB. The eluted SapB was dialyzed overnight against 20 mM PBS pH 7.4 to remove imidazole and NaCl. After dialysis, the pure SapB solution was quantified by Bradford assay. For binding studies of resin bound SapB, 900 µL of IMAC resin was added to a SapB solution in a falcon tube and rotated at 4°C. After two hours, the resin was centrifuged down, and a portion of the supernatant was used for SapB quantification by a Bradford assay. After the non-bound protein solution was decanted off, and the IMAC resin was rinsed once with fresh PBS. The resin was resuspended again in PBS and aliquoted evenly into three different tubes, centrifuged and decanted. The resin in each of the four tubes was then resuspended in a 20 mM phosphate solution at a pH of 5, 7, or 9, then centrifuged and decanted. This was repeated until the decanted solution was at the appropriate pH value of 5, 7, or 9. The resin was then resuspended in a 20 mM phosphate solution at the appropriate pH with 10% isopropanol, centrifuged and decanted. A
Bradford assay on the decanted solution showed undetectable amounts of protein was removed from the resin with the 10% isopropanol wash. A solution of CoQ10 in pure isopropanol (1 mg / mL) was then added to the 20 mM phosphate solution (of appropriate pH value) to make a 10% isopropanol in aqueous phosphate solution with a final CoQ10 concentration of 0.1 mg / mL. 1 mL of each 10% isopropanol/aqueous CoQ10 solution was added to the pelleted SapB IMAC affinity resin. The tubes were then rotated at 4°C for 40 minutes, then centrifuged and decanted and washed twice with 10% IPA in PBS of the appropriate pH. CoQ10 was quantified in the combined non-bound fractions by HPLC-MS. The four samples were then resuspended in fresh non-CoQ10 containing 10% isopropanol/phosphate solution of the appropriate pH, centrifuged, and decanted. The process was repeated once more to ensure non-bound CoQ10 was removed from the SapB IMAC resin. CoQ10 was quantified from the combined washings. The resin was then washed multiple times with 10% IPA / PBS pH 9 in order to elute the CoQ10. 15 washings of 1.5 mL each, were combined and quantified for CoQ10 by HPLC.

5.3 *Sporidiobolus johnsonii*

5.3.1 Microorganism

The yeast reported in this paper was initially collected (BioSym Technologies of Iowa) in Oklahoma, USA and ultimately designated Sj0801. The species was purified to homogeneity by successive plating on enriched media agar (see media recipes below). Each stage of species purification consisted of selecting six
colonies to inoculate six separate flasks of enriched media to be grown for 100 hours at which time a glycerol stock was made before analyzing for CoQ10 production. After extraction, the best CoQ10 producing strain was determined by 1H NMR and TLC spot intensity under UV light and I2 staining. The glycerol stock of the best CoQ10 producing strain was used to streak an agar plate for the next round of species purification. After three rounds of selection, Sporidiobolus johnsonii was independently verified by ribosomal sequencing by SeqWright (Houston, Texas) and Microcheck (Northfield, Vermont).

5.3.2 Media and culture conditions

A single colony (Sporidiobolus johnsonii) was picked from an agar plate and incubated in 5ml enriched media (malt extract 5g/L, yeast extract 5g/L, potassium phosphate monobasic 1g/L, 4-hydroxybenzoic acid 0.5g/L, pH=5.65) overnight at 28°C at 250 RPM. This 5 ml overnight culture was then used to inoculate a 50 ml fresh enrich media and incubated overnight at 28°C at 250 RPM. The culture was harvested the next morning.

5.3.3. Genomic DNA isolation and preparation

Genomic DNA was isolated using the Epicentre Biotechnologies, MasterPure™ Complete DNA and RNA Purification Kit. The isolation process was conducted as describe in the manufacture’s protocol. The isolated DNA was run on 0.5% agarose gel for secondary purification. The genomic band was exercised and DNA was isolated using the QIAEX II Gel Extraction Kit (500). Subsequently,
DNA was precipitated out to obtain a \(1.8 \frac{A_{260}}{A_{280}}\) ratio. Precipitation procedures are as follow: two volumes of 100% ethanol and sodium acetate was added to make the final concentration of 0.3M sodium acetate. The sample was then placed at -80°C for 1 hr, and spun down at 4°C for half hr. 70% cold ethanol was added to wash sample 3 times. The sample was left at room temperature to dry for 5 mins. Finally, TE buffer was added to resolublize DNA pellet. The genomic DNA was sent to Aptmatrix, NY for genomic sequencing.
Chapter 6 Future work

6.1 Purification of CoQ10 from a fermentation extract using SapB affinity resin

On the industrial scale, CoQ10 is usually produced by microbial fermentation. However, the isolation and purification process of CoQ10 is more expensive than the production/fermentation of CoQ10, and toxic solvents are commonly used [1]. These factors were the driving force for the development of the SapB-IMAC affinity system to purify CoQ10. In this study, *S. johnsonii* was selected as the strain to study the production of CoQ10. While *S. johnsonii* has been proven as a high natural CoQ10 producer, it also produces a triacyl glyceride (TAG) as a fermentation byproduct [2]. TAG are major component of membranes; this hydrophic association makes the purification of CoQ10 from *S. johnsonii* difficult and expensive [3]. We have already demonstrated the SapB resin has the ability to bind with CoQ10 in solution. However, the SapB resin has not been used to purify CoQ10 from an actual fermentation extract.

6.2 Cyrstallization of CoQ10 bound with SapB

There is no crystal structure of CoQ10 either as a free molecule or bound in any protein. The crystal structure of SapB is known [4]. It would be interesting to have a crystal structure of SapB binding CoQ10, which would provide insight into the SapB and CoQ10 binding mechanism.
6.3 Genetic engineering of the yeast *Sporidiobolus johnsonii* for Coenzyme Q10 production

We have recently shown that the yeast, *S. johnsonii* is a high natural producer of CoQ10 (10 mg CoQ10 per gram dry cell weight), making it the highest known yeast producer of CoQ10. This increased CoQ10 production was achieved with the addition of a biosynthetic intermediate, para-hydroxy benzoic acid (HBA) [2]. The CoQ10 yield is the highest reported to date by any yeast, and was accomplished initially with no genetic manipulation. It would be interesting to genetically modify the yeast *S. johnsonii* to investigate the production of CoQ10 as compare to the non-genetic modify strain. In order to focus on the best place for genetic manipulation, the biosynthetic pathway (Figure 1), of CoQ10 must be considered [5]. CoQ10 is produced from HBA and isoprene units. For CoQ10 production in *S. johnsonii*, HBA is added directly to the fermentation media, which makes genetic manipulation of the HBA biosynthesis unlikely to result in a significant increase in CoQ10 production. The other portion of CoQ10, the prenyl tail, whose production is a major limiting factor in CoQ10 production. The decaprenyl tail is produced from individual prenyl units. *S. johnsonii* also produces carotenoids (β-carotene, torulene, and torularhodin) from the individual isoprene units. Reduction of carotenoid production through gene knock-out will increase the available isoprene units for CoQ10 production. Increased utilization of the isoprene units will be accomplished by over-expressing the COQ2 gene, encoding para-hydroxybenzoate-polyprenyl transferase, which catalyzes that final steps of CoQ10 biosynthesis [6]. Coupling this isoprene availability with
increased isoprene utilization will increase CoQ10 production in *S. johnsonii*.

![Diagram of biosynthetic pathway for CoQ10]

Figure 1. Biosynthetic pathway for CoQ10. *p*HBA is added to the media. PS = phytoene synthase. Coq2 = Coq2 enzyme.

### 6.3.1. Knocking out PS

The first committed step towards carotenoid biosynthesis is formation of phytoene by phytoene synthase (PS)[7]. Knock out of PS will result in complete elimination of carotenoids. Elimination of carotenoids production should not result in large negative side effects as carotenoids are primarily thought to function as photo and oxidation protection. CoQ10 also acts as a photo and oxidation protection, and should perform the same function. The initial strain used is designated Sj0801. The PS knock out strain will be named SjPS\(^i\). After successful generation of the SjPS\(^i\) strain, the SjPS\(^i\) will be fermented in the bioreactor and evaluated for CoQ10 content. Results from the SjPS\(^i\) strain will be compared to previous results from Sj0801.
6.3.2 Over-expressing COQ2

With more isoprene units available for CoQ10 production (a result of the PS knock out), it will be desirable to make *S. johnsonii* more efficient at using the isoprene units. A decaprenyl pyrophosphate synthase (dpps) takes the individual isoprene units and elongates them into the decaprenyl unit. Then a polyisoprenyl transferase, known as COQ2, then covalently attaches the decaprenyl and the HBA [6]. This ligation of the HBA and prenyl tail is considered a major bottleneck in biosynthesis of CoQ10. Overexpression of the COQ2 enzyme will result in increased CoQ10 production in *S. johnsonii*, especially given the isoprene and HBA availability previously established. The SjPS\textsuperscript{J} strain will be used for the insertion of the COQ2 gene, resulting in a new *S. johnsonii* strain to be designated SjPS\textsuperscript{J}COQ2\textsuperscript{+}.

6.3.3 Expected outcomes and potential pitfalls

By increasing the availability of prenyl units and the utilization of those prenyl units by COQ2, the yield of CoQ10 will greatly increase. Also, the genetic manipulation proposed here will permit efficient production of CoQ10 with shortened fermentation time and reducing expense. One possible negative effect of the PS gene knock out (reduced carotenoid production) is the possibility of oxidative stress, photo\textsuperscript{J}damage, and overall lower cell viability. These problems can be overcome by fermenting in the dark, as carotenoids have been shown unnecessary in dark growth conditions\textsuperscript{10}. Additionally, antioxidants can be added to the media in order to provide added protection and increased cell viability. The
gene knock out process can be difficult. If no quick results are obtained from gene
knock out, then we will switch to over expressing the COQ2 gene first as our
group has experience in overexpressing recombinant proteins in yeast.
References:


Summary

CoQ10 plays a key role in energy production in humans. Cells require a great deal of energy and are very sensitive to CoQ10 levels. CoQ10 has great clinical potential in diseases such as Parkinson's, chronic heart failure, and Ménière's. Statins also lower CoQ10 levels, so CoQ10 supplementation is often required. CoQ10 is in great demand, but producing it on an industrial scale presents some challenges. Currently, production is via bacterial or yeast fermentation. High-performance liquid chromatography is the purification method of choice, but the co-elution of the fatty acid triacylglycerol makes it difficult and expensive. The aim of my work was to develop a protein-binding purification method for CoQ10 by taking advantage of the body’s natural CoQ10-binding protein (SapB), which binds CoQ10 at selective pH values.

My project was broken down into three stages: (1) the expression and purification of SapB, (2) the characterization of SapB, and (3) the assay studies of CoQ10 and SapB binding. First, the pET27b+_SapB plasmid was constructed and transformed into E. coli for expression. SapB expression was performed by means of fermentation, where the pH was maintained at 7, dissolved oxygen was maintained at 35%, and glucose was added to the culture before it was induced with isopropyl β-D-1-thiogalactopyranoside. After 24 hours of fermentation, the culture was harvested. Second, SapB was purified by immobilized metal affinity chromatography (IMAC), using a Ni^{2+} IMAC column. The sample was bound to the column in 0.5 M NaCl, 20mM PBS, at pH 7.5 and eluted with 0.5 M NaCl, 20
mM PBS, and 250 mM imidazole at pH 7.5. The yield of SapB was 100mg/L. The bound fractions were then run on a SDS-PAGE gel. Upon staining with comassie stain, a 12kDa band was shown and excised for trypsin digestion, followed by MALDI-MS. A non-trypsin digested MALDI-MS was conducted. Studies of the structure of the recombinant SapB were also performed, using circular dichromism (CD), melting CD up to 90°C, and CNBr-based MALDI-MS to localize disulfide bonds.

In the third stage, CoQ10 was added to the SapB-bound IMAC resin at pH 5 and rotated for 30 minutes. The resin was then equilibrated to pH 9 to elute the CoQ10. All the washed fractions were combined, analyzed and quantified for CoQ10 by HPLC. The eluted fraction was also analyzed and quantified via HPLC.

We successfully demonstrated that SapB could be used to construct a reusable solid affinity matrix for CoQ10 purification in a pH-dependent manner, which is a great improvement over the current CoQ10 purification methods used by industry. I was part of this project for two years, and I am the second author of a 2011 paper that was accepted by the journal *Analytical Biochemistry*. In addition, this work was presented at two conferences: at the Undergraduate Research Symposium of the 4th Annual Western New York-American Chemical Society Conference, and at Syracuse University’s Spring 2011 McNair Colloquium.
Since Spring of 2011, I have been working on a project, funded by an Arnold and Mabel Beckman Scholarship, to genetically engineer a specific yeast, \textit{S. johnsonii} to increase production of CoQ10. We have recently shown that the yeast, \textit{S. johnsonii} is a high natural producer of CoQ10. The increased CoQ10 production was achieved with the addition of a biosynthetic intermediate, \textit{para-hydroxybenzoic acid} (HBA), was accomplished with no genetic manipulation. The hypothesis of my new project is that CoQ10 production can be increased through genetic manipulation of \textit{S. johnsonii}. In order to focus on the best place for genetic manipulation, the biosynthetic pathway of CoQ10 must be considered. CoQ10 is produced from HBA and isoprene units. For CoQ10 production in \textit{S. johnsonii}, HBA is added directly to the fermentation media, meaning genetic manipulation concerning the HBA will not result in a significant increase in CoQ10 production. The other portion of CoQ10 is the prenyl tail and production is a major limiting factor in CoQ10 production. The decaprenyl tail is produced from individual prenyl units. \textit{S. johnsonii} also produces carotenoids from the individual isoprene units. Reduction of carotenoid production through gene knock\textbullet out will increase the available isoprene units for CoQ10 production. Increased utilization of the isoprene units will be accomplished by over\textbullet expressing the COQ2 gene, encoding \textit{para-hydroxybenzoate-polyprenyl transferase}, which catalyzes that final step of CoQ10 biosynthesis. Coupling this isoprene availability with increased isoprene utilization will increase CoQ10 production in \textit{S. johnsonii}. 
This project is broken down into three steps. First, the genomic DNA of *S. johnsonii* was isolated and sent to Aptmatrix, NY for sequencing. Second, the gene that is responsible for the carotenoid production will be knocked-out through zinc finger nuclease technology. Finally, the COQ2 gene will be cloned into an expression vector and transformed into the cell for expression.