

**Cloning and Overexpression of Yeast
Cystathionine γ -Lyase**

by

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Submitted in Partial Fulfillment of the Requirements
for the Degree of
Masters of Science
in the
Chemistry
Program

YOUNGSTOWN STATE UNIVERSITY

December, 2012

Cloning and Overexpression of Yeast Cystathionine γ -Lyase

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Thesis Abstract

Cystathionine γ -lyase (CGL) is a protein involved in the conversion of methionine to cysteine as part of the trans-sulfuration pathway. Inactivation of the enzyme, possibly by oxidation of reactive site residues, has been linked to cataractogenesis. This research isolated the cystathionine γ -lyase gene (*CYS3*), modified it with an XhoI and BlnI restriction site and enterokinase sequence (creating a modified *CYS3*, *mCYS3*, gene), and ultimately spliced it into a pET-15b plasmid to create a recombinant plasmid. Then transformed the recombinant plasmid into BL21 *E. coli* cells. This enabled the production of bacterial cells capable of generating μ g amounts of CGL for future metal catalyzed oxidation (MCO) research.

Acknowledgments

I would like to start by thanking my wife, Sondra Nicole Raby, who has exhibited extraordinary patience as she helped and supported me throughout my pursuit of my Master's degree. Without her, I never would have been able to accomplish all that I've done here.

I would have been unable to get this procedure completed if it were not for the aid and advice of my adviser, Dr. Michael Serra, who has shown more patience with this experimental procedure than I thought a human being could exhibit. Thanks as well to Drs. Mincey and Balendiran for agreeing to serve on my thesis committee, reviewing my thesis and attending my defense.

Thanks also belong to my lab mates, particularly Cory Muraco and Nikki Patton. They've provided a great deal of help over the past two years, from aid in learning where all the equipment was located and how to use it to moral support then things went wrong.

I have also received immense amounts of help from Julio 'Ed' Budde in the Department of Biological Sciences in running the sequencing reactions on my samples. I am also thankful to Dr. Heather Lorimer of the Department of Biological Sciences for the yeast genome sample that she provided. It was an invaluable help in isolating the gene for my research.

I appreciate the financial support from the YSU School of Graduate Studies, and thank them for that support. Lastly, I would like to thank the faculty and staff of the Chemistry Department at Youngstown State University. They have proven invaluable as I've done the research described as follows, and enabled me to accomplish as much as I have these past few years.

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List of Symbols and Abbreviations

ATP.....	adenosine triphosphate
CGL.....	cystathionine γ -lyase protein
CYS3.....	cystathionine γ -lyase-3 gene
DNA.....	deoxyribonucleic acid
EDTA.....	ethylenediaminetetraacetic acid
Fe.....	iron
g.....	gravity
H ₂ S.....	hydrogen sulfide
H ₂ O ₂	hydrogen peroxide
HO•.....	hydroxyl radical
IPTG.....	isopropyl- β -D-thiogalactopyranoside
LB.....	Luria-Bertani
LOOH.....	lipid peroxide
mCYS3.....	modified cystathionine gamma lyase
mM.....	millimolar
NADPH.....	nicotinamide adenine dinucleotide phosphate
NSAIDs.....	non-steroidal anti-inflammatory drugs
NO.....	nitrous oxide
O ₂ ^{•-}	superoxide radical
PCR.....	polymerase chain reaction
RNA.....	ribonucleic acid
ROS.....	reactive oxygen species
RPM.....	rotations per minute
SDS-PAGE.....	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC.....	super optimal broth with catabolite repression

CHAPTER I: INTRODUCTION

Reactive oxygen species (ROS) include both free radicals such as the superoxide radical ($O_2^{\cdot-}$) and the hydroxyl radical (HO^{\cdot}) and non-radicals such as hydrogen peroxide (H_2O_2) and lipid peroxides (LOOH). These species can be generated through endogenous means such as aerobic respiration or taken in from external sources.¹ There are numerous biological activities that produce ROS, such as the production of superoxide radicals during NADPH oxidase reactions and as a result of activities including immune cell activation, inflammation, and aging.² When such ROS are either generated inside the body or taken internally, there is the possibility for oxidative damage to biological molecules.

One of the major negative effects of such oxidants is to greatly decrease the efficiency of proteins within the body, both in the short term and through potential cumulative effects over the long term.³ Oxidative species are able to modify the structure of individual amino acids and correspondingly alter the overall tertiary structure of proteins. The areas affected by these oxidative conditions are determined by the nature of the oxidant and the structure and properties of the protein being oxidized. Some research has found that histidine and lysine are particularly susceptible to oxidation, suggesting areas within affected proteins to look for possible oxidative damage.⁴

These oxidative effects have been linked to numerous diseases and other biological problems. Some physiological problems linked to oxidized proteins include premature aging, Alzheimer's disease, Parkinson's disease, and Huntington's disease.⁵ The association between the production of ROS and various diseases have made the study of oxidative damage an area of research interest for years.⁶ Further

research of ROS may lead to the discovery of methods to counteract their oxidative effects and minimize the damage that they cause.

Reactive Oxygen Species and Free Radicals

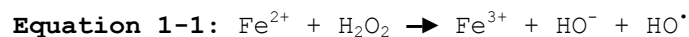
Free radical ROS are particularly damaging to biological systems, with the hydroxyl radical being the most reactive. Free radicals are highly reactive chemical systems containing a single, unpaired electron.⁷ These oxidants are harmful to the body and undesirable for living entities. Free radicals abstract electrons from molecules, generating new free radicals. This continues until two free radicals react or the free radicals are eliminated by antioxidant reactions in the organism.

This level of reactivity presents numerous problems. The generation of free radicals can lead to structural damage by reacting with atoms found in proteins, DNA, RNA, and other biologically important molecules. These reactions, including the breaking of existing atomic bonds and the formation of new bonds, can lead to alteration of those molecules and decreases in their function. These alterations can range from superficial changes to rendering protein completely non-functional, leading to numerous problems within living organisms exposed to such free radicals.

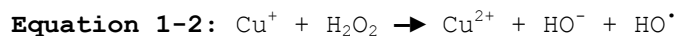
Metal Catalyzed Oxidation of Proteins

One of the most important mechanisms leading to the oxidation of proteins is metal catalyzed oxidation (MCO). This method of oxidation relies on an endogenous metal to serve as an electron donor or acceptor in order to induce the formation of a free radical or other ROS.⁸

One of the ways in which these MCO reactions occur in living systems is through the Fenton reaction⁹, where hydrogen peroxide reacts with Fe(II) to generate Fe(III) and the highly reactive hydroxyl radical, HO[•] (Equation 1-1).



Similar reactions are possible with the Cu(I) ion¹⁰, which can also react with hydrogen peroxide in order to generate the Cu(II) ion and the hydroxyl free radical (Equation 1-2).



The hydroxyl radical produced by both reactions is a particularly powerful reagent, causing oxidative damage to proteins, DNA, and lipids. In enzymes, this can lead to the loss of the enzyme's catalytic activity, the formation of protein-protein cross-links, peptide bond cleavage, and increased susceptibility to proteolysis.¹¹

Not all oxidative damage is the result of MCO systems. Research by Nappi and Vass demonstrated how nitric oxide could serve as a donor to the hydrogen peroxide molecule to generate the hydroxyl radical.¹² While such alternative methods are possible, many oxidation events are linked to MCO conditions, making this an important area of research.

Antioxidants in the Body

Antioxidants are small molecules or enzymes that minimize the harmful effects caused by ROS to biological molecules.¹³ One such antioxidant is ascorbic acid (also known as Vitamin C), which is a mild reducing agent. It is capable of reacting with a ROS to generate its own stable radical ion, the ascorbyl radical, which is stabilized by conjugated pi orbitals in its structure. The ascorbyl radical can then donate an electron to another radical species, generating the dehydroascorbate molecule. Overall, two ROS are neutralized by the ascorbic acid reactions (Figure 1-1).

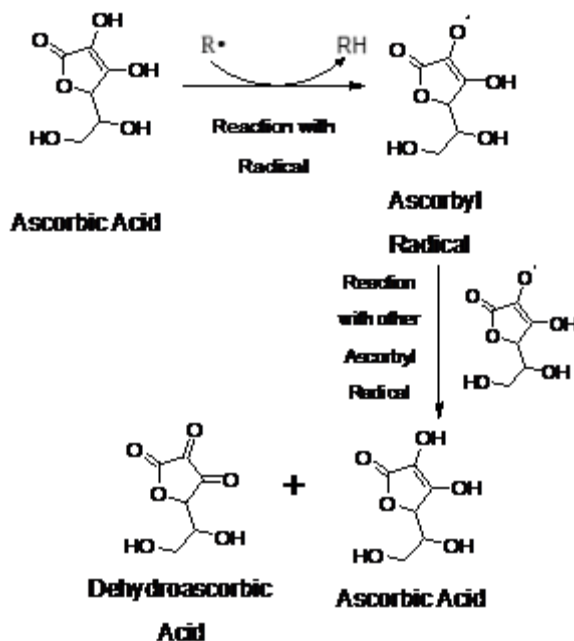
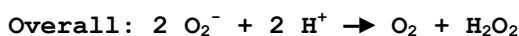
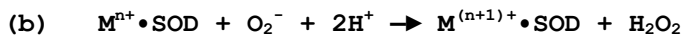
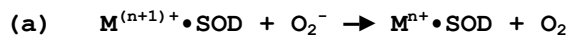


Figure 1-1: Ascorbic acid reactions to eliminate free radicals

Another antioxidant, superoxide dismutase, converts two superoxide anions into oxygen and hydrogen peroxide molecules through a pair of half reactions (See Equation 1-3). In step (a), the oxidized metal cofactor of the superoxide dismutase is reduced by a one electron transfer from one superoxide ion. In step (b), the reduced metal cofactor is oxidized again while converting another superoxide and two protons into a hydrogen peroxide molecule. Because of the reaction of superoxide dismutase with the superoxide anions, the potential damage of the anions is limited, as the anions have less opportunity to react with and damage other molecules or tissues within the cell.

Equation 1-3: The Superoxide Dismutase Reaction



Where M can be Cu (n = 1), Mn (n = 2), Fe (n = 2) or Ni (n = 2)

Glutathione, (Figure 1-2), is another commonly encountered antioxidant. Glutathione is a tripeptide composed of cysteine, glutamate and glycine, and it serves many purposes in the body, including regulation of the nitric oxide cycle and iron metabolism.¹⁴ Cysteine's thiol group, found within glutathione, serves as the reducing agent, enabling the glutathione to react with and neutralize oxidizing agents in the body.

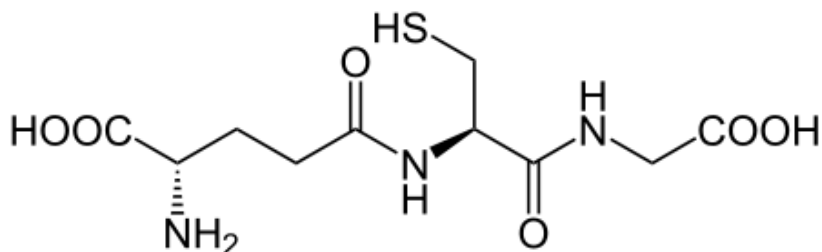


Figure 1-2: Glutathione, an important biological molecule in anti-oxidant reactions.

Glutathione functions as an antioxidant by reducing hydrogen peroxide and organic peroxides to water and an alcohol. Two molecules of glutathione are converted to glutathione disulfide in the process (Figure 1-3).

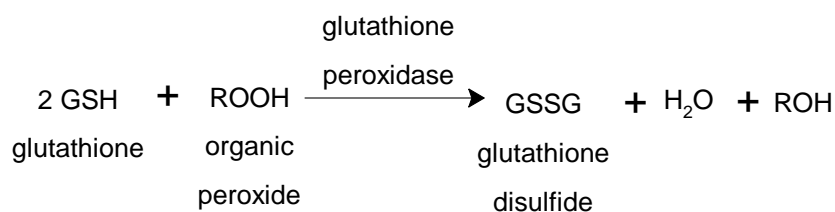


Figure 1-3: The antioxidant reaction involving glutathione and catalyzed by glutathione peroxidase.

This process prevents the transformation of peroxides to free radicals, which could cause damage to other biological molecules.¹⁵ Production of glutathione is important in keeping the level of peroxides to a minimum and preventing any damage from such peroxides from occurring.

The reverse reaction, the regeneration of glutathione from glutathione disulfide, is catalyzed by the enzyme glutathione reductase. This is one method used to maintain a steady supply of glutathione. As organisms age, their ability to regenerate glutathione by the glutathione reductase reaction decreases.¹⁶ Older organisms increasingly rely on the production of cysteine via the trans-sulfuration pathway, which converts the essential amino acid methionine to cysteine, and the subsequent generation of fresh glutathione. Given the nature of glutathione as a tripeptide, it is possible to decrease the level of glutathione production by lowering the levels of any one of the three amino acids that are required in its synthesis. By doing so, the amount of glutathione that is produced will be decreased, and the amount of oxidative damage that occurs would be subsequently increased.

Cystathionine γ -Lyase

Cystathionine γ -lyase (CGL) is part of the trans-sulfuration pathway that converts the amino acid methionine to cysteine. CGL catalyzes the final step in this pathway, specifically the conversion of the intermediate cystathionine into cysteine, with the production of α -ketobutyrate and ammonia as side products (Figure 1-4).

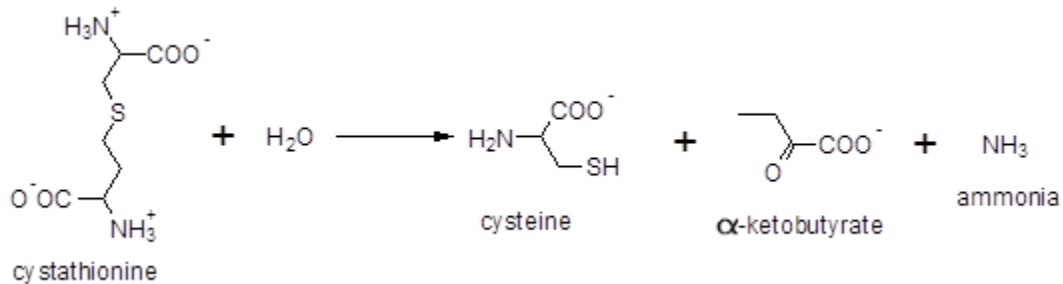


Figure 1-4: The reaction catalyzed by CGL, the conversion of cystathionine to cysteine, α -ketobutyrate, and ammonia.

CGL has been isolated from numerous species, including bacteria,¹⁷ yeast,¹⁸ and humans.¹⁹ In yeast and bacterial species, CGL is most often used in the reverse process, producing methionine from cysteine. In all species in which the enzyme has been isolated, each subunit is approximately 400 amino acids in length. The quaternary structure of CGL consists of four identical subunits²⁰ (a homotetramer), as shown in Figure 1-5. Each monomer requires one molecule of pyridoxal phosphate (PLP) as a cofactor.

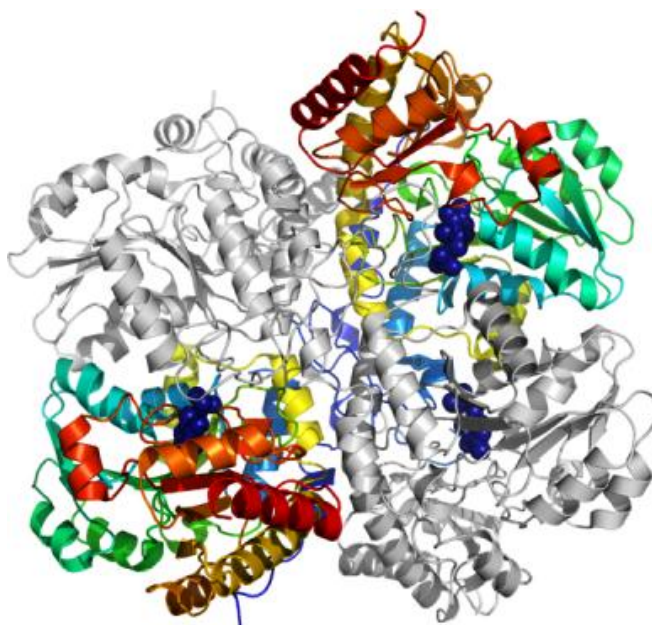


Figure 1-5: Quaternary Structure of Cystathionine γ -Lyase (CGL) from humans. CGL functions as a homotetramer and requires pyridoxal phosphate as a cofactor

The cofactor of CGL, PLP (Figure 1-6), is a derivative of vitamin B₆. Vitamin B₆ and its derivatives also serve other purposes in the synthesis and activity of glutathione. As shown in the simulations conducted by Nijhout *et al*, restricting the levels of vitamin B₆ in organisms led to decreased activity of the glutathione transporter protein and increased oxidative stress, increased amounts of ROS and decreased ability of the organism to handle them.²¹

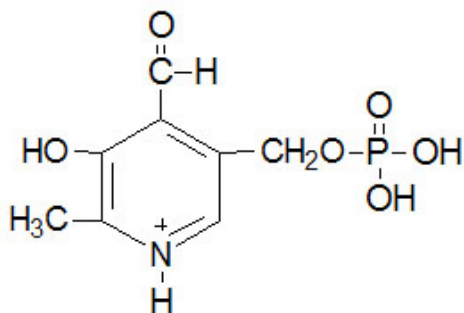


Figure 1-6: The structure of pyridoxal phosphate, the coenzyme required by CGL for its function.

CGL also catalyzes the generation of hydrogen sulfide (H_2S) (Figure 1-7). Because CGL is used to help modulate the production of sulfur containing proteins, it is used in the liver to help generate hydrogen sulfide, which serves as a neuromodulator *in vivo*.²² H_2S also serves to dilate blood vessels, serving a similar role to nitrous oxide (NO). By breeding mice that lacked the ability to generate CGL, it has been possible to more definitely link the activity of CGL to the dilation of blood vessels and the anti-inflammatory action currently being performed medically using non-steroidal anti-inflammatory drugs (NSAIDs).²³

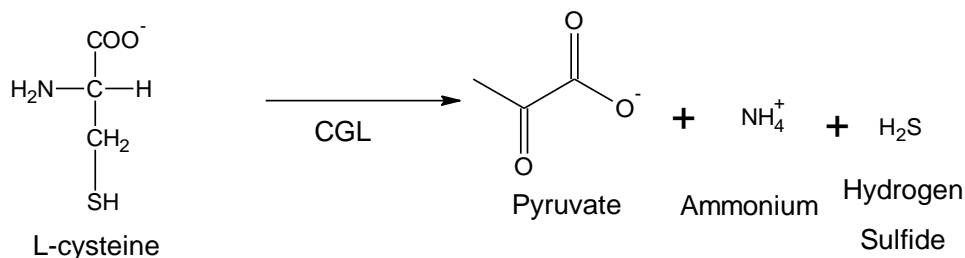


Figure 1-7: The CGL catalyzed reaction that generates Hydrogen Sulfide

A deficiency of CGL and the resulting decrease in the supply of cysteine is considered as a possible cause of glutathione depletion, as noted by the research of Diwakar and Ravindranath.²⁴ Such glutathione depletion is linked to several types of adverse conditions, including

surgical stress, cancer, and cystathionuria, a condition that is caused by the accumulation of cystathionine in the blood plasma.²⁵ There has also been increased speculation on the link between lowered cysteine and glutathione levels that result as animals and humans age, and the aging process itself. Conditions that are known to lower the glutathione level, such as fasting, lead to a slowing of the glutathione regeneration cycle in older animals, resulting in increased biological stress.²⁶

Cataractogenesis and Oxidative Damage

The loss of CGL through oxidative damage has been linked to cataractogenesis.²⁷ In the lens of the eye, there are proteins called crystallins. These proteins are not regenerated, and must last the lifetime of the organism. As organisms age, damage to the crystallins increase, due in part to the oxidation and subsequent destruction of enzymes that serve a role in preventing the formation of free radicals. Such enzymes include CGL and methionine sulfoxide reductases, which serves to catalyze the reduction of oxidized methionine residues to their normal, non-oxidized form.²⁸

One study looked specifically at aged rat eye lenses, noting the total loss of CGL activity in about half of the older rat population surveyed while the younger rats had substantially higher CGL activity and much lower levels of cataractogenesis. Sastre *et al* proposed that the amino acid residues in the active site of CGL were damaged by the free radicals, leading to the loss of activity, and connected the declining activity in older rats to their higher cataractogenesis development rates.²⁷ It was noted that as organisms age, increasing amounts of the CGL enzymes are oxidized and destroyed by the body's other anti-oxidative mechanisms, decreasing the amount of cysteine

present in the body. With less cysteine available, glutathione production declines, and a downward cycle commences. With lower amounts of cysteine, less glutathione can be produced, and the rate of antioxidant reactions involving glutathione is lessened. Ultimately, the lowered amounts of CGL result in increasing oxidative damage and in this particular case, more damage to the crystallin proteins.

Statement of Research Problem

This research is focused on the enzyme Cystathionine β -Lyase (CGL), also known as cystathionase. CGL will be expressed to test its sensitivity to Reactive Oxygen Species (ROS) systems in the body, to test a hypothesis about such ROS and cataractogenesis. The research will be in the same vein as the research reviewed by Stadtman and Levine, to determine how oxidative environments can affect the structure and function of proteins.²⁹ CGL will ultimately be exposed to a metal catalyzed oxidation (MCO) system to determine where the oxidative damage occurs in CGL and what effect that oxidative damage has on the enzyme's activity.

Chapter II: Materials and Methods

Materials

The primers used in this procedure were all prepared by Integrated DNA Technology, Inc., in Coralville, IA. The GoTaq[®] Green Master Mix was obtained from Promega. The Cyclo-Pure[™] Agarose Gel-Extraction Kit, the 2X Protein Dye Loading Buffer, Blue BANDit Protein Stain, Coomassie[®] Brilliant Blue R-250, 25X TAE Buffer Liquid Concentrate, tryptone, isopropyl- β -D-thiogalactopyranoside (IPTG), yeast extract, TE buffer, agar, electrophoresis buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS, pH 8.3-8.7), and streptomycin sulfate were all purchased from Amresco, Solon, OH. The ethidium bromide monoazide, ampicillin sodium salt, and sodium chloride were obtained from Sigma Aldrich. The EcoRI restriction enzyme, the EcoRI buffer, the XhoI restriction enzyme, the BlnI restriction enzyme, the NEBuffer 4, the Quick Ligation Kit, Bovine Serum Albumin (BSA) 10 mg/mL and the BL21 Chemically Competent Cells were all obtained from New England Biolabs. The TOPO[®] TA Cloning[®] Kit for Sequencing (with pCR[®]4-TOPO[®] vector), the One Shot[®] TOP10[™] Chemically Competent *E. coli* cells, the One Shot[®] Mach 1[™] Chemically Competent *E. coli* cells and the SOC (Super Optimal broth with Catabolite suppression) media were all obtained from Invitrogen. Beckman-Coulter was the source of the Genome Lab[™] DTCS Quick Start Kit and the CEQ 2000 XL DNA Analysis System. The Quick Start Master Mix, QIAprep Miniprep Kit, and the Buffer EB were all obtained from Qiagen. The Mini-Protean TGX 12% Gel and the Precision Plus Standards molecular weight marker were both obtained from Biorad. Amicon[®] was the source of the Ultra-4 Centrifugal Filter Devices. The LB plates were obtained from Fischer Scientific. The yeast genome was prepared by Dr. Heather Lorimer of the Department of Biological Sciences at YSU.

Methods

Section 1: Isolation of the CYS3 Gene from the Yeast Genome

To isolate the *CYS3* gene from the yeast genome, the primers CYS3F and CYS3R (Figure 2-1) used in the reaction were diluted to a concentration of 10 μM using Buffer EB (10 mM Tris-Cl, pH 8.5). The yeast genome used as the template had an initial concentration of 1 $\mu\text{g}/\mu\text{L}$ and was diluted with EB Buffer to 100 $\text{ng}/\mu\text{L}$, 10 $\text{ng}/\mu\text{L}$, and 1 $\text{ng}/\mu\text{L}$ yeast DNA solutions.

CYS3F: 5'- CAC ACA AGA CAA AAC CAA AAA-3'

CYS3R: 5'- AAG GCA GAG ACG TGG CAC T-3'

Figure 2-1: Sequences of CYS3F and CYS3R primers

The three PCR reactions mixtures were prepared by mixing 12.5 μL GoTaq[®] Green Master Mix, 1 μL CYS3F, 1 μL CYS3R, 2 μL of each yeast genome DNA Template dilution and 8.5 μL sterile water. The samples were run using program CYS3 on the TC-312 thermal cycler (Techne[®] model P-6200-252). After an initial heat step at 95°C for two minutes, the samples then went through thirty cycles of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 48°C, and 80 seconds of extension at 72°C. Following the thirty cycles, the samples were held at 72°C for fifteen minutes for a final extension step before being kept at 4°C until collected.

The samples were analyzed on a 1% agarose gel run for 52 minutes at 85 V. The gel was then soaked in a solution of 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide dissolved in 1X TEA Buffer. The PCR reaction was then scaled up to 50 μL using 25.0 μL GoTaq[®] Green Master Mix, 2.0 μL CYS3F primer (10 μM), 2.0 μL CYS3R primer (10 μM), 4.0 μL of yeast genome DNA Template at 10 and 1 $\text{ng}/\mu\text{L}$, and 17.0 μL sterile water. The *CYS3* gene was amplified using program CYS3 as before.

The samples were analyzed on a 1% agarose gel that was run for 60 minutes at 65 V. The gel was soaked in a solution containing 0.5 µg/mL ethidium bromide dissolved in 1X TEA Buffer, and the bands at ~1300 bp were extracted from the gel and isolated using the Cyclo-Pure™ Agarose Gel-Extraction Kit, following the manufacturer's procedure. The extracted gene samples were characterized by a 1% agarose gel as before, to determine the concentration of the isolated genes.

To verify that the isolated gene was indeed the CYS3 gene, an EcoRI digest was performed. The EcoRI digest was prepared by mixing 2 µL of each isolated CYS3 gene sample (~40 ng/µL), 2 µL BSA solution (at a 1 mg/mL concentration), 2 µL EcoRI buffer solution, 1 µL EcoRI enzyme, and 13 µL sterilized water. The samples were incubated for 1 hour at 37°C. The digest was analyzed on a 1% agarose gel as on page 13.

Section 2: Addition of Restriction Sites by the PCR Overlap Extension Method

The isolated CYS3 gene was modified by the addition of an XhoI restriction site and enterokinase sequence to the 5' end and a BlnI restriction site to the 3' end. This was done by using the CGL 5' and CGL 3'-BLPI primers (Figure 2-2).

CGL5':5'-CAT ATG **CTC GAG** GAC GAC GAC GAC AAG ATG ACT CTA CAA GAA TCT-3'

CGL 3'-BLPI: 5'- AGC CGG ATC **GCT CAG CTT** AGT TGG TGG CTT G -3'

Figure 2-2: Sequences of CGL 5' and CGL 3'-BLPI primers. The bold portions are the XhoI site (CTC GAG) in the upper sequence, and the BlnI site (GCT CAGC) in the lower sequence. The underlined sequence is the enterokinase sequence (GAC GAC GAC GAC AAG).

A PCR mixture was prepared for both isolated gene samples, consisting of 1 µL of each isolated CYS3 gene sample (with a concentration of approximately 40 ng/µL), 1 µL CGL 5' primer (diluted to 10 µM with EB buffer), 1 µL CGL 3'-BLPI (diluted to 10 µM with EB

buffer), 10 μ L GoTaq[®] Green Master Mix, and 7 μ L sterile water. The samples were run on the thermal cycler in a two cycle method named CGLBLP, beginning with an initial denaturation step at 95°C for 2 minute. The first cycle consisted of five rounds of a denaturation step at 95°C for 1 minute, an annealing step at 32°C for 30 seconds, and an extension step for 30 seconds at 72°C. The second cycle consisted of thirty rounds, each beginning with a denaturation step at 95°C for 1 minute, an annealing step of 66°C for 30 seconds, and an extension step at 72°C for 80 seconds. A final extension step at 72°C for 10 minutes was performed after the completion of all thirty cycles. The resulting samples were visualized on a one percent agarose gel as on page 13.

Scaled-up PCR mixtures were prepared for both gene samples, composed of 3 μ L of each unaltered CYS3 gene template (approximately 40 ng/ μ L), 3 μ L CGL 5' primer (10 μ M), 3 μ L CGL 3'-BLPI (10 μ M), 25 μ L GoTaq[®] Green Master Mix, and 16 μ L sterile water. The samples were run in the thermal cycler using the CGLBLP program as before. The resulting samples were characterized on a one percent agarose gel as on page 13.

The band at about 1300 bp of each sample was extracted and isolated using the Cyclo-Pure[™] Agarose Gel-Extraction Kit as on page 13, yielding 50 μ L of each of the two samples, the modified CYS3 gene, *mCYS3*. The concentration of the isolated genes was determined to be 40 ng/ μ L, via a run on a 1% agarose gel as on page 13.

Section 3: Cloning of mCYS3 into pCR[®]4-TOPO[®]..

The *mCYS3* gene was spliced into the pCR[®]4-TOPO[®] plasmid to generate the *mCYS3/pCR* recombinant plasmid. The concentration of the isolated *mCYS3* gene was estimated to be 40 ng/ μ L. From this

concentration, the amount of *mCYS3* gene needed for splicing was calculated to be:

(Concentration Of Plasmid)	(Size Ratio Of Gene and Plasmid)	(Molar Ratio of Gene to Plasmid)	(Concentration of Gene)	
10 ng PCR [®] 4-TOPO [®]	1.3 kb <i>mCYS3</i>	6 moles <i>mCYS3</i>	1 μ L <i>mCYS3</i>	= ~0.5 μ L <i>mCYS3</i>
1 μ L PCR [®] 4-TOPO [®]	4.0 kb PCR [®] 4-TOPO [®]	1 mole PCR [®] 4-TOPO [®]	40 ng <i>mCYS3</i>	

The TOPO[®] TA Cloning reaction solutions were prepared by mixing 0.5 μ L (approximately 20 ng) of each of the *mCYS3* gene solutions, 3.5 μ L sterile water, 1.0 μ L salt solution (provided in the cell transformation kit; consisting of 1.2 M NaCl and 0.06 M MgCl₂ in sterile water), and 1.0 μ L TOPO[®] vector (10 ng/ μ L). The reaction mixture was incubated at room temperature for 5 minutes. 2 μ L of each reaction mixture was transferred to a vial of One Shot[®] TOP10[™] Chemically Competent *E. coli* cells. The vials were incubated on ice for 10 minutes. They were then heat shocked at 42°C for 30 seconds. The vials were then returned to ice immediately and kept on ice for 5 minutes. To each tube, 250 μ L of room temperature SOC media (containing 2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) was added. The tubes were shaken horizontally for one hour at 37°C. From each tube, 20, 60 and 100 μ L aliquots were obtained and spread on Luria-Bertani (LB) plates (0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) tryptone and 1.5% (w/v) agar) with ampicillin (0.1 mg/mL) and were incubated at 37°C overnight.

Several colonies from each plate were selected, and each colony was transferred to a fresh LB and ampicillin plate and grown overnight at 37°C. One colony from each of these plates that showed growth was transferred with a sterile toothpick into 2 mL aliquots of LB broth

(containing 0.5% (w/v) yeast extract, 1% (w/v) tryptone and 1% (w/v) tryptone) with ampicillin (0.1 mg/mL) and grown overnight at 37°C with shaking.

Six colonies were selected and the plasmids from the overnight growth were isolated according to the QIAprep Miniprep Kit protocol. Each of the isolated *mCYS3*/pCR plasmids were characterized on a 1% agarose gel as on page 13. To confirm that each of the plasmids contained the proper-sized gene, digest mixtures were prepared for each of the isolated plasmids, combining 1 μ L (approximately 30 ng/ μ L for each plasmid) of the *mCYS3*/pCR plasmids, 1 μ L BSA (10 mg/mL), 2 μ L NEBuffer 4, 1 μ L XhoI restriction enzyme, 1 μ L BlnI restriction enzyme, and 14 μ L sterilized water. These digests were incubated at 37°C for one hour. The digests were characterized on a 1% agarose gel as on page 13. The sample from colony G, *mCYS3_pCR_G*, showed bands appropriate in size to be both the pCR[®]4-TOPO[®] plasmid and *mCYS3* gene.

Section 4: Sequencing of CYS3 in *mCYS3*/pCR

The isolated *mCYS3*/pCR from colony *mCYS3_pCR_G* served as a template for sequencing. The sequencing solutions were prepared as shown in Table 2-1 using the Genome Lab[™] DTCS Quick Start Kit from Beckman-Coulter. The samples were run in the thermal cycler according to a thirty cycle program consisting of a 20 second denaturation step at 96°C, a 20 seconds annealing step at 50°C, and a 4 minutes extension step at 60°C.

Table 2-1: Gene Sequencing Sample Mixtures, *mCYS3*/pCR Samples (All volumes expressed in μ L)

Sample Number	1	2	3
<i>mCYS3</i> /pCR plasmid (20 ng/ μ L) <i>mCYS3_G TOP10</i>	8.0	0	8.0
Distilled H ₂ O	2.0	9.5	8.0
pUC-18 Template	0	0.5	0
Primer T3	2.0	0	0
Primer T7	0	0	2.0
-47 Sequencing Primer	0	2.0	0
DTCS Master Mix	8.0	8.0	8.0

15 μL of the stop solution/glycogen mixture was prepared from 6 μL of 3M sodium acetate, 6 μL of 100 mM $\text{Na}_2\text{-EDTA}$, and 3 μL of 20 mg/mL glycogen. 5 μL of this solution was transferred to each of three labeled 0.5 mL microcentrifuge tubes. The PCR mixtures were transferred to the appropriately labeled tubes and mixed thoroughly, at which point 60 μL of -20°C 100% ethanol was added to each tube. The tubes were centrifuged at 13,000 rpm for 15 minutes at 4°C . The ethanol was then removed from each sample via pipette, and the pellet was rinsed twice with 200 μL of -20°C 70% ethanol in water. Following each rinse, the tubes were centrifuged for 2 minutes at 13,000 rpm at 4°C and the supernatant was carefully removed via pipette. After the second rinse, the solutions were vacuum-dried for 10 minutes, and dissolved in 40 μL of sample loading solution. The samples were analyzed by a Beckman-Coulter CEQ 2000 running a LFR-105 protocol.

Section 5: Isolation of the mCYS3 Gene

The plasmid digestion was scaled up to 50 μL to isolate the modified gene. A 10 μL (20 ng/ μL) portion of the *mCYS3/pCR* plasmid (Sample *mCYS3_pCR_G*) was mixed with 5 μL NEBuffer 4, 5 μL BSA (10 mg/mL), 2 μL XhoI restriction enzyme, 2 μL BlnI restriction enzyme, and 26 μL of sterilized water. The sample was incubated at 37°C for one hour. The digest was examined with a 1% agarose gel as on page 13, and the gene was isolated via the Cyclo-Pure™ Agarose Gel-Extraction Kit as described on page 13.

Section 6: Isolation and Double Digestion of pET-15b plasmid

The plasmid chosen for gene expression was pET-15b (Figure 2-3). The pET-15b plasmid is known for its use in gene expression, particularly within the BL21 *E. coli* strain. The pET-15b system was chosen because it is a source of the T7 transcription sequence, which

can be triggered by T7 RNA and leads to the production of a high level of the desired protein. The pET-15b plasmid can be induced using IPTG, which will cause the bacteria containing the plasmid to generate sizable amounts of the desired protein, potentially generating up to 50% of the cell's protein content as the target protein. The pET-15b plasmid contains the selective marker for ampicillin, enabling the monitoring of the success of the plasmid transformation using ampicillin-containing broth and plates.

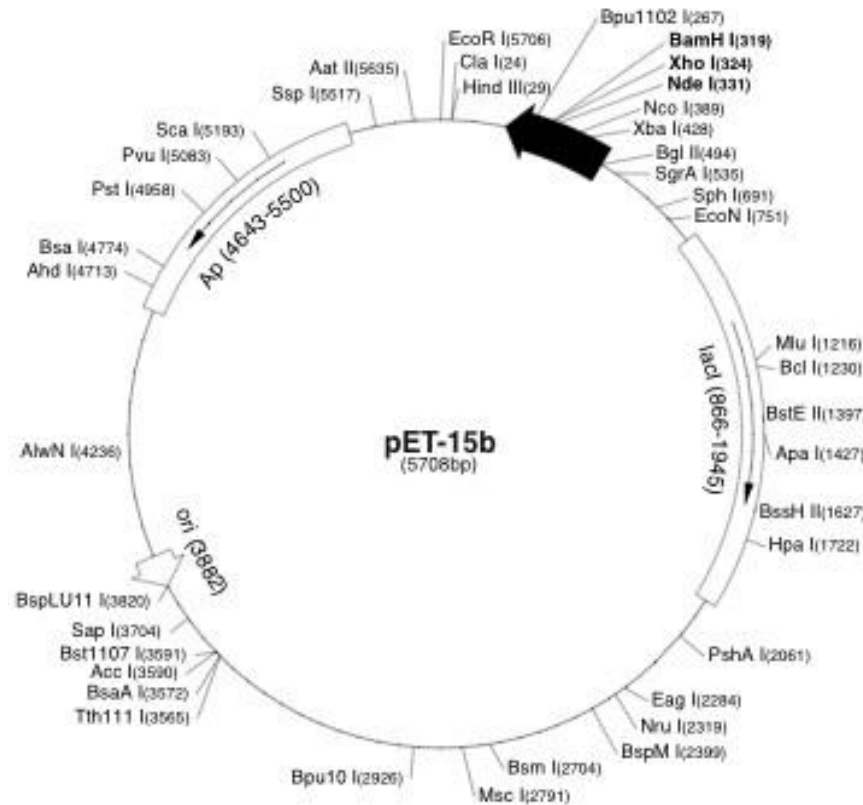


Figure 2-3: Structure of pET-15b, target plasmid for transformation

A sample of pET-15b plasmid at a concentration of 500 ng/ μ L was diluted to 50 ng/ μ L using TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). A 2 μ L aliquot of the 50 ng/ μ L pET-15b plasmid was added to an ice-cold vial of One Shot[®] Mach 1[™] Chemically Competent *E. coli* cells, which

then incubated on ice for 10 minutes. The cells were heat-shocked at 42°C for 30 seconds, then returned to ice immediately and kept on ice for 5 minutes. To the tube, 250 µL of room temperature SOC media was added, and the tube was shaken horizontally for one hour at 37°C. From the tube, 10, 30 and 50 µL aliquots were obtained and spread on LB plates with ampicillin (0.1 mg/mL). The plates were incubated at 37°C overnight.

Several colonies from each plate were transferred to fresh LB with ampicillin (0.1 mg/mL) plates, which were then incubated at 37°C overnight. One colony from each of the plates showing growth was transferred with a sterile toothpick into a test tube containing a 2 mL aliquot of LB with ampicillin (0.1 mg/mL) broth. These colonies were allowed to grow overnight at 37°C with shaking. Plasmids from the overnight growth were isolated via the QIAprep Miniprep Kit. These samples were examined using a 0.8% agarose gel run for 52 minutes at 85 V and stained with 0.5 µg/mL ethidium bromide in 1X TEA buffer.

The plasmids were digested with XhoI and BlnI to linearize the isolated plasmids. A pair of digests were prepared, taking 4 µL of the pET-15b plasmid solution (15 ng/µL), 2 µL of NEBuffer 4, 2 µL of BSA (10 mg/mL), 1 µL XhoI restriction enzyme, 1 µL BlnI restriction enzyme, and 10 µL of sterilized water. These samples were digested at 37°C for one hour, and then characterized with a 1% agarose gel as on page 13.

The double digest was scaled up, using 10 µL of the pET-15b plasmid from each sample (15 ng/µL), 5 µL NEBuffer 4, 5 µL BSA (10 mg/mL), 2 µL XhoI restriction enzyme, 2 µL BlnI restriction enzyme, and 26 µL sterile water. These solutions were digested at 37°C for one hour, and then characterized on a 1% agarose gel as on page 13. The digested plasmid was then extracted from the agarose using the Cyclo-

Pure™ Agarose Gel-Extraction Kit as on page 13. The extracted gene samples were characterized by a 1% agarose gel as on page 13.

**Section 7: Transfer of *mCYS3* Gene into *pET-15b* (*mCYS3/pET-15b*)
Plasmid**

The *mCYS3* gene was spliced into the doubly-digested *pET-15b* plasmid, creating the *mCYS3/pET-15b* recombinant plasmid. 3 µL of doubly-digested *pET-15b* (~15 ng/µL), 7 µL of isolated *mCYS3* gene (~20 ng/µL), 10 µL of 2X Quick Ligation Reaction Buffer, and 1 µL of T4 DNA Ligase (both included in the Quick Ligation Kit) were combined. The solution was centrifuged for 15 seconds, allowed to incubate at room temperature for 5 minutes, and then chilled on ice for ten minutes. Two sets of One Shot® Mach 1™ Chemically Competent *E. coli* cells were thawed on ice. To one set of cells, 2 µL of the ligation mixture was added, while 5 µL of ligation mixture was added to the other. Each set of cells was incubated on ice for 30 minutes, and then heat shocked at 42°C for 2 minutes. The solutions were chilled on ice for an additional 5 minutes, at which point 500 µL of room temperature SOC Media was added to each tube. The test tubes were then shaken horizontally for 1 hour at 37°C. After incubation, portions (100, 60, and 20 µL) of each sample were spread onto LB and ampicillin (0.1 mg/mL) plates, and each plate was incubated overnight at 37°C.

Two colonies from each overnight growth plate were selected, and each colony was transferred onto a fresh plate and allowed to grow overnight at 37°C. A total of eighteen colonies selected from this set of plates were isolated using a sterile toothpick and used to inoculate 2 mL aliquots of LB broth with ampicillin (0.1 mg/mL), which were grown overnight with shaking at 37°C. Plasmids were isolated from six of the overnight growths via the QIAprep Miniprep Kit. Each of the isolated

samples were characterized by electrophoresis on a 1% agarose gel as described on page 13.

The isolated plasmids were doubly digested with XhoI and BlnI restriction enzymes. A 4 μ L aliquot of each of the six plasmid samples was taken (~20 ng/ μ L), to which was added 2 μ L of NEBuffer 4, 2 μ L BSA (10 mg/mL), 1 μ L XhoI enzyme, 1 μ L BlnI enzyme, and 10 μ L distilled water. The samples were digested for one hour at 37°C. The resulting digested samples were characterized on a 1% agarose gel as on page 13.

Section 8: Transformation of BL21 Cells

Three 25 μ L aliquots of chemically competent BL21 *E. coli* cells were transferred to three separate, labeled test tubes. A 2 μ L aliquot (~10 ng/ μ L) of one of the *mCYS3/pET-15b* samples (those isolated from colonies *mCYS3_pET_C*, *mCYS3_pET_L*, and *mCYS3_pET_R*) was added to each tube of BL21 cells, and the solution was mixed by strumming the tubes with a finger. The three tubes were incubated on ice for a total of 30 minutes, heat shocked at 42°C for 30 seconds and placed on ice for 5 minutes. Each tube had 250 μ L of room temperature SOC media added and was shaken horizontally at 37°C for 60 minutes. 20, 60, and 100 aliquots of each sample were spread onto LB plates containing ampicillin (0.1 mg/mL) and allowed to grow overnight at 37°C.

Several colonies from each set of overnight growth were transferred to individual fresh LB plates containing ampicillin (0.1 mg/mL) and allowed to grow overnight at 37°C. From each set of the second overnight growth, one colony was transferred using a sterile toothpick into a 2 mL aliquot of LB broth containing ampicillin (0.1 mg/mL) and grown overnight at 37°C.

Plasmids from the overnight growth were isolated via the QIAprep Miniprep Kit. Each of the isolated samples were characterized on a 1% agarose gel as on page 13. To confirm that the *mCYS3/pET-15b* samples

had both the appropriate pET-15b plasmid and the *mCYS3* gene present, a double digest was performed. 4 μ L of each plasmid sample was obtained (~20 ng/ μ L), and mixed with 2 μ L NEbuffer 4, 2 μ L BSA (10 mg/mL), 1 μ L XhoI enzyme, 1 μ L BlnI enzyme, and 10 μ L distilled water. The mixtures were incubated at 37°C for 1 hour, and then characterized with a 1% agarose gel in the same manner as on page 13.

The genes were sequenced using conditions found in Table 2-2. The sequence of primers 1F, 2F, 1R, and 2R are given in Figure 2-4, and their corresponding position in the *mCYS3* gene is represented in Figure 2-5. Each of these samples, including the control (Sample 1) were then run in the thermal cycler following a thirty cycle program of 20 seconds of denaturation at 96°C, 20 seconds annealing at 50°C, and 4 minute extension at 60°C, before holding at 4°C overnight.

Table 2-2: Gene Sequencing Sample Mixtures, *mCYS3*/pET-15b Samples

(All volumes expressed in μ L)

Sample Number	1	2	3	4	5	6	7
Distilled Water	9.5	6.0	6.0	6.0	6.0	6.0	6.0
<i>mCYS3</i> /pET-15b	0	4.0	4.0	4.0	4.0	4.0	4.0
pUC-18 Template	0.5	0	0	0	0	0	0
-47 Sequencing Primer	2.0	0	0	0	0	0	0
Primer T3	0	2.0	0	0	0	0	0
Primer T7	0	0	2.0	0	0	0	0
Primer 1F	0	0	0	2.0	0	0	0
Primer 1R	0	0	0	0	2.0	0	0
Primer 2F	0	0	0	0	0	2.0	0
Primer 2R	0	0	0	0	0	0	2.0
DTCS Master Mix	8.0	8.0	8.0	8.0	8.0	8.0	8.0

Primer 1F: 5' - GAT TTG TTG AAC GAT CTA CCT CAA TTG - 3'

Primer 2F: 5' - AGA CAA GCT GCC CTC AGC -3'

Primer 1R: 5' - GAT CCA GAC CAA TTT GGT GTT TT- 3'

Primer 2R: 5' - GTC TGC TGC CAA GAA TTC AGC - 3'

Figure 2-4: Sequences of Primers 1F, 2F, 1R, and 2R

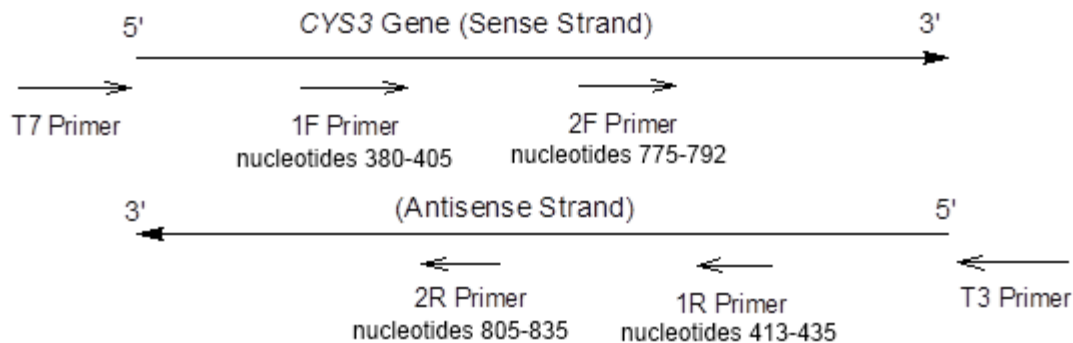


Figure 2-5: Illustration of Portion of mCYS3 Gene Matched by Each Primer

For each of the samples, 5 μ L of the stop solution/glycogen mixture was produced, using 2 μ L of 3M sodium acetate, 2 μ L of 100 mM Na₂-EDTA, and 1 μ L of 20 mg/mL glycogen. Each sample was then prepared and sequenced with the Beckman-Coulter CEQ 2000 as on page 19.

Section 9: Expression of the mCYS3 Gene

20 μ L of the mCYS3/pET-15b in BL21 *E. coli* cells sample were added to a 14 mL portion of LB broth with ampicillin (0.1 mg/mL). The bacteria were grown overnight at 37°C with shaking of the solution. A 4 mL aliquot of the overnight growth was transferred to a 20 mL sample of LB broth, and the absorbance of the solution was taken every 20 minutes (Table 2-3) to determine when the bacteria had reached their exponential growth phase.

Table 2-3: Measurement of Absorbance of *E. coli* growth at 595 nm

Minutes Since Addition	Absorbance at 595 nm
0	0.10764
20	0.16374
40	0.29018
60	0.76844
80	0.76844
100	1.19900
120	1.51220
140	1.76550
160	1.70390
180	1.67710

From this data, it was determined that the BL21 *E. coli* cells entered their exponential growth phase when the absorbance measurement was between 0.15 and 0.20. A fresh (100 mL) sample of LB and ampicillin (0.1 mg/mL) broth was prepared. 2 mL of an overnight growth were added, and the cells were grown at 37°C with shaking. The absorbance of the solution was monitored every fifteen minutes, and when the absorbance reached 0.17812, 50 mL of the sample was set aside as the 'Uninduced' sample, and 30 µL of 1 M IPTG were added to the remaining 50 mL 'Induced' portion. Both samples were grown overnight at 37°C with shaking at 200 rpm.

After overnight growth, the cells were harvested by centrifugation at 6,000 × g at 4°C for ten minutes. The pelleted cells were resuspended in 10 mM Tris buffer with 2 mM EDTA (pH 7.0) solutions, and stirred for one hour on ice. The suspension was centrifuged for 20 minutes at 11,000 × g, then stirred on ice. To maximize the amount of isolated protein, the pellets were resuspended in 10 mM Tris buffer with 2 mM EDTA (pH 7.0). The resuspended cells were treated to six cycles of 30 seconds of sonication and 60 seconds of cooling on ice, in order to lyse open the remaining cells. The lysed portion of each pellet was combined with the earlier supernatant of the associated sample and then centrifuged again at 11,000 × g for ten minutes. The supernatant solutions were treated with 1% (w/v) streptomycin solution, which was added drop-wise with stirring until 10 mL had been added to each sample. The solutions were then stirred for an additional 10 minutes, and centrifuged once more at 11,000 × g for 20 minutes.

Both samples were concentrated from ~50 mL to 0.2 mL with the Ultra Centrifugal Filter Devices from Amicon. Each sample was loaded into a filter device to concentrate the portions of the sample mixture

greater in size than 3 kilodaltons, and spun in the centrifuge for repeated 30 minutes spins at $11,000 \times g$. When the samples had been concentrated to 1 mL, 500 μL , and a final volume of 200 μL , the concentration of protein in the solutions was determined by running a Bradford Assay. The samples were incubated at room temperature for ten minutes, and the absorbance of each was measured at 595 nm to determine the concentration of the samples.

50 μL of each sample were placed into separate 1.5 mL microcentrifuge tubes, to which 50 μL of 2X protein loading dye was added. The tubes were placed in a heat block set to 100°C for three minutes, then spun for 30 seconds at 10,000 rpm in a microcentrifuge. The ready-made SDS-PAGE gel was obtained and loaded into the BioRad MiniProtean II electrophoresis system. To lanes three and six were loaded 20 μL of the Uninduced sample, while 20 μL of the Induced sample was loaded into lanes four and seven. To lanes one and ten were loaded 5 μL of the Precision Plus molecular weight marker. The gel was run at 200 V for sixty minutes.

When the gel had finished running, it was unloaded from the BioRad MiniProtean II electrophoresis system, placed into a plastic container, and washed with 100 mL of deionized water. The container was heated for one minute in a microwave, the water discarded, and the water wash was repeated twice more. After the wash, 70 mL of Blue BANDit solution from Amresco was added to the container with the gel. The solution was microwaved for 1 minute and allowed to stand for 15 minutes. The stain solution was then poured off, and 100 mL of distilled water was added to the container for 10 minutes, before being poured off. Another 100 mL of water was added, and the container was allowed to stand for 30 minutes. The gel was placed back into the

container with 100 mL of fresh distilled water, and allowed to destain overnight.

Chapter III: Results

Section 1: Isolation of the CYS3 Gene from the Yeast Genome

The CYS3F and CYS3R primers were used to isolate the CYS3 gene from the yeast genome. Three samples were prepared, and were labeled as 100 ng/ μ L, 10 ng/ μ L, or 1 ng/ μ L, according to the concentration of yeast DNA used. An agarose gel showing the PCR results of a small scale run are shown in Figure 3-1a. The PCR procedure was scaled up to 50 μ L using the 10 ng/ μ L and 1 ng/ μ L concentrations of the yeast genome. The resulting band was eluted from an agarose gel (Figure 3-1b). The isolated genes were examined by running a third agarose gel (Figure 3-1c). To help verify the identity of the isolated gene, an EcoRI digest was performed. The CYS3 gene has one EcoRI restriction site whose digestion will generate bands of 900 kb and 400 kb. (Figure 3-2)

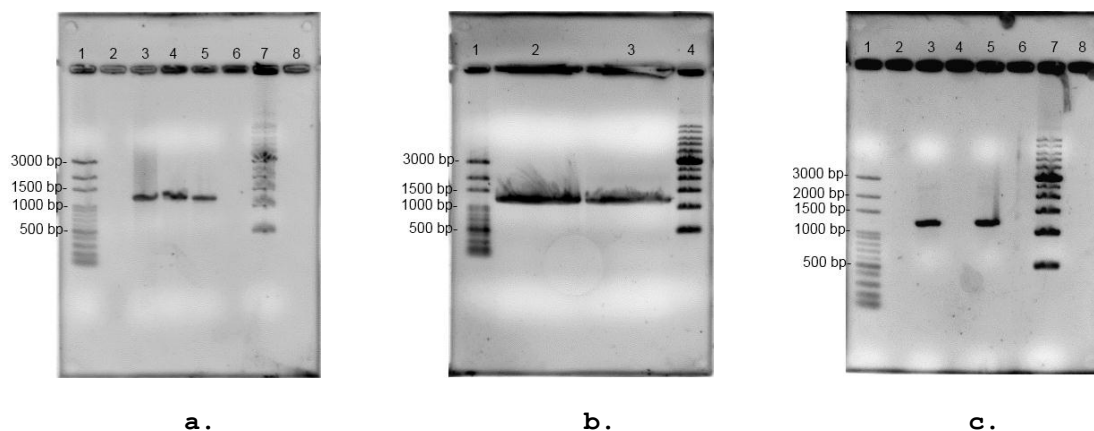


Figure 3-1: (a) Small-scale PCR preparation of the CYS3 gene (size: ~1300 bp) from the yeast genome. Lane 1: 100 bp ladder; Lane 3: CYS3 gene from 0.1 μ g/ μ L sample; Lane 4: CYS3 gene from 1 μ g/ μ L sample; Lane 5: CYS3 gene from 10 μ g/ μ L sample; Lane 7: 1 kb ladder. (b) Large-scale PCR preparation of the CYS3 gene from the yeast genome. Lane 1: 100 bp ladder; Lane 2: CYS3 gene from 1 μ g/ μ L sample; Lane 3: CYS3 gene from 10 μ g/ μ L sample; Lane 4: 1 kb ladder. (c) An agarose gel showing the results of eluting the CYS3 gene from the scaled-up PCR experiment. Lane 1: 100 bp ladder; Lane 3: CYS3 gene from 1 μ g/ μ L sample; Lane 5: CYS3 gene from 10 μ g/ μ L sample; Lane 7: 1 kb ladder.

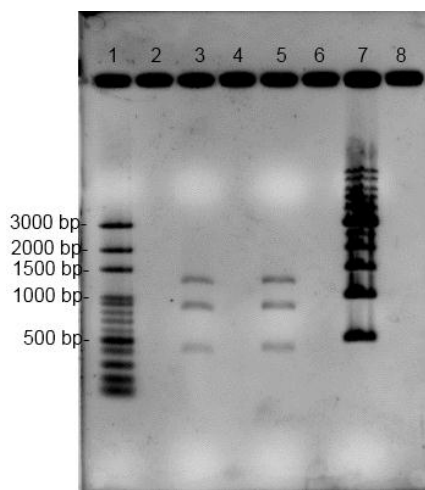


Figure 3-2: Digestion of the isolated *CYS3* gene using the *EcoRI* restriction enzyme Lane 1: 100 bp ladder; Lane 3: *CYS3* gene from 1 $\mu\text{g}/\mu\text{L}$ sample; Lane 5: *CYS3* gene from 10 $\mu\text{g}/\mu\text{L}$ sample; Lane 7: 1 kb ladder.

Section 2: Addition of Restriction Sites by the PCR Overlap Extension Method

Primers CGL 5' and CGL 3' - BLPI were used to modify the *CYS3* gene by adding an *XhoI* restriction site and enterokinase recognition sequence on the 5' end and a *BlnI* restriction site on the 3' end of the gene, creating the *mCYS3* gene. The small scale PCR results (Figure 3-3a) were scaled up (Figure 3-3b). The gene was eluted from the gel and the isolation was confirmed by running an agarose gel (Figure 3-3c).

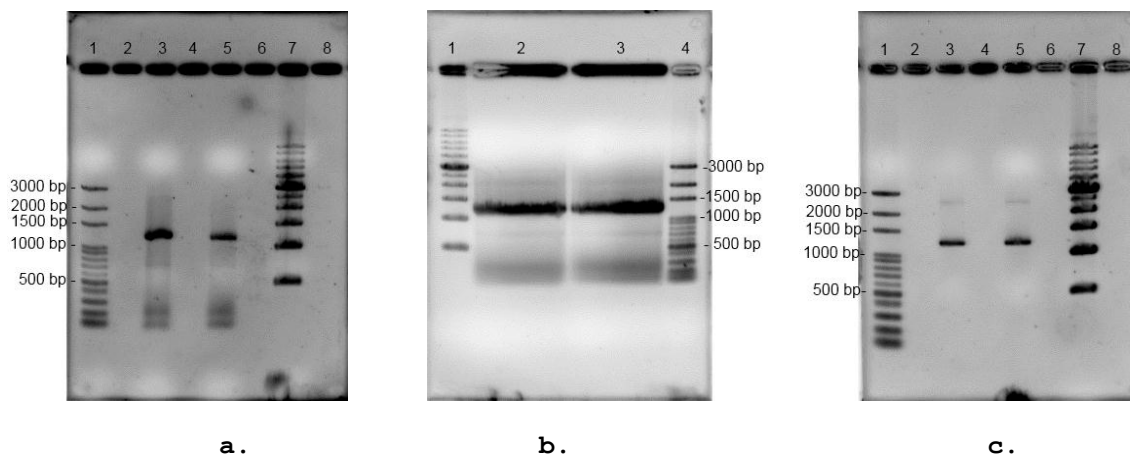


Figure 3-3: (a) An agarose gel for the small scale PCR preparation of the *mCYS3* gene with an enterokinase recognition sequence and XhoI and BlnI restriction sites. Lane 1: 100 bp ladder; Lane 3: *mCYS3* gene produced from 1 µg/µL isolated gene; Lane 5: *mCYS3* gene produced from 10 µg/µL isolated gene; Lane 7: 1 kb ladder. (b) An agarose gel of the large scale preparation of the *mCYS3* gene with an enterokinase sequence and XhoI and BlnI restriction sites. Lane 1: 100 bp ladder; Lane 2: *mCYS3* gene produced from 1 µg/µL isolated gene; Lane 3: *mCYS3* gene produced from 10 µg/µL isolated gene; Lane 4: 1 kb ladder. (c) An agarose gel showing the results of eluting the *mCYS3* gene from the scaled-up PCR experiment. Lane 1: 100 bp ladder; Lane 3: *mCYS3* gene produced from 1 µg/µL isolated gene; Lane 5: *mCYS3* gene produced from 10 µg/µL isolated gene; Lane 7: 1 kb ladder.

Section 3: Splicing of *mCYS3* into pCR[®]4-TOPO[®]

The isolated *mCYS3* gene was transferred into the pCR[®]4-TOPO[®] plasmid. The recombinant DNA, *mCYS3*/pCR, was used to transform One Shot[®] TOP10[™] Chemically Competent *E. coli* cells. The cells were grown and the plasmids were isolated from the cells (Figure 3-4a). A double digest using XhoI and BlnI restriction enzymes was used to verify the incorporation of appropriate length fragments into the pCR[®]4-TOPO[®] plasmid (Figure 3-4b).

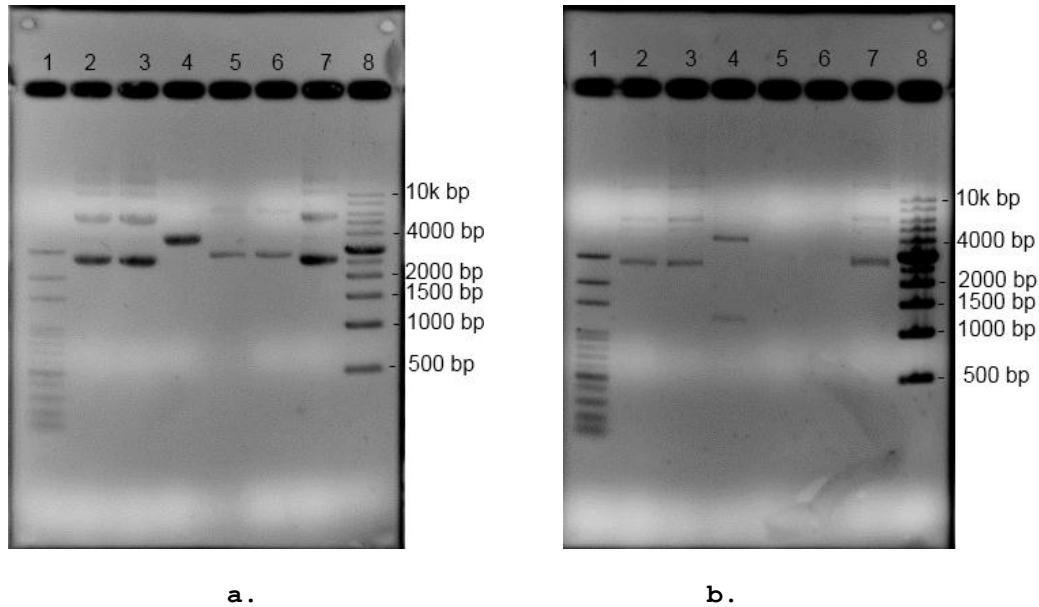


Figure 3-4: (a) Initial isolation of pCR[®]4-TOPO[®] plasmids containing the *mCYS3* gene. Lane 1: 100 bp ladder; Lane 2: Sample *mCYS3_pCR_B*; Lane 3: Sample *mCYS3_pCR_D*; Lane 4: Sample *mCYS3_pCR_G*; Lane 5: Sample *mCYS3_pCR_J*; Lane 6: Sample *mCYS3_pCR_M*; Lane 7: Sample *mCYS3_pCR_O*; Lane 8: 1 kb ladder. (b) Double digest of *mCYS3/pCR* plasmid with XhoI and BlnI restriction enzymes. Lane 1: 100 bp ladder; Lane 2: Sample *mCYS3_pCR_B*; Lane 3: Sample *mCYS3_pCR_D*; Lane 4: Sample *mCYS3_pCR_G*; Lane 5: Sample *mCYS3_pCR_J*; Lane 6: Sample *mCYS3_pCR_M*; Lane 7: Sample *mCYS3_pCR_O*; Lane 8: 1 kb ladder.

Section 4: Sequencing of *CYS3* in *mCYS3/pCR*

To confirm that the gene spliced into the plasmid (*mCYS3_pCR_G*) that had been isolated was the *mCYS3* gene, a sequencing reactions were run (Figure 3-5).

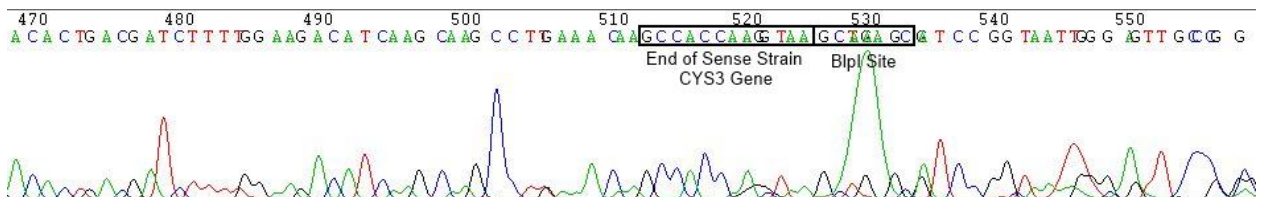


Figure 3-5: Partial electropherogram results of the *mCYS3* gene sequence, showing the BlnI site that was added at the 3' end of the *CYS3* gene.

Section 5: Isolation of *mCYS3* Gene

The *mCYS3* gene was excised from the *mCYS3*/pCR plasmid using a double digest consisting of XhoI and BlnI restriction enzymes (Figure 3-6). The gene was then extracted from the agarose gel.

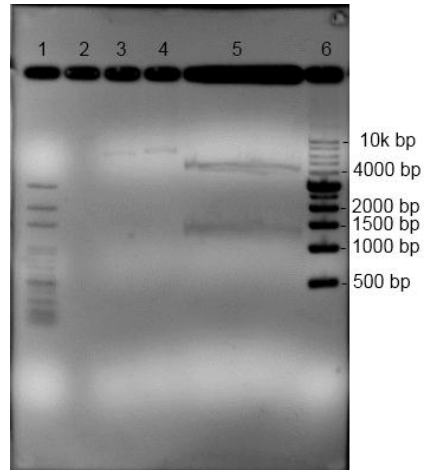


Figure 3-6: Double digest of recombinant *mCYS3*/pCR plasmid with XhoI and BlnI restriction enzymes. Lane 1: 100 bp ladder; Lane 5: Double digested *mCYS3*/pCR plasmid; Lane 6: 1 kb ladder (Lanes 3 and 4 are identified in Figure 3-13.)

Section 6: Isolation of *pET-15b* plasmid

The *pET-15b* plasmid was used to transform One Shot[®] Mach 1[™] Chemically Competent *E. coli* cells. The plasmid was then isolated from the transformed bacteria cells and run on an agarose gel (Figure 3-7a). The plasmid was linearized by a double digest with XhoI and BlnI restriction enzymes and examined by agarose gel electrophoresis (Figure 3-7b). A 50 μ L digestion mixture containing 200 μ g of *mCYS3*/pCR plasmid was examined by agarose gel electrophoresis (Figure 3-7c) and then eluted from the gel (Figure 3-7d).

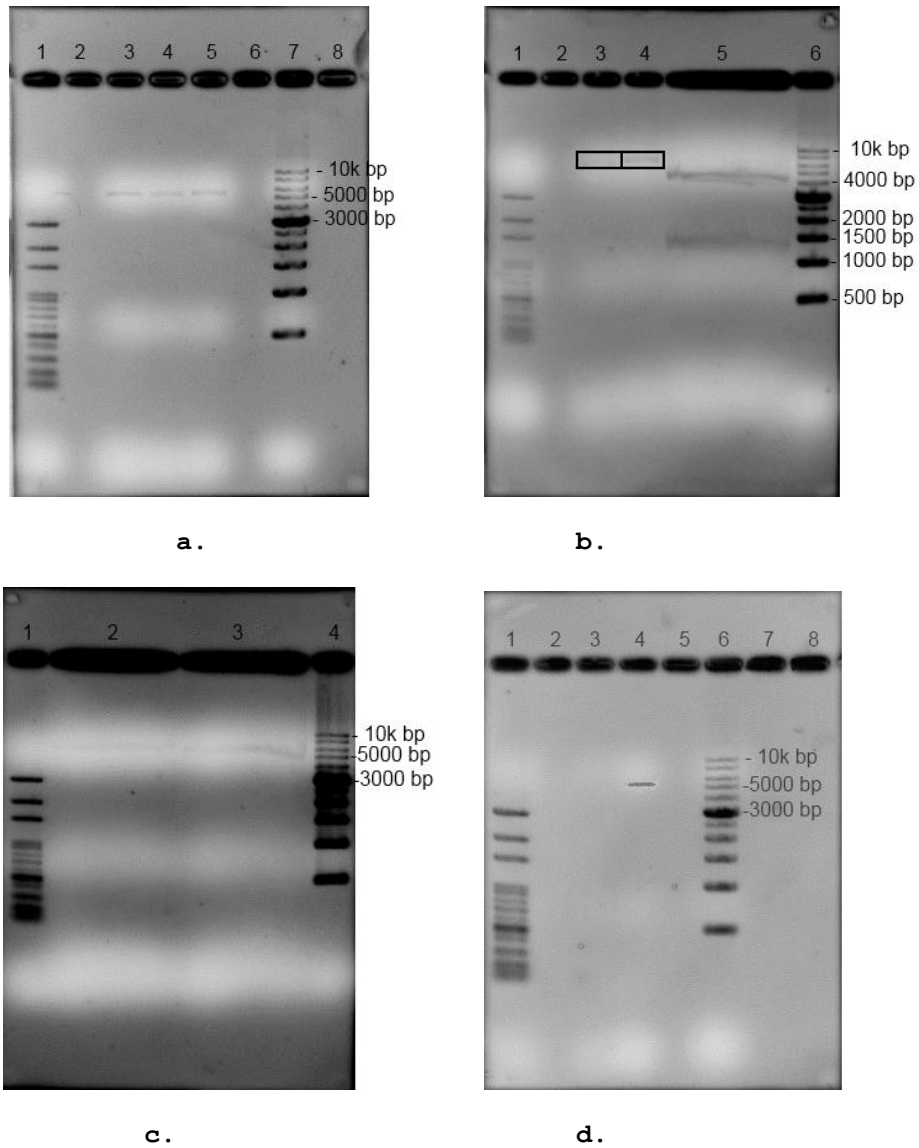


Figure 3-7: (a) Initial run of isolated pET-15b plasmids. Lane 1: 100 bp ladder; Lane 3: Sample C pET-15b; Lane 4: Sample D pET-15b; Lane 5: Sample E pET-15b; Lane 7 1 kb ladder. (b) Small scale double digest of pET-15b with XhoI and BlnI restriction enzymes. Lane 1: 100 bp ladder; Lane 3: pET-15b Sample E; Lane 4: pET-15b Sample D; Lane 6: 1 kb Ladder. (Lane 5 is identified in Figure 3-6.) (c) 50 μ L double digest of pET-15b samples. Lane 1: 100 bp ladder; Lane 2: pET-15b Sample D; Lane 3: pET-15b Sample E; Lane 4: 1 kb Ladder. (d) Verification of pET-15b plasmid isolation. Lane 1: 100 bp Ladder; Lane 3: pET-15b Sample D; Lane 4: pET-15b Sample E; Lane 6: 1 kb Ladder

Section 7: Splicing of *mCYS3* Gene into the *pET-15b* (*mCYS3/pET*) Plasmid

The isolated *mCYS3* gene was transferred into the doubly digested *pET-15b* plasmid to generate *mCYS3/pET* plasmid. These plasmids were used to transform One Shot[®] Mach 1[™] *E. coli* cells. The recombinant plasmids were isolated and characterized via a double digest with XhoI and BlnI restriction enzymes (Figure 3-8).

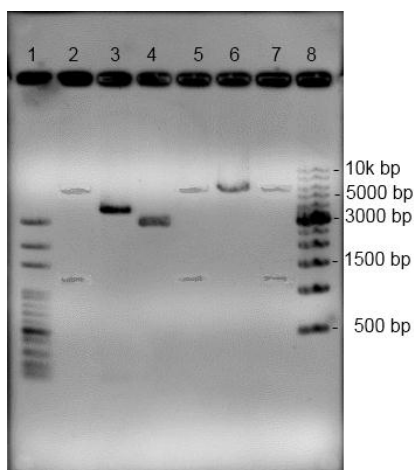


Figure 3-8: Double digest of isolated *mCYS3/pET* plasmids. Lane 1: 100 bp Ladder; Lane 2: Sample *mCYS3_pET_C*; Lane 3: Sample *mCYS3_pET_F*; Lane 4: Sample *mCYS3_pET_I*; Lane 5: Sample *mCYS3_pET_L*; Lane 6: Sample *mCYS3_pET_O*; Lane 7: Sample *mCYS3_pET_R*; Lane 8: 1 kb Ladder.

Section 8: Transformation of BL21 Cells

The *mCYS3/pET* plasmids were used to transform BL21 cells. The transformed BL21 cells were grown overnight and their plasmids extracted. The *mCYS3/pET* plasmids were digested with XhoI and BlnI restriction enzymes to verify the gene and plasmid size (Figure 3-9). The plasmids were also sequenced (Figure 3-10).

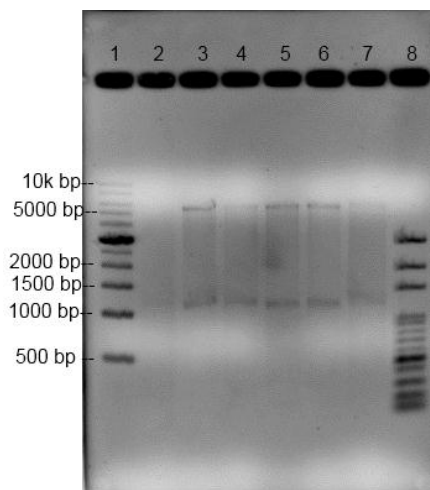


Figure 3-9: Double digest of isolated *mCYS3*/pET plasmids with XhoI and BlnI restriction enzymes. Lane 1: 1 kb Ladder; Lane 2: Sample *mCYS3*_pET_BL21_B; Lane 3: Sample *mCYS3*_pET_BL21_F; Lane 4: Sample *mCYS3*_pET_BL21_I; Lane 5: Sample *mCYS3*_pET_BL21_K; Lane 6: Sample *mCYS3*_pET_BL21_N; Lane 7: Sample *mCYS3*_pET_BL21_R; Lane 8: 100 bp Ladder.

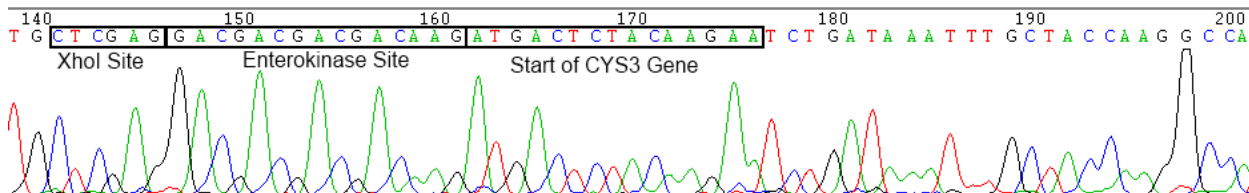


Figure 3-10: Partial electropherogram results of the *mCYS3* gene sequences from *mCYS3*/pET. Showing the presence of the XhoI site and enterokinase site at the 5' end of the *mCYS3* gene.

Section 9: Expression of the *mCYS3* Gene

The transformed cells were used to generate the CGL protein. Protein production by the transformed cells was monitored in the presence (Induced) and absence (Uninduced) of IPTG. The cells were lysed and the lysates were collected and concentrated. The concentrate was examined by SDS-PAGE (Figure 3-11).

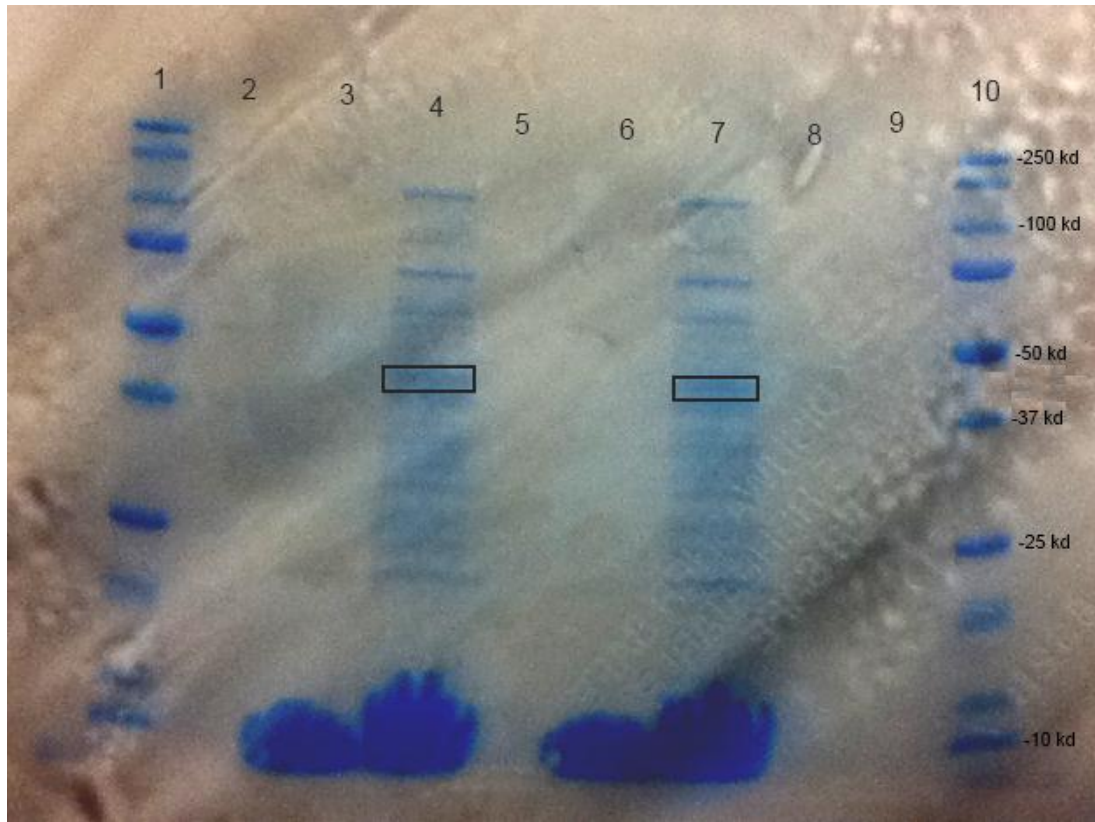


Figure 3-11: SDS-Page results of Induced and Uninduced protein production. Lane 1: Precision Plus Molecular Weight Marker; Lane 3: Uninduced Protein Sample; Lane 4: Induced Protein Sample; Lane 6: Uninduced Protein Sample; Lane 7: Induced Protein Sample; Lane 10: Precision Plus Molecular Weight Marker

Chapter IV: Discussion

Metal catalyzed oxidation (MCO) represents a major means by which proteins can be structurally altered and inactivated. By oxidizing individual amino acids in a protein, the structure can be changed, potentially preventing the protein from performing its intended function. In most cases, the altered proteins end up targeted for degradation by the proteasomes. The cell must then synthesize new proteins or suffer from the decreased protein activity.

There are several antioxidant mechanisms used to counteract or prevent the production of ROS. The tripeptide glutathione, for example, reacts with organic peroxides to convert them to water and alcohol. These peroxides would otherwise serve as a source of free radicals, which are capable of damaging biological tissue and can lead to numerous diseases and premature aging. As organisms get older, their primary means of producing glutathione increasingly depends on the trans-sulfuration pathway, raising their dependence on this pathway and its production of cystathionine.¹⁶ This higher dependence also increases the effect of any oxidative damage to the proteins within this pathway on the organism at large. Oxidizing the proteins involved in anti-oxidation processes potentially renders the organism more vulnerable to further oxidation.

Cystathionine γ -lyase (CGL) was chosen as the focus for this research due to its position within the trans-sulfuration pathway. CGL converts cystathionine into cysteine with the side products of α -ketobutyrate and ammonia. It's a valuable part of the trans-sulfuration pathway, and is increasingly important to organisms as they age. Focusing on the CGL enzyme allows us to view the effect of damage to this pathway on the oxidation resistance of the organism at large.

One particular area where glutathione, and the trans-sulfuration

pathways that generate its component cysteine, proves particularly important is in the lens of the eyes. Crystallins are proteins in the lenses that are required for sight. Antioxidant reactions are particularly valuable in protecting the crystallins from being damaged, as the crystallins are not regenerated and must last for the organism's lifetime. Damage to the active site amino acid residues of the CGL protein caused by free radicals has been hypothesized to be the cause of more crystallin damage as organisms get older.²⁷ Oxidative damage to CGL leads to less cysteine production, which leads in turn to less glutathione production. With less glutathione available, more oxidative damage to the crystallin proteins are then possible, leading to the potential formation of cataracts. This research focuses on the oxidation of CGL to examine the potential link to cataractogenesis,

The cystathionine γ -lyase gene (*CYS3*) was obtained from the yeast genome using appropriate primers and the polymerase chain reaction (PCR). A digest of the PCR product with EcoRI was performed, followed by agarose gel electrophoresis. The resulting product generated two fragments of ~400 kb and ~900 kb as expected for the *CYS3* gene (Figure 3-2). The isolated *CYS3* gene served as the template for adding an XhoI site and enterokinase sequence to the 5' end of the gene and a BlnI site to the 3' end. Specialized primers were used in conjunction with PCR to produce the modified *mCYS3* gene in sizable quantities.

The *mCYS3* gene was cloned into the pCR[®]4-TOPO[®] vector to produce the recombinant *mCYS3*/pCR plasmid, and the plasmids were used in turn to transform One Shot[®] TOP10[™] Chemically Competent *E. coli* cells. The recombinant plasmids were isolated after an overnight growth in LB broth containing ampicillin. The *mCYS3*/pCR plasmids were characterized via double digest with the XhoI and BlnI digestive enzymes, Figure 3-

4b, and by sequencing.

The recombinant plasmid was grown to µg quantities, digested with XhoI and BlnI enzymes, and the *mCYS3* gene was extracted from the gel for sub-cloning into pET-15b. The pET-15b plasmid was similarly grown to µg quantities, double-digested with XhoI and BlnI, and extracted from the gel. The *mCYS3* gene was then sub-cloned into the pET-15b plasmid, and the resulting *mCYS3*/pET recombinant plasmid was then used to transform One Shot[®] Mach 1[™] *E. coli* cells. These cells were grown and the plasmid extracted and characterized with a double digest using XhoI and BlnI restriction enzymes (Figure 3-8). The isolated *mCYS3*/pET plasmids were used to transform BL21 cells.

At this point, the transformed BL21 cells were induced with IPTG to examine expression of CGL production. The results showed what appeared to be a higher concentration of the CGL protein than of the other proteins in the cell, but further testing is needed to verify the overproduction of CGL.

The procedure described here is the last of several attempts at transforming BL21 cells with the *CYS3* gene capable of expressing CGL. For the first several attempts at sub-cloning the CGL gene into pET-15b, a CGL gene containing the XhoI restriction site at both the 5' and 3' ends was used, with an enterokinase sequence in front of the gene at the 5' end. It was hoped that doing so would simplify the task of splicing the CGL gene into pET-15b. However, this was not the case, as the gene and pET-15b plasmid did not ligate. After several attempts, the CGL gene was instead modified by introducing two different restriction enzyme sequences, specifically those of the XhoI sequence followed by the enterokinase site at the 5' end and BlnI at the 3' end.

Another issue arose when the sequence of the *mCYS3* gene was

determined. It was discovered that an extra codon had been added to the *mCYS3* sequence. It was necessary to go back to the yeast genome and isolate the gene anew, with fresh primers, as described in the Materials and Methods section.

There was one more major obstacle that needed to be overcome before the gene could be properly isolated, spliced and expressed. Several attempts at preparing and isolating the *mCYS3* gene met with initial success, only to have the isolated plasmids decline in concentration and quality over time. Numerous attempts were made at determining the source of the difficulty with the samples. It was eventually determined that the source of water was the problem, and it was likely due to the way it was stored. When another source for distilled water was used, the procedure went much more smoothly, as described in the Materials and Methods section.

Chapter V: Conclusion

CGL was chosen as the target for the MCO focused research due to its connection to cataractogenesis. Seeing where that structure is oxidized under MCO conditions and how those oxidation conditions affect the tertiary structure and activity of the protein will provide useful information about the specific effects of such oxidation on CGL, and possibly, the development of cataracts.

The procedure performed here served as a means of cloning the *CYS3* gene into suitable bacteria for overexpression. The sequencing procedures provide proof that the *mCYS3* gene has been successfully spliced into pET-15b and the resulting *mCYS3/pET* plasmid was successfully transformed into the BL21 *E. coli* bacteria. Induction of this plasmid by IPTG within the BL21 bacteria will serve as a method of generating a substantial amount of the CGL protein for use in future oxidation experiments. The results of the SDS-PAGE gel indicate success in that regard, showing a thicker band at the 42 kilodalton mark which corresponds to the mass of a subunit of CGL after induction by IPTG.

The next step in this research will be to perform large-scale expression of the *CYS3* gene to generate mg quantities of the CGL protein. The poly-His tag on the N-terminus of the expressed CGL protein will be employed to purify the protein by passage through a Ni-column. The protein will then be released from the column by cleaving the poly-His tag using enterokinase.

Upon production of a substantial amount of CGL, the protein will then be exposed to a MCO system. The level of activity of the oxidized protein will be compared to the activity level of the unaltered protein, to determine how such oxidation affects the activity of the

CGL protein. MS and MS/MS analysis will be used to determine the extent and specific sites of oxidation. This will establish a link between the sites of oxidation and the loss of activity, and may bolster the connection between cataractogenesis and CGL activity.

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