Expression and Mechanistic Studies on Orotidine-5'-Monophosphate Decarboxylase

by

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Orotidine-5'-Monophosphate Decarboxylase

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Thesis Abstract

Orotidine-5'-monophosphate decarboxylase, ODCase, catalyzes the conversion of OMP to UMP in the pyrimidine biosynthetic pathway. This enzyme is remarkable in both its rate enhancement and lack of a cofactor. For this reason, the mechanism has been under investigation. Currently, a new hypothesis raises the question as to whether the substrate binds with its C6 group or O2 group facing the lysine residue at position 93. To determine this, an isoteric inhibitor along with chemically modified and mutated ODCase clones would provide evidence through ^{15}N labeled NMR samples. This thesis produced the resources necessary to chemically modify the protein by providing an adequate expression system and growth conditions for ODCase WT, CysFree, C93only, and K93C.

Acknowledgements

I acknowledge the efforts set forth from the Youngstown State University Chemistry Department and School of Graduate Studies. Both played integral roles in the development and continuance of my education. I extend my sincere thanks to Dr. Smiley for his support, friendship, and guidance in both research and life. I am grateful for all the friendships encountered and especially for a wonderful lab group. The boys, the original Jas Lab and the Boyfriend Committee will not soon be forgotten for the fun times shared together. I appreciate all the members of my thesis committee for their patience and effort to see me through to graduation. I acknowledge my dear old stock room, the wisdom of Sue, and friendship of Chris who makes an excellent roommate and provided common sense at times I was lacking. I give my most heartfelt thanks to my mom and dad and family. Their constant support and interest, even if they had no clue what I was doing, is appreciated greatly. I especially thank my sister, Kristin, without whom I could not have gotten through these past three years. A small acknowledgement goes to Minxy, Rascal, Izzy, Lucky and Lumber who taught me about cats and that true friends need not speak volumes, but just listen. If they learn to talk I'm screwed. I

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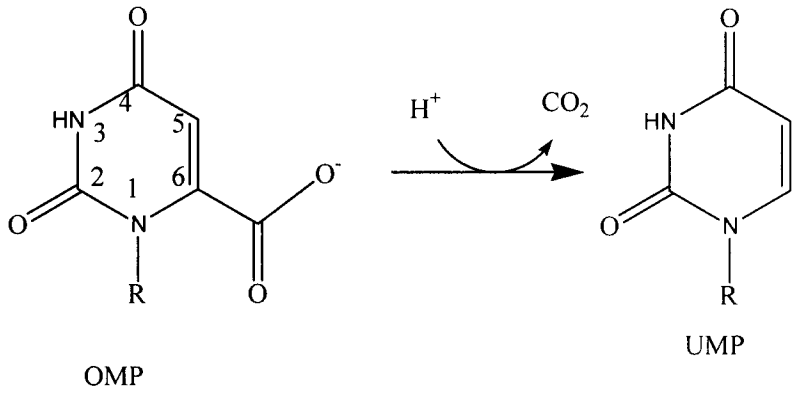
List of Symbols and Abbreviations

α	Alpha
β	Beta
Δ	Delta
γ	Gamma
μ	Micro
μg	Microgram
μL	Microliter
μM	Micromolar
®	Registered
™	Trademark
%	Percent
^{15}N	Isotopically labeled nitrogen
A	Adenosine
Abs	Absorbance
ADP	Adenosine diphosphate
Amp	Ampicillin
Asp	Aspartate
ATP	Adenosine triphosphate
Bp	Base pair
C	Cytosine
CTP	Cytosine triphosphate
D	day
ddNTP	dideoxynucleotide triphosphate
dH_2O	deionized water
dNTP	deoxynucleotide triphohsphate
dUTP	deoxyuridine triphosphate
G	guanidine
H	hour
Lys	lysine
Kb	kilobase
kcal	kilocalories
K_d	dissociation constant
M	Molar
Mg	milligram
Min	minute
ML	milliliter
Mm	millimeter
MM	millimolar
Mol	moles
Ng	nanogram
Nm	nanometer
nmol	nanomole
pmol	picomole

ssDNA	single stranded DNA
T	thymidine
TTP	thymidine triphosphate
U	units
UV	ultraviolet
V	volts
v/v	volume per volume
WT	wild type
W/v	weight per volume

CHAPTER I: INTRODUCTION

Orotidine-5'-monophosphate decarboxylase, ODCase, catalyzes the reaction of orotidine-5'-monophosphate (OMP) to uridine-5'-monophosphate (UMP) in the *de novo* biosynthetic pyrimidine pathway (Figure 1.1).



OMP UMP

Figure 1.1: Reaction catalyzed by ODCase

UMP is a precursor to nucleotides involved in the storage and expression of genetic materials like DNA and RNA.

Nucleotides exist as either purines or pyrimidines based on ring structure (Figure 1.2). guanine and adenine are both purines forming base pairs with pyrimidines cytosine and uracil in RNA. A third pyrimidine is thymine which differs structurally from uracil by a methyl group. Adenine base pairs with thymine in DNA. Nucleotides are involved in catabolism, metabolism and the regulation of many biological pathways. The triphosphate groups provide sources of energy in phosphoryl transfers and upon release of either inorganic phosphate (P_i) or pyrophosphate, (PP_i).

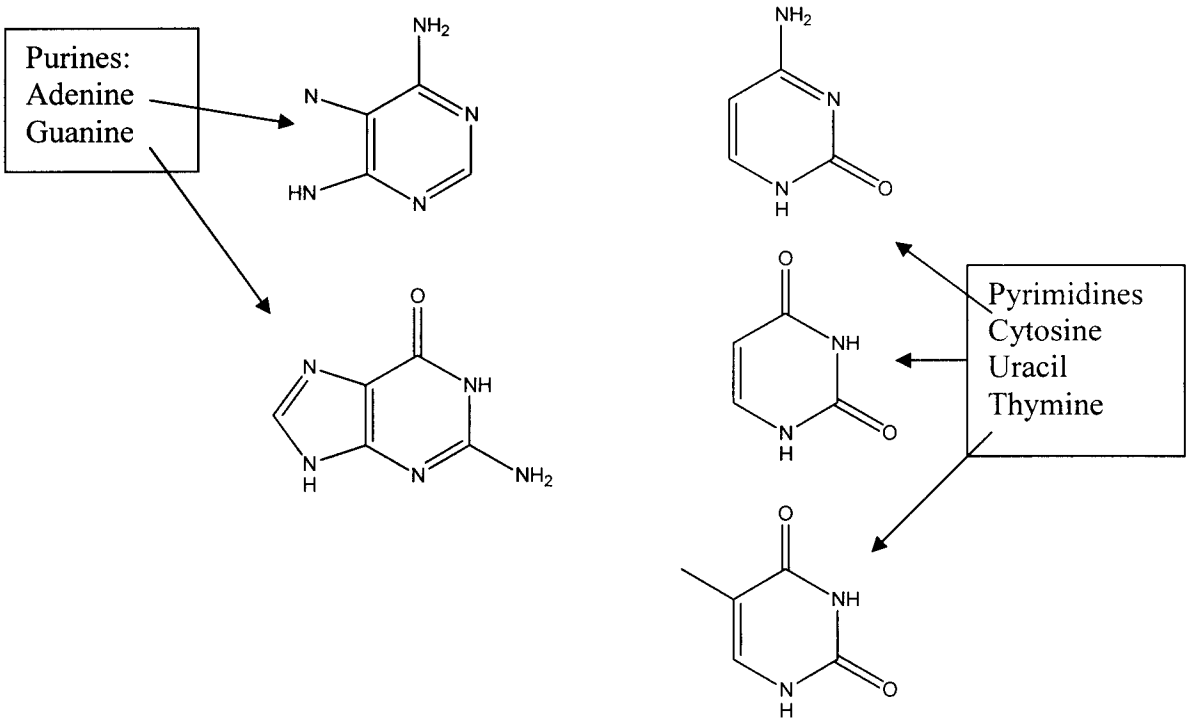


Figure 1.2: Nucleotide Structures[1]

The *de novo* pyrimidine biosynthetic pathway (Figure 1.3) was documented by M.E. Jones upon discovering *Neuorospora crassa* growth in the absence of cytosine and uracil and presence of orotic acid.[2] The six step cytosolic pathway begins with carbamoyl phosphate synthase II catalyzing the formation of carbamoyl phosphate in the reaction of 2 ATP, bicarbonate, and glutamine with the releases of 2 ADP, P_i, and glutamate. In step 2 aspartate transcarbamoylase creates carbamoyl aspartate with the release of P_i. This step is highly regulated by CTP inhibition and ATP and UTP activation. Dihydroorotase completes the ring's closure in step 3 by releasing H_2O and dihydroorotate. Upon the reduction of quinine, dehydroorotate dehydrogenase catalyzes step 4 producing orotate. Step 5's addition of the ribose unit releases PP_i and OMP. The 6^{th} and final step produces UMP with the release of CO_2 as catalyzed by ODCase.

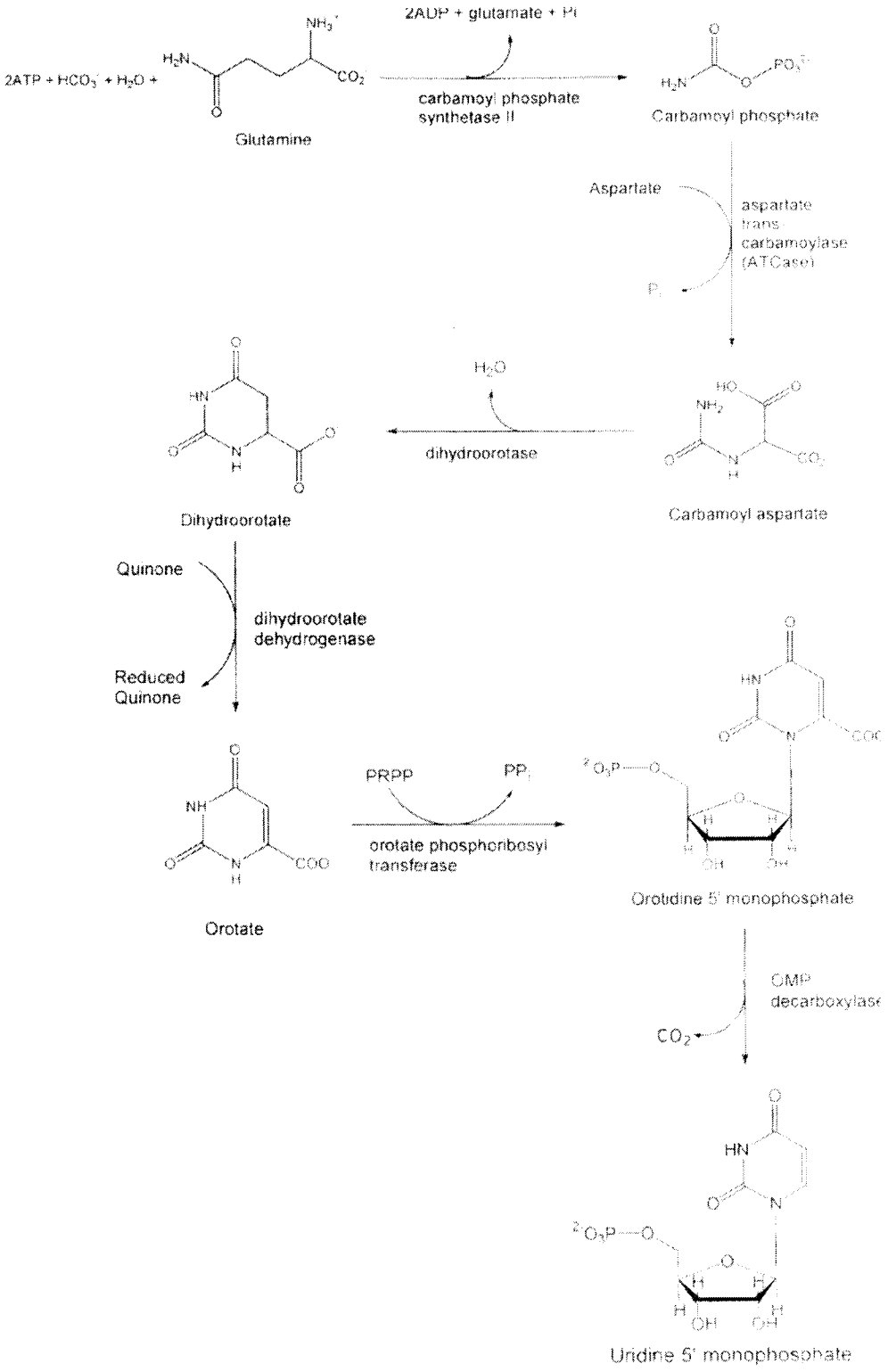


Figure 1.3: Pyrimidine Biosynthetic Pathway

ODCase is interesting to study because of its remarkable catalytic enhancement, one of the greatest rate enhancements known. ODCase catalyzes the reaction at a rate 10^{17} times faster than the uncatalyzed reaction.[3] In addition to its catalytic proficiency and unlike other enzymatic decarboxylations, ODCase requires no metal cofactor.[4] Due to ODCase's extreme rate enhancement and lack of a cofactor, the mechanism becomes of interest. Several mechanisms have been proposed to explain the catalytic proficiency.[5-9] Loss of this enzyme *in utero* is lethal and therefore, not all related deficiencies are known at this time. One inherited disease is orotic aciduria in which there are defects in the eukaryotic bifunctional enzyme catalyzing steps 5 and 6 of pyrimidine synthesis leading to increases in orotic acid. This genetic defect can be overcome with uridine or cytosine treatments.

There are many different types of decarboxylation reactions throughout the study of biochemistry, especially in discussion of metabolism. From amino acid degradation to the Krebs cycle, decarboxylations are a functional part of nature. Many enzymatic reactions involve cofactors for the transfer of functional groups or in stabilization of the transition state. ODCase behaves unlike common decarboxylase reactions leaving its mechanism unknown.

Decarboxylase Reactions

Amino Acid Decarboxylation

One type of decarboxylation occurs with amino acids and requires a pyridoxyl cofactor. Pyridoxyl-5'-phosphate (PLP) helps to stabilize the carbanion created by histidine decarboxylase in the formation of histamine from histidine. Histamine is a leading cause in immunologically related allergy symptoms.

Alpha-Keto Acid Decarboxylation

Decarboxylation of an α-keto acid involves a thiamine containing cofactor. Two examples, both occurring in the citric acid cycle, use thiamine pyrophosphate (TPP). Conversion of pyruvate to acetyl coenzyme A and α-ketoglutarate to succinyl coenzyme A both utilize TPP to complete the reaction.

Beta-Keto Decarboxylation

Oxaloacetate decarboxylase converts oxaloacetate to pyruvate. This type of decarboxylation involves the decarboxylation of a β-keto carboxyl through metal ion stabilization of the enolate.

Oxidative Decarboxylation

The last type of decarboxylation as seen in the pentose phosphate pathway involves decarboxylation through hydride transfer to $NADP^+$ to form NADPH, a reductant in anabolic pathways. 6-phosphogluconate is first oxidized by phosphogluconate dehydrogenase to a ketone followed by decarboxylation of the β CO_2 to produce ribulose-5'-phosphate.

Proposed Mechanisms of ODCase

Protonation at O2

One mechanism involves the formation of a zwitterion or nitrogen ylide with protonation at O2, (Figure 1.4). Beak and Siegel originally proposed this mechanism in 1976.[5] Since then some supporting data has emerged. Smiley *et al.*[10] and Acheson *et al.*[11] used kinetic isotope effects to support protonation at O2 leading to a positive charge on N1. This, in turn, stabilizes the vinyl carbanion. Wu *et al.*[12] studied the role of N1 and further indicated that the stabilization of the carbanion was due to the positive charge

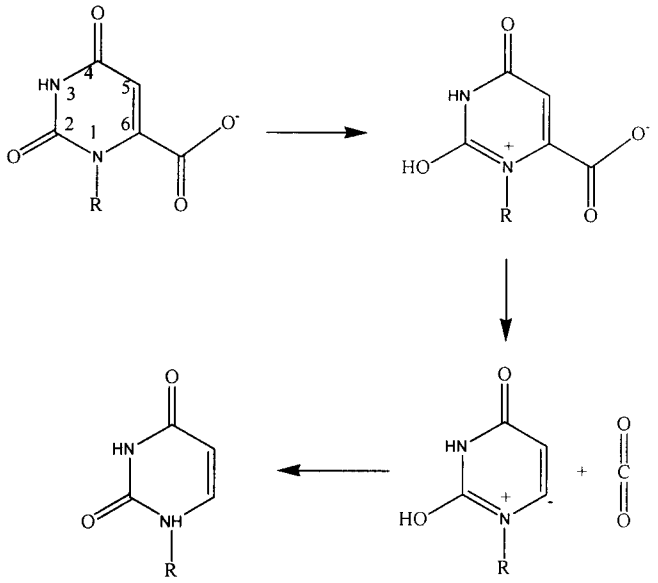


Figure 1.4: Protonation at O2

on N1, which is in agreement with findings from Lundberg *et al.*[13] Other mechanistic studies include the use of inhibitors such as 1-(5'-phospho-ß-D-ribofurunosyl) barbituric acid (BMP) 6-azauridine-5'-phosphate (6-azaUMP) and 2-thioOMP. BMP binds tightly to the active site, even more strongly than OMP.[14] Investigations of bound BMP indicate zwitterion formation. Smiley *et al.* found that 2-thioOMP was unable to bind properly. This indicates a crucial role of O2, possibly protonation through interactions with Lys-93 at the active site (numbering for yeast ODCase).[15] The zwitterion mechanism with protonation occurring at O2 remains a well-supported model for the catalytic proficiency of ODCase.

Protonation at O4

Protonation at O4 would lead to the formation of a carbene intermediate as another mechanism proposed for ODCase (Figure 1.5).

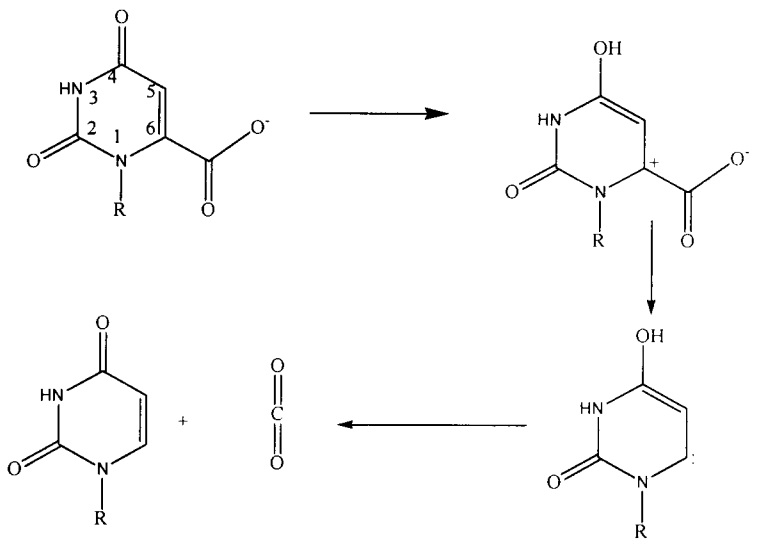


Figure 1.5: Protonation at O4

Protonation at O4 would lead to a carbocation at C6 resulting in decarboxylation, thus avoiding formation of a high energy vinyl carbanion. Lee and Houk[6] originally proposed that in the stabilized carbene, protonation at O4 is concerted with decarboxylation. Later studies demonstrated that the reaction precedes stepwise.[16] Later, Lee *et al.*[7] proposed that a water molecule in the active site was protonated by Lys-93 and in turn that water molecule protonated O4. As the C-C bond breaks, stabilization is achieved via a resonance stabilized carbene. As to protonation at O4 instead of O2, studies indicate O4 protonation is slightly more energetically favored because it is more basic and intrinsically preferred.[17]

Nucleophilic Addition at C5

In the early studies of ODCase a nucleophilic addition at C5 was proposed by Silverman and Groziak[8] (Figure 1.6).

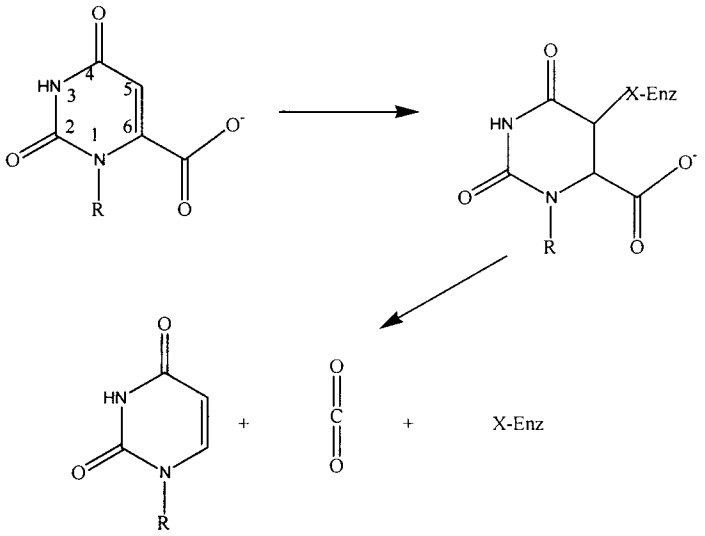


Figure 1.6: Nucleophilic Addition at C5

Silverman and Groziak attempted to explain the rate acceleration as a Michael addition with decarboxylative elimination. The reasoning stemmed from their studies of other covalent addition-elimination catalyzed reactions as precedents. This mechanism is ruled out because crystal studies indicate no nucleophiles exist at the active site[18].

Protonation at C6

Another mechanism involves protonation at C6 (Figure 1.7).

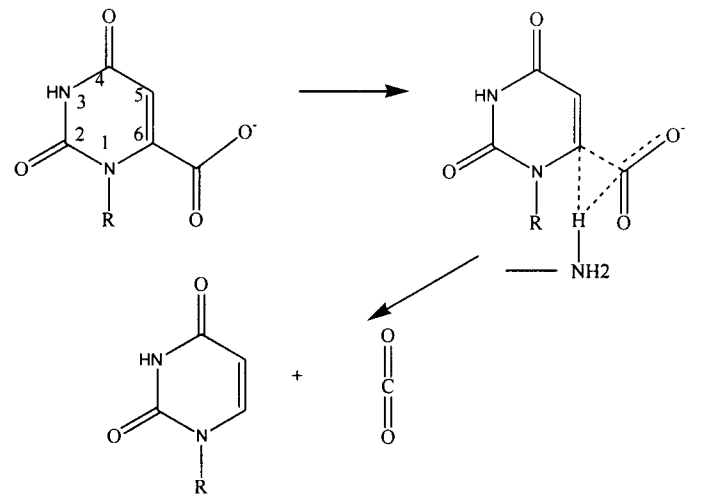


Figure 1.7: Protonation at C6

Groups supporting this mechanism believe that the electrostatic repulsions of the C6 carboxylate group with a nearby Asp residue at the active site initiates decarboxylation.[9,19] Supporters of C6 protonation argue that Lys-93, which provides the proton for protonation, is within proton donating distance of C6, not O2, O4, or C5.[9,18-20]

Zwitterion mechanism: protonation at O2

Basing a mechanism entirely on the presence of active site amino acid residues seen in crystal structures can be faulty, because it does not take into account all the events that may take place at that active site. The recent mechanisms proposed from the crystal structures suggest OMP binds with C6 facing the Lys-93 residue based on the binding of inhibitors. If OMP binds with the pyrimidine ring rotated 180° from that of the inhibitors in the crystal structures, the O2 becomes near the Lys-93 residue ready for protonation. This most closely supports the zwitterion mechanism. In fact, proponents of O4 protonation claim they cannot rule out O2 protonation.[17] Lundberg *et al.*[13] report that protonation at C6 is unlikely due to a high energy barrier, 20 kcal/mol. To rule out O4 protonation, Smiley *et al.*[15] did substitutions at O4 which yielded no difference to the active site with decarboxylation still occurring. Other studies indicate that a Michael addition to C5[14] was not likely, ruling out that mechanism.[10-11]

Thesis Overview

This thesis aids the investigation into the mechanism of ODCase through various expression systems and mutations. The ODCase gene was derived from the yeast strain *Saccharomyces cervisiae* (*S. cereviseae*). From there the gene was previously cloned into the vector pGU2. Expression was first attempted through ESP® Yeast Protein Expression and Purification System and ESP® Yeast Protein Expression Vectors

purchased from Stratagene. The gene was cloned into the vector pESP-1 then transformed into *Schizosaccharomyces pombe* (*S. pombe*). Growth in yeast proceeds at a decreased rate when compared to bacterial growth. For this reason along with a lack of definitive expression evidence and discontinuance of product, the ODCase gene was then expressed in Affinity® Protein Expression and Purification System and Affinity® Protein Expression Vectors also purchased from Stratagene based on success with 3-hydroxyanthranilic acid dioxygenase (3HAO)[20] and alkaline lipase.[21] In the system by Stratagene, the gene is cloned into the vector pCal-n and transformed into *Escherichia coli (E. coli)* strain XL1-Blue followed by the BL21 (DE3) strain. The bacterial system allowed for rapid and efficient growth of protein.

The ODCase gene underwent mutagenesis for future mechanistic studies using ^{15}N-labeled NMR experiments. One mutation, known as K93C, was first made at University of North Carolina, Chapel Hill, by Smiley and Jones.[22] The abbreviation K93C represents that at the 93rd position, a lysine as represented by it one letter abbreviation K is changed to a cysteine as represented by its one letter abbreviation, C. They found that mutating the lysine residue at the 93rd position resulted in loss of activity of the enzyme. They further revealed that with chemical modification using 2-bromoethylamine and creating a lysine analog (S-(2-aminoethyl) cysteine) activity was restored. Based on this observation and the works of Fred C. Hartman and other collaborates,[23-26] it was determined that the mechanism might become more apparent through use of changes in proton shifts in isotopically labeled NMR experiments.

The ODCase gene contains four naturally occurring cysteine residues. Attempting isotope labeling at the 93rd position with ^{15}N 2-ethylbromoamine could result

in alteration of all five cysteine residues and create unwanted signals on an NMR spectrum. To eliminate this possibility, a new mutant, C93only, was devised. The ODCase C93only mutant consists of serine residues at positions 33, 56, 155, and 263 instead of the naturally occurring cysteine residues. Previous mutagenesis work contributed an ODCase CysFree mutant in which there are no cysteine residues and the 93rd position remains unaltered with a lysine residue. ODCase CysFree serves as a control for the C93only mutant after aminoethylation when looking at changes in ODCase activity.

The goal of this thesis is to demonstrate an acceptable system for expression of mutant and WT ODCase allowing ease in purification, and rapid growth procedures.

CHAPTER II: USEFUL TECHNIQUES AND THEORY

Introduction

Several different biochemical techniques appear at several steps throughout the research process. It is more efficient to include an introduction to the theory behind these techniques and provide standard running conditions here rather than repeatedly throughout the chapters. When mentioned later in this thesis, variances in conditions will be noted. The techniques mentioned here appear alphabetically for simplicity.

Agarose Gel Electrophoresis

Introduction

Agarose gel electrophoresis is a technique that separates small quantities of DNA for verification and purification purposes. Agarose gel electrophoresis uses an electric field and an agarose matrix to separate DNA by size. Once the electric field is applied, the DNA begins to migrate toward the anode due to negatively charged phosphate groups. Friction during movement through the agarose matrix causes size separation with smaller molecules traveling farther on the gel than larger ones. The bands are visualized using ethidium bromide as a staining reagent which attains enhanced fluorescence by intercalating between the DNA.

Materials

1 kb DNA Ladder (New England Biolabs), TAE, ethidium bromide, Biorad electrophoresis unit, sample buffer. Sample buffer consists of 20 % v/v Ficoll, 0.1 M Na_2EDTA pH 8.0, 1 % w/v SDS (both from Fisher), and 0.25 % v/v bromophenol blue (Baker). 10x TAE consists of 0.4 M Tris-base (Amresco), 0.2 M glacial acetic acid (Fisher), and 0.01 M EDTA(sodium salt).

Standard Conditions

About 100 ng of DNA is adequate for visualization on a gel and is prepared with 5 μL of sample buffer which can be visualized while the gel is running. A known marker such as a 1 kb DNA Ladder is used for reference in determining the size of the samples. Normally 3 μL is adequate for visualization. The samples are loaded into wells on the gel, placed into the apparatus and the unit is filled with 1x TAE until the gel is covered. The current is set at 100 V for a smaller gel and 120 V for a larger gel. Depending on the size of the sample, the blue markers are either off the gel (larger bands) or until they migrate 2/3 the length of the gel (smaller bands). The apparatus is turned off and the gel is placed in an ethidium bromide/TAE mix for 5-10 min. After staining, the gel is visualized on a fluorescent field and can be saved for a picture. At this time, if gel slice purification is warranted, it can be done easily during visualization.

Bradford Assay

Introduction

The Bradford assay is a colorimetric assay used to determine the concentration of protein. Bovine serum albumin (BSA) of a known concentration is used as a standard. A standard plot of protein mass versus absorbance is generated to determine the unknown concentration of the protein. This works because Coomassie Brilliant Blue dye binds protein in an acidic solution shifting the absorbance wavelength from 465-595 nm. Bradford assays are therefore measured at 595 nm and the samples turn a blue color indicating bound protein. Binding is completed after 5 min with contaminations occurring after 1 h. It is important to use protein concentrations that give absorbance readings between the lowest and highest measured standard spectra. Absorbances over

1.0 are rejected because the direct relationship of absorbance to protein concentration is lost. Often it is useful to perform dilutions to obtain an accurate reading. When generating a plot, it is useful to run standard samples in duplicate for verification of results. Sometimes the points are not completely linear and can be eliminated from the plot.

Materials

Bradford reagent (consists of Coomassie Brilliant Blue) provided by Biorad. Sodium chloride and cuvettes purchased from Fisher. Bovine serum albumin standard (2 mg/ml) purchased from Pierce. Eppendorf supplied the 1.5 mL tubes. The diode array spectrophotometer was from Hewlett Packard 8453 and envisioned on a Gateway 2000 Vivitron computer.

Standard Conditions

The following are added to 1.5 mL concial tubes and mixed by inversion.

Sample	Bradford Reagent	0.15 M NaCl	0.5 mg/mL BSA
1 (blank)	900 μL	100 μL	0 μL
2	900 μL	95 μL	5 μL
3	900 μL	90 μL	10 μL
4	900 μL	80 μL	20 μL
5	900 μL	70 μL	30 μL

Table 2.1: Bradford Standard Assay

It is common to begin using 15 μL of the sample protein, 85 μL NaCl and 900 μL Bradford reagent. From there it can be determined whether or not a dilution is necessary.

A plot is generated using absorbance at 595 nm on the y-axis and μg of protein for the x-axis. An equation of the line can be determined and using simple algebra, the unknown concentration can be determined. Sample math, data, and graph follow:

μg BSA: 30 μL x 0.5 μg/μL = 15 μg BSA

μg BSA	Abs 595 nm	Abs 595 nm
2.5	0.127	0.129
5.0	0.279	0.277
10.0	0.481	0.483
15.0	0.603	0.605

Table 2.2: Sample Bradford Assay Data

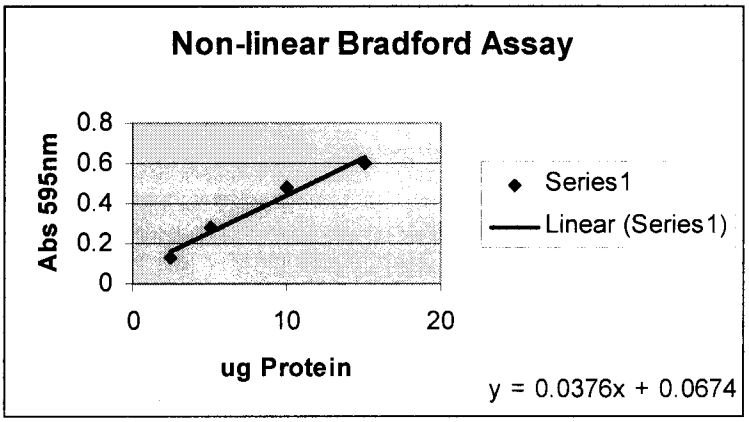


Figure 2.1: Bradford Standard Pl.ot, Poor Example

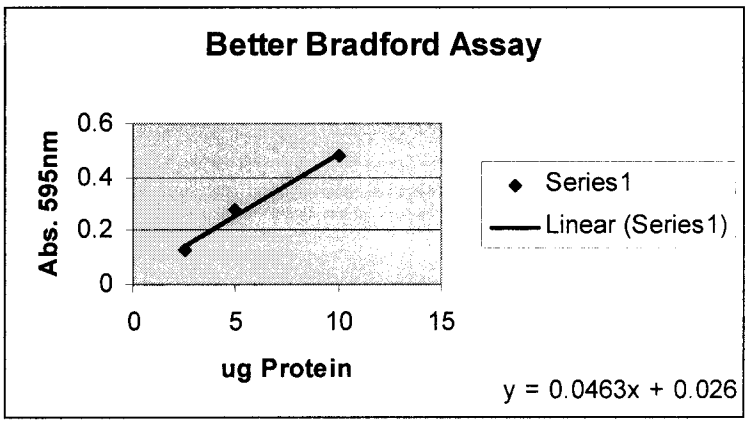


Figure 2.2: Bradford Standard Plot, Better Example

A second graph was created because the original data points do not make the best line, but dropping the last points provide a better graph.

Determining unknown concentration:

A 15 μL sample has an absorbance at 595 nm of 0.395. Using the equation from Figure 2.2, y = 0.0463x + 0.026, the amount of protein is 7.97 μg. Dividing this by the amount used in a 1.0 mL sample, 7.97 μg / 15 μL = 0.531 μg/μL.

Miniprep Purification and Analysis

Introduction

This miniprep procedure purifies small quantities of DNA and is used for screening transformants for changes in plasmid size by visualization on an agarose gel. Solution B lyses the cells when NaOH denatures chromosomal and plasmid DNA and SDS, a detergent, denatures bacterial proteins. The plasmid reanneals and the solution is neutralized with solution C. The chromosomal DNA becomes trapped with potassium and SDS and is precipitated and removed by centrifugation.

Materials

GTE Solution A: 50 mM glucose(Amresco), 25 mM Tris-HCl pH 8.0 (Eastman), 10 mM EDTA; Solution B: 0.2 N NaOH, 1 % w/v SDS (both from Fisher); Solution C: 5 M Acetic Acid pH to 4.8 using KOH (both from Fisher); ethanol, isopropanol (both from Pharmco), 1.5 mL conical tubes (Eppendorf)

Standard Conditions

Colonies from plates are picked and incubated overnight in 5 mL of LB media. 1.5 mL culture is transferred to a 1.5 mL conical tube and centrifuged at high speed for 1 min. It is then resuspended in 100 μL of Solution A, vortexed to mix, followed by the addition of 200 μL of Solution B and inverted several times to mix. After incubating for 5 min at room temperature, 300 μL of solution C is added and set on ice for 5 min

followed by centrifugation at high speed for 5 min. The supernatant is transferred and precipitated with 0.8 mL of isopropanol and reacted for 5 min on ice. The resulting pellet is dried and resuspended in 50 μL 1x TE. The analysis is done using agarose gel electrophoresis with 5-10 μL of sample providing adequate visualization.

Polymerase Chain Reaction, PCR

Introduction

PCR is a quick and efficient technique for molecular cloning and amplifying DNA. Automation with a thermal cycler further increases its appeal. A double-stranded DNA template become separated through heat denaturation. Oligonucleotide primers flank the desired areas of the DNA sequence to be amplified. With a decrease in temperature the primers anneal to each newly produced single strand of DNA at the complementary segment and the polymerase then directs the synthesis of amplification. This process is repeated 30-40 times, potentially doubling the amount of desired DNA each cycle.

For applications of PCR, $Vent_R$® DNA Polymerase is used because of its ability to withstand high temperatures without loss of activity and also for its proofreading exonuclease activity.

Materials

$Vent_R$® DNA Polymerase and ThermoPol Reaction Buffer were ordered from New England Biolabs. Oligonucleotide primers were purchased from IDT. Beckman Coulter provided dNTPs. DNA template came from prior research in the lab. Conical tubes were purchased from Eppendorf.

Standard Conditions

For DNA amplification the following are added in the order listed, sterile diH_2O to complete 100 μL mixture, 1x ThermoPol Reaction Buffer, 200 μM each dNTP, 0.5 μM each oligonucleotide primer, 1 ng of DNA, 1-2 U $Vent_R$® DNA Polymerase. The reaction is carried out in either a 0.2 or 0.5 mL concical tube depending upon the thermal cycler to be used. The tubes are kept on ice until placed into the thermal cycler. The program consists of 35 cycles at 95°C for 1.5 min, 60°C for 2 min, 75°C for 2 min with holding at 4°C. Most often the products are purified and verified on an agarose gel using 5-10 μL of the PCR mix.

Quantification of DNA

Introduction

Oftentimes it is important to determine the concentration of a sample of DNA for use in future experiments and verification. There is a direct relationship stemming from Beer's law $A=\varepsilon lc$ where A is the absorbance, ε is the extinction coefficient (variable depending upon substance), l is equal to 1 cm and describes the pathlength, and c is the concentration in μg/mL. For DNA, whether single or double-stranded, the extinction coefficient never changes allowing one to determine the concentration using a simple ratio.

Materials

A Hewett Packard 8453 spectrophotometer and cuvettes from Fisher are the only materials needed in addition to a sample of DNA.

Standard Conditions

About 10-20 μL of DNA is added to sterile dH_2O. The spectrophotomer absorbs at a wavelength of 260 nm and a spectrum is obtained using water as a blank. The following equations relate the absorbance to concentration for either single or double-stranded DNA.

Double-stranded DNA: A_{260} 1/ 50 μg = A_{260} measured/ x μg

Single-stranded DNA: A_{260} 1/ 37 μg = A_{260} measured/ x μg

QIAGEN® Purification

Introduction

The Qiagen® company provides simplistic kits that enable one to purify plasmids and other DNA samples quickly and with high efficiency. This project used both QIAquick® PCR Purification Kit #28104 and QIAfilter™ Plasmid Midi DNA Purification kit # 12143. The basic principal is DNA binding to the selective QiaResin. The DNA binds in a low pH and salt concentration and elutes when the pH increases. Isopropanol is then used to desalt the DNA and further concentrate it.

Materials

QIAquick® PCR Purification Kit #28104 and QIAfilter™ Plasmid Midi DNA Purification kit # 12143 provide most of the necessary equipment and reagents. Additional supplies are 1.5 mL conical tubes (Eppendorf), ethanol, isopropanol (Pharmco), centrifugal tubes (Labware) and Centrifuge from Sorvall and microcentrifuge from Fisher are also required.

Standard Conditions

Spin Column Purification of PCR product: 5 volumes of PB is added to the PCR product mix (1 volume) and applied to a spin column previously placed in the provided conical tube. This is centrifuged at high speed for min. The flow through is removed and the column is washed with 750 μL of PE. The tube is centrifuged at high speed for 5 min, the flow through is removed and spinning repeated. The column is removed and placed into a sterile 1.5 mL conical tube and eluted with 50 μL EB through centrifugation at high speed for 5 min. The product can be verified on an agarose gel using 5 μL.

Plasmid Purification using QIAfilter™: After overnight incubation of a 50 mL culture spun at 3000 x g for 15 min at 4°C, the pellet is resuspended in 6 mL cold P1 and vortexed until thoroughly mixed. Next, 6 mL of P2 is added and the tube is inverted to mix and incubated at room temperature for 5 min. After incubation, 6 mL of cold P3 is added and inverted a few times to mix. This lysate is immediately added to a capped QIAfilter cartridge and allowed to settle for 10 min at room temperature. At this point a High Speed MidiTip column is equilibrated with 4 mL QBT. The lysate is added using a plunger after the cap is removed. The column is washed with 20 mL QC and the DNA is eluted with 5 mL QF into a sterile 15 mL conical tube. The DNA is precipitated with 3.5 mL isopropanol and incubated at room temperature for 5 min. The solution is run through a QiaMini Module attached to the end of a 20 mL syringe. The module is washed with 2 mL of 95% v/v ethanol. After all liquid is removed the module is attached to a 5 mL syringe and DNA is eluted with 1 mL 1x TE to a sterile 1.5 mL conical tube.

The concentration is then determined using UV spectrophotometry and DNA is verified on an agarose gel using 100 ng.

DNA Sequencing

Introduction

The Sanger dideoxy method of chain termination begins with the use of DNA polymerase to synthesize complementary copies of single-stranded DNA to be sequenced as mentioned in PCR sequencing. Dideoxynucleotides become incorporated into the DNA, thereby halting synthesis. These truncated chains are electrophoresed on a sequencing gel and separate according to size. The automation of this process complete with fluorescent labeling allows for quick and highly efficient sequencing results. A printout is then available through the use of appropriate software thus eliminating the need to mechanically read a gel and piece together the sequence.

Materials

Most reagents provided by Beckman Coulter for use in Dye Terminator Cycle Sequencing including sample loading solution, pUC18 control template, -47 sequencing primer, glycogen, and DTCS premix. DTCS premix contains 10x sequencing reaction buffer, dNTP mix, ddNTP Dye Terminator mixes, polymerase enzyme. DNA template primers purchased from IDT and selectively designed depending upon sequenced sample. Thermal cycler from MJ Research, NaOAc and EDTA from Fisher. Sequencing completed on a CEQ 2000XL DNA Analysis Systems.

Standard Conditions

Protocol followed according to guidelines by Beckman-Coulter. In the case of PCR used in preparing samples for sequencing, only one oligonucleotide primer (1.6 μM)

is necessary. No polymerase, buffer, or dNTP's are added individually, instead a DTCS solution containing these is used. For single-stranded DNA, 150 ng is necessary. Double-stranded DNA requires 250-500 ng with both double and single-stranded DNA volumes not greater than 6 μL. Also, the reaction volume is much smaller at 20 μL. The reaction proceeds differently for 30 cycles consisting of 20 sec at 96°C, 20 sec at 50°C, 4 min at 60°C followed by holding at 4°C.

The samples are then removed from holding at 4°C and 4 μL of equal volumes of 3 M NaOAc and 100 mM EDTA are added to quench the reaction. A 20 μg of glycogen solution (1 μL) is added followed by 60 μL of 95 % v/v cold ethanol. The samples are spun at 4°C at high speed for 15 min. The supernatant is removed with a pipette and the pellet is redissolved in 200 μL of 70 % v/v ethanol. The samples are spun at the same conditions for only 2 min. This step is repeated and the final pellet is vacuum dried in a Savant Speed Vac for 40 min. The pellet is dissolved in 40 μL of sample loading solution and stored at -20°C. Operation of the YSU DNA sequencing center by Diana Arnett in the Biology Department.

The results are contained and interpreted using CHROMAS on the computer and converting the peaks into a formatted printout for use in comparison.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, SDS-PAGE

Introduction

This is another example of electrophoresis used to separate molecules, but this time proteins can be separated. Migration occurs based on size, with smaller proteins migrating further through the matrix than larger proteins. The applied current is used to separate the protein into distinct bands. The two parts of the gel are the stacking and

separating portions. In the stacking or upper part of the gel, the protein bands begin aligning on top of each other according to size. They become separated as they diffuse through the lower gel reservoir. A difference in pH between these two portions of the gel cause protein stacking. When the protein is bound to SDS it configures into a rod-like shape. The large negative charge of SDS masks the protein's charge leading to migration toward the anode.

Materials

Kaleidoscope molecular weight markers and Coomassie Brilliant Blue purchased from Biorad. TEMED, dithiothreitol (DTT), glycerol and Tris-base were purchased from Amresco. Bromophenol blue was purchased from Baker. Tris-HCl from Eastman. Glycine, sodium dodecyl sulfate (lauryl sulfate), acetic acid, and ammonium persulfate from Fisher. Methanol purchased through Pharmco. Stacking gel: 40 % v/v Acrylamide/bis 29:1, 10 % w/v SDS, 1 M Tris-HCl pH 8.8, 1 % w/v ammonium persulfate, 0.02 % w/v TEMED. Separating gel: same as separating gel only 1 M Tris-HCl pH to 6.8. Protein running solution: 196 mM glycine, 0.1 % w/v SDS, 50 mM Tris-HCl pH 8.3. 2x-SDS Buffer: 100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol. Staining solution: 0.2 % Coomassie Brilliant Blue in 45/45/10 % v/v/v methanol, H_2O, acetic acid. Destaining solution: 25/65/10 % v/v/v methanol, H_2O, acetic acid. Gel equipment provided by Biorad including a Power Pak 300.

Standard Conditions

The gel is placed facing the chamber and filled with 1x protein running buffer until the inner and outer chamber are near the top. It is necessary to fill the inner

chamber until the top of the wells is covered. Samples are prepared by mixing with 2x SDS buffer and boiling for 5 min. They are pipetted into individual wells. An electric current of 80 V is applied until the bands cross through to the separating portion of the gel and then it is increased to 120 V. The colorful molecular weight markers enable one to see approximately where on the gel the protein might be. Once the protein has run a desired amount length of the gel and good separation can be obtained, it is removed from the plates and stained in an acetone/methanol/bromophenol blue solution for 5 min. It is then destained with continuous additions of destaining solution consisting of acetic acid and methanol in water. The bands remain stained as the protein binds to the dye and the rest of the gel washes clean.

CHAPTER III: pESP WT AND MUTANTS EXPRESSED BY *Schizosaccharomyces pombe*

Introduction

Most organisms already contain the enzyme ODCase. The small quantity located in the cell is difficult to purify and creates the need to overexpress this enzyme for any investigative purposes. To do this and to introduce mutations, an overexpression/overproduction system is introduced using molecular cloning techniques. In doing so, the gene is located adjacent to a promoter that triggers overexpression of the gene. On a cellular level, the promoter begins in a repressed state. Under repressed conditions, cell growth continues without gene expression and protein synthesis. The application of high concentrations of another metabolite, an inducer, triggers the release of the repressor on the promoter and gene expression and protein synthesis begins in the cells at high rates.

ESP® Yeast Protein Expression and Purification System and ESP® Yeast Protein Expression Vectors by Stratagene allows for quick, high yielding expression and purification of desired protein. In this expression system a glutathione S-transferase (GST) fusion peptide is used as a purification tag. The protein of interest as fused to GST can then be easily purified through affinity chromatography. A one step cleavage reaction releases the protein from GST with bovine enterokinase or thrombin.

S. pombe

Yeast strain SPQ01 is used instead of *E. coli* because of its allowance for post-translational modification. This particular strain of yeast is haploid with a mating type of H^-. The genotype of *leu*$^-$ affects the phenotype in which this mutation renders *S. pombe*

unable to grow on a medium without the presence of leucine. This provides possible screening techniques during transformation.

Plasmid pESP-1

The *S. pombe* host carrying pESP-1 and derivatives has ideal properties such as being a single cell eukaryotic organism with attributes credited to higher eukaryotic organisms including chromosome structure and function, cell-cycle control, and RNA splicing. Some features of pESP-1 include a pUC origin of replication, ampicillin resistance gene, ARSI sequence vector replication, *Leu2*-d gene selection marker, nmt1 promoter and terminator, and a GST gene flanking the multiple cloning site (MCS). The MCS, located between bases 8828-8884, contains restriction sites to enable researchers to clone genes into the vector.

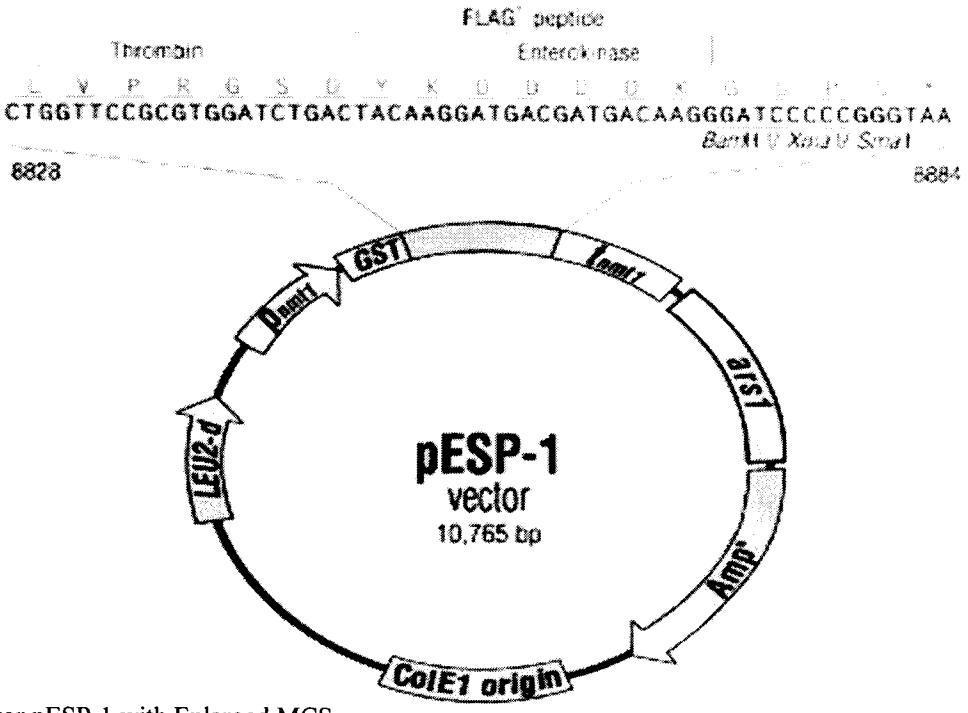


Figure 3.1: Vector pESP-1 with Enlarged MCS

Experimental

This chapter describes the process of transferring ODCase WT, K93C, and CysFree genes into pESP-1 and introduction into *S. pombe*. ODCase WT and K93C genes were originally found in the plasmid pGU2 from past research. ODCase CysFree was created and already in the plasmid pESP from tandem research efforts in the lab and was included in this project at a later time than WT and K93C. Most protocols were adapted from the guidelines described in ESP® Yeast Protein Expression and Purification System and ESP® Yeast Protein Expression Vectors by Stratagene and ODCase lysis conditions from Bell and Jones[27]. Verification of transformations and cloning were done through double digestion using restriction enzymes and sequencing.

The end of the chapter describes the process of protein expression and enzyme activity. Due to determination of poor protein expression levels and lack of promising data, the ESP® expression system was ceased.

Preparation of pGU2 ODCase WT and K93C

Introduction

The pGU2 ODCase WT and K93C plasmids were obtained from glycerol stocks in *S. cereviseae* and were freshly plated on LB/Amp medium. Colonial growth provided information to the presence of the inserted material in pGU2. A single colony is selected and prepared and analyzed using the miniprep protocol. On the agarose gel, inmodified pGU2 is used as a control to verify if the bands present in the lanes showing pGU2 WT and K93C do contain an insert by appearing slightly raised. Once the presence of the insert is verified, the gene can be cloned into pESP-1 and transferred to *S. pombe*.

Materials

Stocks of pGU2 WT and K93C plasmids were made previously in the lab. Purchases from Fisher include tryptone, NaCl, $CaCl_2$, EDTA, and agarose. Agar and Tris-base were from Amresco. Restriction enzymes, buffers, and ligases were purchased from New England Biolabs. Eastman carried Tris-HCl. Sterile 50 and 15 mL tubes from Labware, Falcon provided the tubes for the transformation. LB medium includes 10 g tryptone, 5 g NaCl, 5 g yeast pH 7.4 with NaOH in 1.0 L. Amp: media plus 0.1 % w/v ampicillin. Agar plates: media plus 0.75 % w/v agar.

Procedure

A sterile inoculating glass rod is lowered into an *E. coli* glycerol stock containing either pGU2 WT or pGU2 K93C and then is streaked across an LB/Amp plate and incubated overnight at 37°C. After the appearance of several colonies, a single colony from each plate of either WT or K93C is inoculated in 5 mL LB/Amp overnight at 37°C with shaking. A miniprep purification step is then performed and the resulting plasmids are analyzed on an 0.8 % w/v agarose gel using 5 μL of sample buffer in addition to either 5 μL of K93C, WT, or pGU2, and 1 kb DNA Ladder.

Results and Discussion

Purification for both pGU2 WT and pGU2 K93C was indicated by the presence of bands on an agarose gel (gel not provided). The colonies from the plates were then used in subsequent steps to complete the expression system.

Transformation

Introduction

The pGU2 plasmids and pESP-1 plasmid are purified using QIAGEN® Plasmid Midi Purification Kit to allow for their ligation and subsequent transformation. Restriction endonucleases are necessary enzymes in molecular cloning due to their aid in ligations and characterization. A restriction site is a 4-8 base sequence of double-stranded DNA that can be cut specifically by a restriction endonuclease, producing an overhang known as a cohesive or sticky end. These cohesive ends allow for complementary base pairing between other digested DNA.

BamHI restriction site:	5'-G↓GATCC-3' 3'-CCATG↓G-5'
XmaI restriction site:	5'-C↓CCGGG-3' 3'-GGGCC↓C-5'

The use of two different restriction sites diminishes the probability of plasmid self-ligation. A ligation uses an ATP dependent enzyme, ligase, to form phosphodiester linkages at insertion sites for plasmid closure.

A transformation is the process by which cells take up exogenous DNA in the presence of $CaCl_2$. The carrier such as *E. coli* strain XL1-Blue, is grown until reaching midlog growth. The cells are treated with $CaCl_2$, enhancing their ability to take up DNA. The plasmid DNA can then efficiently enter the pretreated cells during the period of heat shock. The resulting mixture is incubated in LB broth to allow expression of antibiotic resistance to begin, then plated on LB/Amp plates. If colonies appear it is evident that the plasmid has been taken up due to the lack of an ampicillin resistance gene in XL1-Blue.

The transformants are verified through restriction site double digestion and sequencing. In a double digestion, the plasmid construct of pESP-ODCase-WT and

K93C are digested with the same endonucleases that enabled ligation. The presence of an additional band on the gel appearing at about the size of ODCase indicates that there is an insert. To determine if the insert is correct, DNA sequencing is employed using primers for the ODCase gene and the Sanger dideoxy chain termination method of sequencing.

Materials

Supplies purchased from NEB include: NEB buffer-4, buffer U, BamHI, Sma1, XmaI, EcoR1, 10x BSA, T4 DNA ligase and buffer. Oligonucleotide primers were purchased from IDT. Purchases from Fisher include petrie dishes, tryptone, NaCl, $CaCl_2$, EDTA, Sodium acetate, phenol/chloroform/isoamyl alcohol and agarose. Amersco provides DTT and yeast extract. Ethanol, methanol, isopropanol all were purchases from Pharmco and Tris-HCl from Eastman. Eppendorf supplied 1.5 mL conical tubes and Falcon supplied 15 mL round bottom tubes. $CaCl_2$-Tris solution: 10 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5, 1 % w/v DTT.

Preparation for Ligation: A 50 mL culture of pGU2 K93C was grown overnight in LB/Amp at 37°C. The cells were then collected by centrifugation at 3000 x g in a Sorvall centrifugation and then purified according to QIAGEN®. The DNA was then visualized on a 0.8 % w/v agarose gel. The purified DNA was then ready to be amplified using PCR and primers coordinating to the restriction sites of BamHI for start and XmaI for stop. Following verified successful PCR, the amplified products are then purified using a QIAquick PCR Purification Kit and again verified that the purification was successful.

The next step was digestion of the amplified products with restriction enzymes. The restriction reaction mix consists of a total volume of 20 μL using 1 μg of PCR purified product, 1/10 total volume of 10x NEB Buffer-4, 1-2 U of both BamHI and XmaI and diluted with sterile diH_2O. The reaction is incubated at 37°C for 2 h and the enzymes are heat killed for 20 min at 80°C. At this time, pESP-1 is also digested for ligation using the same endonuclease restriction sites.

The ODCase WT and K93C genes are ligated to the vector pESP-1. The reaction mixture consists of 1 μM purified restricted ODCase K93C or WT (done separately), 100 ng restricted digestion product pESP-1, 1 U T4 Ligase, 1x T4 Ligase Buffer, and diluted to 20 μL with sterile dH_2O. The reaction proceeds 10 min at room temperature followed by placement on ice. Positive and negative control ligations are also performed. The positive control consists of only pESP-1 and the negative control contains no DNA.

Transformation: The ligated pESP WT and pESP K93C products are then transferred into the *E. coli* strain XL1-Blue. The first step is to prepare competent cells for the transformation using calcium chloride. A toothpick from a freezer stock of XL1-Blue is incubated in 10 mL LB overnight at 37°C with shaking. A flask of 50 mL LB/Amp is inoculated with 0.5 mL of the overnight culture and grown for 3 h at the same conditions. The cells are then centrifuged at 3000 x g, 4°C, for 10 min. The pellet is resuspended in 25 mL cold $CaCl_2$-Tris and kept on ice for 15 min. After another round of centrifugation, the cells are resuspended in a final volume of 1 mL $CaCl_2$-Tris and placed on ice until ready for use.

Chilled polypropylene round bottom tubes receive 0.2 mL of $CaCl_2$ treated cells. No DNA is added to the negative control and 1 μL of pESP-1 Qiagen purified is added to

the tube for the positive control. 5 μL of the ligation reaction is added and the tubes are placed on ice for 5 min. They are then heated at 42°C for 2 min and returned to ice. Upon the addition of 1 mL of LB, the tube is incubated at 37°C for 1 h with shaking. The mixture is then plated onto LB/Amp plates and incubated overnight at 37°C.

Individual colonies are selected from the experimental plates for pESP WT, pESP K93C, and pESP CysFree. All are incubated overnight in a test tube containing 5 mL LB/Amp. A preparation and analysis on an agarose gel is then performed with the mini-preps possibly containing the correct insert. First, a 0.7 % agarose gel is run using purified pESP-1 as the standard and running it against the possible ligated insertions. If the ligations went correctly, then the band of DNA should appear larger on the gel than the unmodified pESP-1. The mini-preps appearing to contain the insert were further purified, double digested, and run on an agarose gel to determine if the insert was present. Double digestion reaction includes 1μg of plasmid, 1/10 total volume of NEB Buffer 4, 1 U of both BamHI and XmaI, 1x BSA and diluted to a total volume of 30 μL with sterile dH_2O. The reaction proceeds for 2 h at 37°C. The entire reaction mixed is run on an agarose gel to see if there is a band for ODCase.

The presence of an additional band indicates the presence of an insert. However, to further verify the insert as ODCase K93C, CysFree and WT, sequencing is used according to the standard guidelines of Beckman-Coulter. The ODCase primers are as follows:

Start primer

5'- GGGAAAGGGGATCCATGTCGAAAGCTACATATAAGGAA-3'

Stop primer

3'-GGGAAAGGCCCGGGTTAGTTTTGCTGGCCGCATCTTCT-5'

Results and Discussion

For the Qiagen purification process of pGU2 WT and pGU2 K93C constructs, the agarose gel ran at 100 V until the blue marker reached 2/3 the length of the gel. Ethidium bromide staining showed the presence of purified pGU2 K93C and pGU2 WT in comparison to known molecular weights and the weights of the bands indicated on the DNA ladder (gel not provided).

Both gels for the PCR products and the PCR purified products were run at 100 V until reaching 2/3 the length of the gel. After staining, both gels showed the presence of one band between 0.5 and 1 kb on the DNA ladder. A negative control reaction containing no plasmid DNA was also run on the gel and the lack of any bands in this lane indicates no contaminations. To ensure proper digestions prior to ligation, an agarose gel is run at 100 V until the indicator reaches 2/3 the length of the gel. A positive control is run under the same conditions which contains a known insert to verify the activity of the enzymes. The appearance of an additional band for the control indicates that the restriction enzymes worked successfully.

After the ligation and transformation, the plate containing the positive control pESP-1 had several colonies of growth and covered almost the entire plate. The experimental plate containing the transformed cells appeared to have colonial growth as well. The negative control plate with no DNA had no colonies and this determined that there was proper transformation.

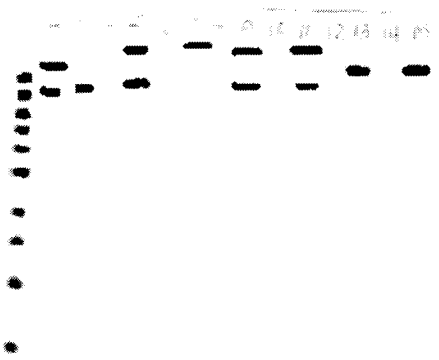


Figure 3.2: Agarose Gel of Purified Plasmid for Screening

Figure 3.2 shows Qiagen purified plasmid DNA run for two samples each of pESP WT, pESP K93C, and pESP CysFree. Lane assignments are as follows: 1) 5 μL DNA Ladder, 2) 100 ng pESP-1, 3) 100 ng 3HAO, 4) 0.037 μg K93C-A, 5) 0.37 μg K93C-A, 6) 0.015 μL K93C-B, 7) 0.15 μL K93C-B, 8) 0.0515 μg WT-A, 9) 0.515 μg WT-A, 10) 0.035 μg WT-B, 11) 0.35 μg WT-B, 12) 0.0234 μg CysFree-A, 13) 0.234 μg CysFree-A, 14) 0.0265 μg CysFree-B, 15) 0.265 μg CysFree-B. Designations of A or B indicated different sources. This gel demonstrates the possibility that each sample contains the insert due to their raised appearance compared to purified pESP-1 without insert.

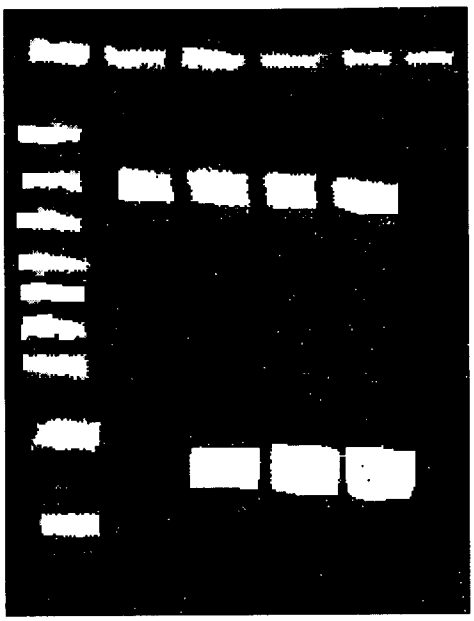


Figure 3.3: Double Digest pESP

Figure 3.3 shows the results of the double digestion of pESP WT, pESP K93C, and pESP CysFree (lanes 3-5 respectively). The gel demonstrates an additional band in lanes 3-5 between the two lower DNA Ladder bands (lane 1) that are not found in lane 2 representing pESP-1. From this information it is fairly clear that the plasmids have been properly constructed. This is then further analyzed through sequencing.

At times when no bands appear in a known plasmid construction, it is necessary to test the restriction enzymes for activity. XmaI denatures easily and can be substituted in the double digestion for verification of insert with SmaI. One simple way to test the restriction enzymes is to run a control along with the experimental digestion to see if the known ligation will cut. If it does not cut, then individual tests can be done on both restriction enzymes to determine if one or both are not active. This is done by running separate reactions for each restriction enzyme and comparing these results on a gel with

uncut pESP-1. The enzyme is determined to be inactive if the double band of purified pESP-1 is not reduced to a single band.

When it is evident from a miniprep that a sample contains an insert, Qiagen purification yields DNA for restriction digestion to produce a band signifying the insert's presence. If the DNA will not sequence with known working primers for ODCase, the DNA may contain too high of a salt concentration for sequencing. XmaI can still cut DNA in the presence of excess salt but the restriction enzyme, EcoRI cannot. To test for high salt concentration using EcoRI, the sequence is searched for the number of restrictions sites, including any caused through mutagenesis reactions. None of the mutations resulted in generation of an EcoRI restriction site. Therefore, pESP WT, pESP K93C, and pESP CysFree constructs should cut identically with EcoRI and produce 4835, 3802, 1200, 779, 88, and 65 kb size bands.

For the digestion, NEB supplied Buffer U with EcoRI. The reaction contained 0.5μg plasmid, 1 μL 10x BSA, 3 μL Buffer U, 2.5 μL EcoRI and was diluted to a final volume of 30 μL with sterile dH_2O. The reaction proceeds for 2 h at 37°C followed by analysis on an agarose gel with pESP-1 as a control.

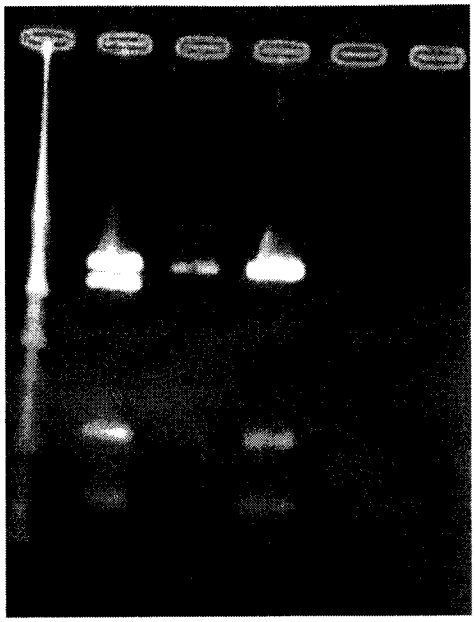


Figure 3.4: EcoRI Digestion

A similar cutting pattern for EcoRI digestion on pESP-1 and both pESP WT samples was represented (Figure 3.4). Neither pESP K93C nor pESP CysFree showed any digestion revealing that the enzyme was indeed inhibited by high salt concentrations. To rectify the situation, the samples were ethanol precipitated using equal volume of 25/24/1 v/v/v phenol/chloroform/isoamyl alcohol buffered with Tris followed by vortexing for 1 min then ice for 1 min and repeating. Next 1/10 the sample volume of 3 M NaOAc is added and mixed again with a vortex. Lastly 2x sample volume plus 1/10 original sample volume of 100 % cold ethanol is added and the tube is inverted to mix and stored 2 h at -20°C. Digestion was then repeated with the reduced salt pESP K93C and pESP CysFree plasmid constructions and was successful.

Sequencing showed the insertion of ODCase WT and ODCase CysFree into pESP-1. All ODCase K93C mutants did not have the mutation and were therefore simply ODCase WT.

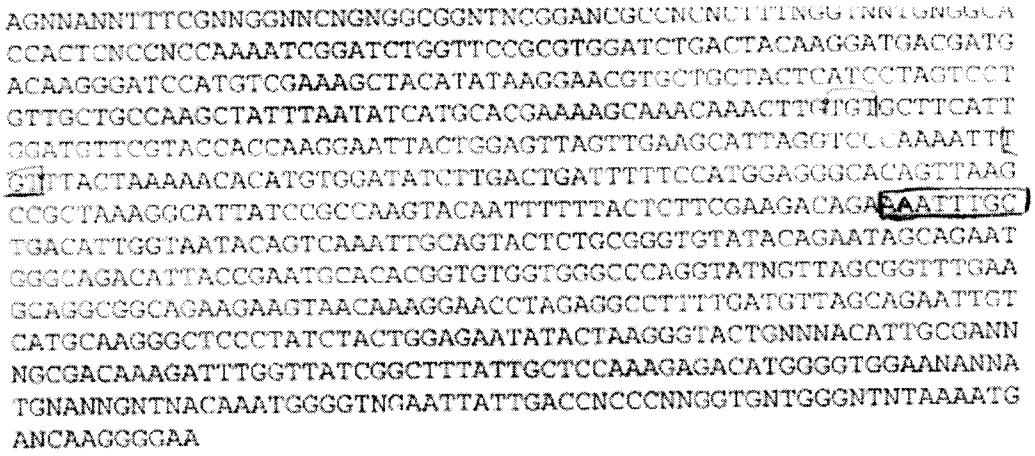


Figure 3.5: Sequencing Results pESP K93C

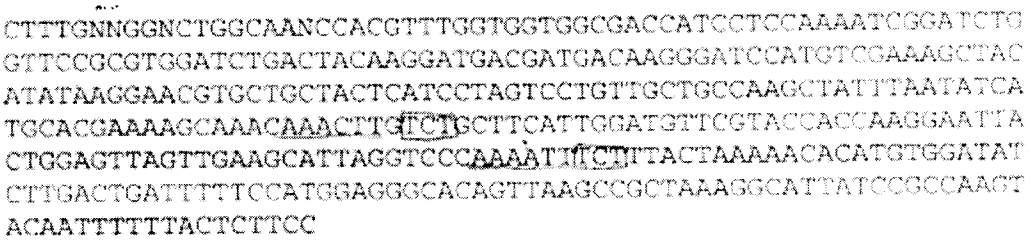


Figure 3.6: Sequencing Results pESP CysFree

Several attempts prior to these sequencing results revealed various errors. First attempt at transforming pESP-1 and ODCase WT resulted in ligating pESP-1 to itself! Other problems involved incorrect primers which were then replaced. The sequence of ODCase gene is known and is:

```
      ATGTCGAAAG CTACATATAA GGAACGTGCT GCTACTCATC CTAGTCCTGT
60    TGCTGCCAAG CTATTTAATA TCATGCACGA AAAGCAAACA AACTTG[TGT]G Cys33
120   CTTCATTGGA [TGT]TCGTACC ACCAAGGAAT TACTGGAGTT AGTTGAAGCA Cys56
180   TTAGGTCCCA AAATTTGTTT ACTAAAAACA CATGTGGATA TCTTGACTGA
240   TTTTTCCATG GAGGGCACAG TTAAGCCGCT AAAGGCATTA TCCGCCAAGT
300   ACAATTTTTT ACTCTTCGAA GACAGA[AAA]T TTGCTGACAT TGGTAATACA Lys93
360   GTCAAATTGC AGTACTCTGC GGGTGTATAC AGAATAGCAG AATGGGCAGA
420   CATTACGAAT GCACACGGTG TGGTGGGCCC AGGTATTGTT AGCGGTTTGA
480   AGCAGGCGGC GGAAGAAGTA ACAAAGGAAC CTAGAGGCCT TTTGATGTTA
540   GCAGAATTGT CATGCAAGGG CTCCCTATCT ACTGGAGAAT ATACTAAGGG
600   TACTGTTGAC AT[TGC]GAAGA GCGACAAAGA TTTTGTTATC GGCTTTATTG Cys155
660   CTCAAAGAGA CATGGGTGGA AGAGATGAAG GTTACGATTG GTTGATTATG
720   ACACCCGGTG TGGGTTTAGA TGACAAGGGA GACGCATTGG GTCAACAGTA
780   TAGAACCGTG GATGATGTGG TCTCTACAGG ATCTGACATT ATTATTGTTG
840   GAAGAGGACT ATTTGCAAAG GGAAGGGATG CTAAGGTAGA GGGTGAACGT
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900   TACAGAAAAG CAGGCTGGGA AGCATATTTG AGAAGATGCG GCCAGCAAAA Cys263
960   CTAA
```

Mutations and insertion is then verified by comparing the sequencing results to the known sequence. In the case of pESP-1 ligated to itself, the sequence was entered into a data base and found that it was pESP-1. For mutation determination, one of two methods is employed. One way to compare sequences is to use the online database provided by NCBI which will align and compare the two sequences. The other method is to format the sequence in a manner that frames are known and locate the position of possible mutations and comparing the nucleotides for a particular amino acid.

For determination of K93C: possible nucleotides that code for cysteine are TGT and TGC. Codons for lysine are either AAA or AAG with AAA appearing in ODCase. In the case of sequencing for K93C, only the codon for lysine was found indicating that no mutation was present. Therefore, more mutagenesis is required for ODCase K93C.

For the case of CysFree: The cysteine residues were changed to serine residues. The codons for serine are TCN with N being any nucleotide. Using this the sequencing primer only had to ensure that the second nucleotide was a cytosine. It was evident that such mutations were made at positions 33, 56, and 155. Due to limitations of sequencer and the proximity of the last cysteine residue to the stop codon, mutation C263S was not clearly evident, but seen in prior sequencing efforts.

Protein Expression

Transformation from pESP-1 to *S. pombe* SPQ01

Introduction

The strain of *S. pombe* provided by Stratagene had to be prepared for the final transformation from the pESP-1 vector in XL1-Blue to *S. pombe*. The yeast is prepared

and made into competent cells to be used in the transformation. The transformants are verified by their ability to grow on minimal media. The resulting colonies are then used to make 20% v/v glycerol stocks and stored at -70°C.

Materials

TE was provided in QIAGEN Plasmid Midi Purification kit. 1 M LiAc, EDTA, calcium chloride, and glucose were purchased from Fisher. The Stratagene kit contains *S. pombe*, and EMM powder. Peptone was purchased from Amresco. Agar and yeast came from Amresco. Thiamine, Tris-HCL and adenine sulfate were purchased from Eastman. DMSO was obtained from an independent biological laboratory and carrier DNA (salmon sperm) was previously obtained. 50%w/v PEG 3350 came from Sigma.

TE-LiAc solution consists of 1x TE and 1x LiAc. TE-LiAc-PEG solution consists of 1x TE, 1x LiAc, and 40 % w/v PEG. YPAD media consists of 20 g peptone, 10 g yeast, 40 mg adenine sulfate, 2.0 % w/v glucose in 1 L pH 5.8. EMM media contains 32 g of EMM powder in 1 L.

EMM consists of 14.7 mM potassium hydrogen phthalate, 15.5 mM Na_2HPO_4, 93.5 mM NH_4Cl, 111 mM glucose, 20 mL/L of 50x salts, 1 mL of 1000x stock vitamins, 0.1 mL of 10,000x minerals. 50x salt consists of 0.26 M MgCl, 4.99 mM $CaCl_2$, 0.67 M KCl, and 14.1 mM Na_2SO_4. Vitamins 1000x stock consists of 4.2 mM pantothenic acid, 81.2 mM nicotinic acid, 55.5 mM myo-inositol, 40.8 μM biotin. Minerals 10,000x consists of 80.9 mM boric acid, 23.7 mM $MnSO_4$, 13.9 mM $ZnSO_4$, 7.4 mM $FeCl_2$, 2.47 mM molybdic acid, 6.02 mM KI, 1.6 mM $CuSO_4$, and 47.6 mM citric acid. Autoclave conditions are 121°C for 15 min followed by a few drops of chlorobenzene/dichloroethane/chlorobutane (1:1:2) as a preservative.

Procedure

Protocols followed according to those from Stratagene. A toothpick stock of *S. pombe* is added to 2.0 mL of YPAD media and incubated at 30°C overnight. Plates of EMM-thiamine with agar are then plated with *S. pombe* and allowed to grow 2-3 d until colonies appear. Colonies about 2-3 mm in diameter are placed into 1 mL of YPAD and vortexed until clump-free. This is then added to a 50 mL flask of YPAD and incubated 18-24 h at 30°C with shaking. The 50 mL culture is then transferred to 300 mL of YPAD in a 1-2 L flask and incubated for 3 h at the above mentioned conditions. The cells are harvested by centrifugation at 1000 x g, for 5 min at room temperature. The pellet is resuspended in 1.5 mL of freshly prepared TE-LiAc.

In a hot water bath, 1 mL of carrier DNA is boiled for 20 min. Into prechilled conical tubes by Falcon, 100 µL of yeast cells are added using wide bore pipette tips followed by 100 µg, 5.3 µL of carrier DNA. For each experimental tube, 100 ng of pESP ODCase construct is added. For a positive control, 100 ng pESP-1 Qiagen purified is added. The negative control contains no additional reagents. All tubes are mixed by using wide bore pipettes. To the tubes, 600 µL of TE-LiAcPEG is added and mixed by vortex. The tubes are incubated for 30 min at 30°C with shaking. After incubating, 70 µL of biological grade DMSO is added and mixed gently followed by heat shock at 42°C for 2 min. The tubes are returned to ice for 10 min and centrifuged thereafter for 10 sec at 1000 x g. The supernatant is removed and the pellet is resuspended in 0.5 mL of 1x TE. Using wide bore tips, about 200 µL are added to EMM-thiamine agar plates and incubated 4-6 d at 30°C.

Results and Discussion

Prior to the transformation, the colonies produced by *S. pombe* were verified by checking under a microscope to ensure that it was yeast as confirmed by the presence of fission. It is also indicative of yeast colonies to appear creamy in color and raised compared to bacterial colonies. For the transformation, a positive control of *S. pombe* resulted in numerous colonies of growth. A negative control lacking DNA contained no colonies and indicated successful transformation parameters. The experimental plates contained fewer colonies than the positive control.

Induction and Enzymatic Activity

Introduction

The growth of protein can be a time consuming process, especially for growing yeast. The purpose of induction is to allow the cells to reach a high density before inducing the expression of the gene of interest. This is usually controlled in *E. coli* using the *lac* operon. When repressed the gene produces a protein that binds to the promoter and no protein is produced. In the case of *S. pombe*, induction is suppressed with the addition of thiamine. It should be apparent from comparison of induced and noninduced protein samples whether the protein is being expressed adequately. An enzyme assay provides insight into the purification of the protein and whether or not the induction was complete.

Due to the simplicity of the Stratagene expression system, all that is required is one-step affinity column purification. Affinity chromatography works on the basis of protein interactions of a solid phase bound ligand with the desired protein in mobile

phase. The protein binds to the ligand and is washed off when a higher affinity substance is introduced or salt concentration and pH conditions are varied.

Materials

EMM powder, YES powder, and GST Elution Buffer provided by Stratagene. Thiamine, adenine, histidine, leucine, uracil, and lysine hydrochloride were purchased from Eastman. NaCl and KCl were both from Fisher. Purchases from Amresco include glucose and agar. OMP was synthesized independently in the lab. Other items include conical tubes from Eppendorf, sterile tubes from Labware, 0.5 mm glass beads from Biospec, triton and KH_2PO_4 from Baker, and Na_2HPO_4 from Merck. YES media requires 35 g in 1 L. YES powder consists of 0.5 % w/v yeast extract, 3.0 % glucose, and 50-250 mg adenine, histidine, leucine, uracil, and lysine. PBS buffer: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4. GST elution buffer contains 10 mM glutathione and 50 mM Tris-HCl pH 8.0. PBST: PBS plus 1.0% w/v triton. PI: 1 μg/mL aprotinin, 1 μM pepstatin A, 100 μM leupeptin, 1 μg/mL chymostatin most of which were purchased through either Amresco or Sigma.

Procedure

A single colony from an EMM-thiamine agar plate is selected and incubated in 10 mL of YES medium overnight at 30°C with shaking. In the morning, 1 mL is transferred to 10 mL of fresh YES media and grown at the above conditions for 5 h. The time was determined through development of a doubling time plot of growth in YES media using cell density and Klett flasks. A Klett flask containing YES medium and a colony from a plate was grown according to the protocol, but the density was read several times over the course of 9 h. The cells were collected by centrifugation at 1000 x g for 5 min at room

temperature. The pellet was washed with sterile water and resuspended in 10 mL EMM broth. One tube contains 5 mM thiamine and is noninduced, suppressed, with all tubes growing 18-24 h at 30°C. The cells are collected and spun using a Sorvall centrifuge at 1000 x g for 5 min at 4°C. The resulting pellet is resuspended in100 µL PBST + PI. For cell lysis, about 0.25 g of 0.5 mm glass beads are added and bead beating occurs for 5 min at 1 min intervals followed by 1 min on ice. The cells are centrifuged at 12,000 x g for 5 min at 4°C and the supernatant is stored at -80°C until future use.

To begin column purification, the resin must be prepared by placing 1.5 mL in a 15 mL conical tube and centrifuging it for 5 min at 500 x g. The pellet is washed twice with 10 bed volumes of PBS buffer and mixed thoroughly through inversion. The final pellet is resuspended in 1 mL of PBS buffer and transferred to a 1.5 mL conical tube. From thawed cell lysate, 0.5 mL is mixed on a rocking bed at 4°C for 30 min. The mixture is then centrifuged at 500 x g for 5 min and resuspended in 5 bed volumes of PBST and again centrifuged at the above conditions. This process is repeated an additional 4 times. Lastly, 1 bed volume of GST elution buffer is added and incubated at room temperature for 5 min followed by centrifugation at 500 x g for 5 min. The supernatant is kept and these steps are repeated 3 more times, pooling all supernatants followed by protein quantification using the Bradford assay.

The ODCase enzyme assay is performed by setting the wavelength on the spectrophotometer to absorb at 286 nm. In a cuvette, 900 µL of diH_2O is added to 20 µL of 50 mM Tris-HCl pH 8.0 and 30 µL enzyme to obtain a blank spectrum. The substrate and enzyme amounts can vary by each assay but it is standard to begin using 50 µL of 1 mM OMP and 30 µL of enzyme. Immediately upon the addition of OMP the absorbance

is measured and is continually measured for a few min in timed intervals usually 10 sec. The absorbance decreases as OMP is converted to UMP by the enzyme.

Results and Discussion

Time	Hours	Klett Units
10:00	0	0
12:30	2.5	43
15:00	5.0	88
15:30	5.5	109
16:30	6.5	146.5
19:00	9.0	310

Table 3.1: Growth Time Determination in pESP

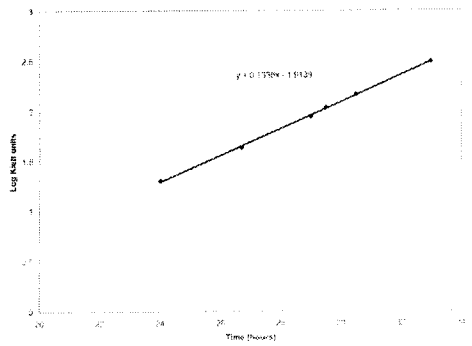

Figure 3.7: *S. pombe* Growth Determination

$$\Delta y\ 1 = \Delta x\ 1/0.1335$$

$$= \Delta x\ 7.5$$

$$\Delta y\ (\log 2) = \text{doubling time}$$

$$\Delta y\ (0.3) = \Delta x\ (7.5 \times 0.3)$$

The doubling time is 2.25 h.

To determine if induction occurred, the protein was run on an SDS-PAGE gel using 6x SDS Buffer. Concentrations were obtained using Bradford assay. The gel showed no difference between induced and uninduced samples. Therefore, a medium

scale induction was applied. This time the proper amount of lysis was determined by beating the cells and removing 100 μL for investigation of protein concentration each time until no more increases in protein occurred. Using the Bradford method for determining concentrations it was found that the protein is properly released from the cells after 1 min of lysis.

Fraction/minute	Concentration μg/μL
1	1.8
2	2.1
3	1.8
4	1.5
5	1.8

Table 3.2: Lysis Determination

The medium scale growth was repeated and the results were run on an SDS-PAGE gel using 50 μL protein and 50 μL 2x SDS Buffer mix and running 5, 10, 20, and 30 μL aliquots of this on the gel for each induced and uninduced. All three ODCase constructs underwent attempted purification followed by subsequent enzymatic activity assays. Unfortunately, only ODCase-WT appeared on an SDS-Page gel.

The enzymatic assays were performed by Dr. Smiley and revealed little or no activity for either induced or uninduced ODCase-WT.

Time min	Abs_{286} 5 μL induced	Abs_{286} 5 μL uninduced	Abs_{286} 20 μL induced	Abs_{286} 20 μL uninduced
0.16	0.2834	0.2611	0.2321	0.1836
0.33	0.2837	0.2605	0.2316	0.1725
0.50	0.2838	0.2621	0.2321	0.1660
0.66	0.2835	0.2616	0.2314	0.1600
0.83	0.286	0.2603	0.231	0.1590
1.0	0.286	0.2595	0.2303	0.1581
1.16	0.2856	0.2592	0.2301	0.1585
1.33	0.2859	0.2593	0.2310	

1.5	0.2853	0.2589		
1.66	0.2861	0.2589		

Table 3.3: pESP WT Enzyme Activity

To determine enzymatic activity a plot of Abs versus time is constructed and the equation of the line is determined. The plot for 5 μL induced was incomprehensible and therefore will not be included.

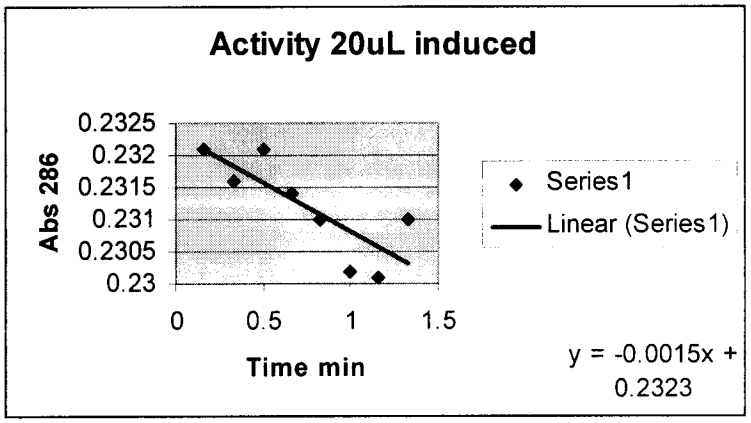

Figure 3.8: pESP WT Activity 20 μL Induced

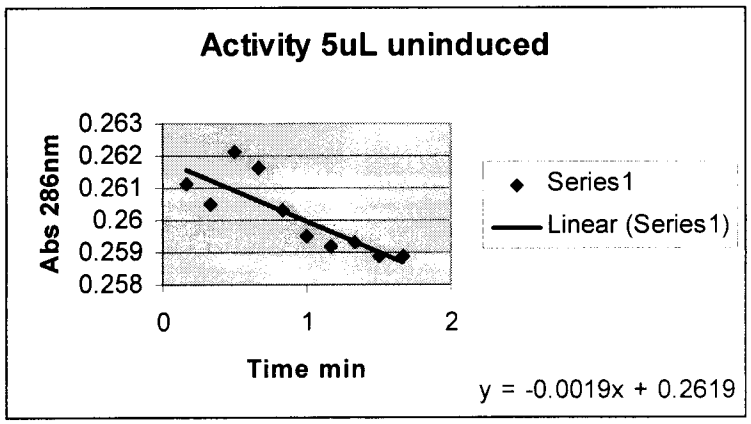

Figure 3.8: pESP WT Activity 5 μL Uninduced

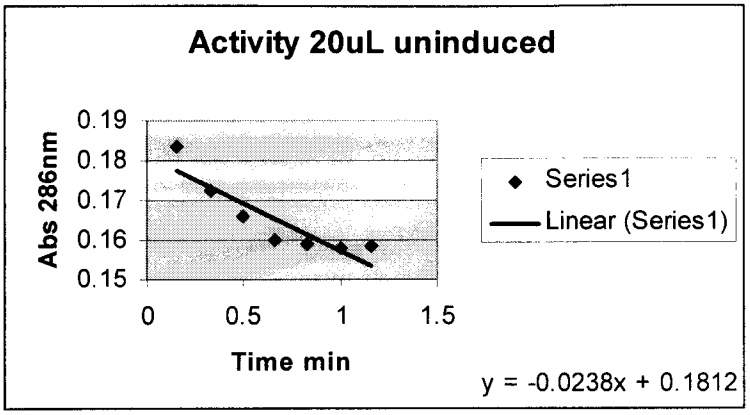

Figure 3.10: pESP WT Activity 20 μL Uninduced

Activity is determined through the following equations with the slope of the line equal to ΔA/min.

To obtain μM/min: $\Delta A/min/0.00225M^{-1}$ where 0.00225 is the coefficient for OMP.

To obtain nmol min^{-1} μg^{-1}: μM/ min/ μg (as obtained from Bradford assay)

CHAPTER IV: MUTAGENESIS

Bacteriophages provide excellent vehicles for molecular cloning techniques. These filamentous phages are either single-stranded or double-stranded circles of DNA packed into phage particles. The particles secrete from their carrier cells into the lysate. One particular bacteriophage, M13 contains several derivatives to allow for different desired cloning purposes and ease of sequencing. The DNA is inserted into a region known as a polylinker. This region contains several restriction sites much like an MCS. These sites are located in an inessential region of the genome.

M13 is male specific in *E. coli* and contains the F factor. M13 is a long, thin-tubed, capsid virus. The single-stranded circular DNA consists of 6407 nucleotides that encode for 10 proteins. There also is an intergenic, (IG), space between the second and fourth genes, origins of (+) or (-) strand DNA, signal for packaging (+) strands to phage, and a transcription terminator. M13 derivatives have no limit in size of inserted DNA.

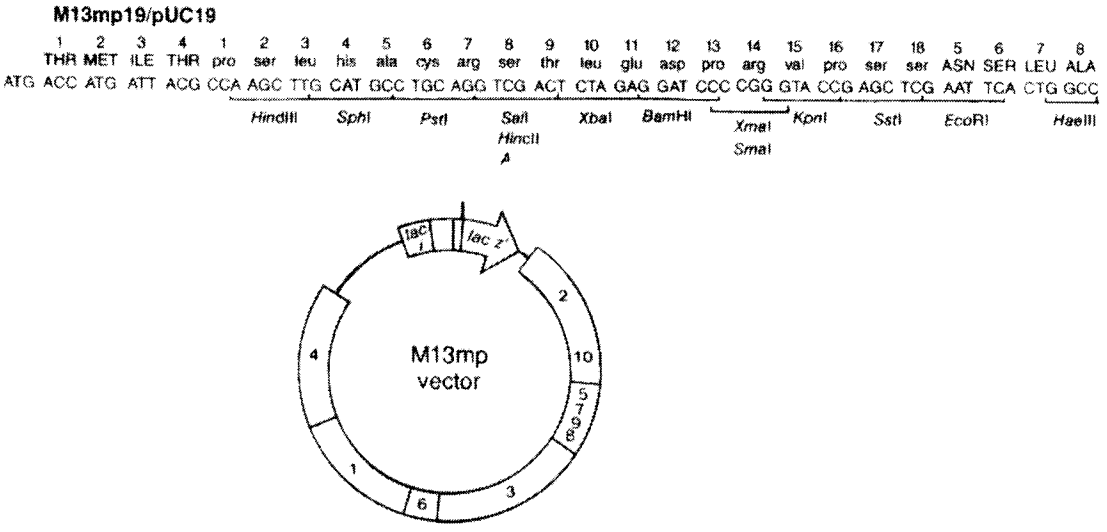

Figure 4.1: M13mp19 Phage Vector

When the phage infects bacteria the resulting infection is usually not lethal but does slow cell growth. The longer length of time for cell growth results in the appearance of a clear "lawn" of plaques. This is the area where the phage particles multiplied and lysed the bacterial cells. The center of a plaque contains the infected cells used for transfection.

The M13mp19 derivative was used in the ODCase mutagenesis reactions because of past success with this phage transfecting *E. coli* strain CJ236. The CJ236 strain contains uracil nucleotides instead of thymine for rapid and efficient recovery of mutated DNA. The ssDNA must be isolated from individual plaques for use in sequencing. Once sequenced, the DNA containing the correct mutations will undergo PCR applications leading to cloning into pCal followed by transfer to *E coli.*

K93C Mutations

Introduction

There are multiple ways to perform mutagenesis. One method is site directed mutagenesis. In site directed mutagenesis, particular nucleotides are changed resulting in different codons for different amino acids. Chemical and enzymatic methods for mutagenesis can produce 100 % complete mutagenesis through the use of PCR site directed mutagenesis. In this method, an oligonucloetide primer containing the mismatched bases becomes hybridized to the template DNA. When synthesis begins, the polymerase synthesizes new DNA containing the mutation.

The method utilizing M13mp19 was used for changing ODCase WT to K93C and ODCase CysFree to C93only. Care is taken that uracil nucleotides are present in the DNA instead of thymine. This allows for rapid and efficient recovery once the mutants

are created. The *E. coli* strain CJ236 has the genotype of *dut*$^-$, *ung*$^-$. The dut$^-$ indicates the absence of dUTPase which leads to elevated levels of dUTP. In the pyrimidine pathways, dUTP competes with TTP for DNA incorporation. Thus, an elevated level of one nucleotide over another would lead to its incorportation. The *ung*$^-$ represents a lack of uracil N-glycosylase which under normal conditions, removes uracil from DNA (recall that RNA contains uracil whereas DNA contains thymine). The uracil in DNA is not mutagenic because it has the same coding potential as thymine. The duplex DNA created in the second strand synthesis with the mutagenic oligonucleotide is introduced into a *dut*$^+$ *ung*$^+$ strain, and the original template strand carrying the original sequence is repaired according to the mutagenic sequence of the second strand. This leads to higher rates of successful site-directed mutagenesis. This method is preferred by some investigators because the T7 DNA polymerase in the M13 method is usually less error prone than the thermostable polymerase in the PCR method.

Materials

For mutagenesis reactions: T4 PNK and buffer, T7 polymerase and buffer, T4 ligase and buffer and M13mp19 phage were all purchased from NEB. The oligonucleotide containing the K93C mutation was purchased from IDT. Beckman and Coulter supplied dNTP's in 10 mM concentrations. NaCl, citric acid, EDTA, and HCl were all purchased from Fisher. 20x SSC (salt sodium citrate): 0.15 M NaCl, 0.022 M citrate, pH 7.0 with HCl.

For *E. coli* transformations: Purchases from Fisher include Petri dishes, tryptone, NaCl, $CaCl_2$, EDTA, Sodium acetate, phenol/chloroform/isoamyl alcohol and agarose. Amresco provides DTT and yeast extract. Ethanol, methanol, isopropanol all

were purchases from Pharmco, PEG from Sigma, and Tris-HCl from Eastman. Eppendorf supplied 1.5 mL conical tubes and Falcon supplied 15 mL round bottom tubes. $CaCl_2$-Tris solution: 10 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5, 1 % w/v DTT.

Procedure

For site directed mutagenesis: A 1 % w/v agarose gel was used to determine the presence of uracil containing DNA in the freezer stocks of ODCase WT and ODCase CysFree in M13, carried in the host CJ236. Approximately 100 ng of each were ran at 100 V until the markers reached 2/3 the length of the gel. After visualization using UV fluorescence, it was evident that the DNA was present, and that CysFree had a small amount of RNA present which would not interfere with subsequent work.

Kinase enzymes are involved in the transfer of phosphoryl groups from a phosphoryl donor, typically ATP, to a phosphoryl acceptor. Polynucleotide kinase (PNK) removes a phosphoryl group from the γ position on ATP and transfers it to the 5' hydroxyl terminus on a polynucleotide. As in the case of using PNK preceding ligation reactions, PNK can be used in the addition of a 5' phosphoryl group to an oligonucleotide to allow ligation following second strand synthesis. For mutagenesis using the K93C oligonucleotide, PNK is used prior to mutagenesis.

The kinase reaction mix consists of 12.5 μL sterile dH_2O, 1x PK Buffer, 14 ng of K93C oligonucleotide, 10 μM ATP and 1 U of T4 PNKase added in the written order. The reaction proceeds for 1 h at 37° C and then is quenched with 3 μL of 100 mM EDTA followed by heat inactivation at 70° C for 20 min. One μg of phage M13mp19 DNA is added in addition to 1.3 μL of 20x SSC. The reaction mix is centrifuged at high speed, heat inactivated, followed by cooling to room temperature and then ice. This mixture of

template DNA and mutagenic oligonucleotide is used in the following second strand synthesis.

For the mutagenesis reaction, four reactions were carried out including a negative control lacking DNA, a positive control of just M13mp19, the ODCase WT to K93C, and ODCase CysFree to C93only. A 100 μL reaction mix consisted of sterile dH_2O, 1x T7 Polymerase Buffer, 1x T4 Ligase Buffer, 1 μg of DNA, 0.15 mM dNTPs, 1 U of both T4 ligase and T7 polymerase. The reaction proceeds for 5 min on ice followed by 5 min at room temperature and is finally incubated at 37°C for 2 h. After incubation, the reaction is quenched with 3 μL of 500 mM EDTA and stored at -20°C until use.

Introduction of duplex DNA into *E. coli*: The mutated DNA needs to be expressed for reproduction and future transformation. The DNA mixture from the second strand synthesis reaction is transformed into *E. coli* strain XLI-Blue and the ssDNA is isolated and verified for the desired mutated sequence before becoming transferred into pESP-1. Without first being expressed in *E. coli*, the mutants cannot be manipulated nor verified that the mutagenesis reaction occurred successfully. A transformation is the process by which cells take up exogenous DNA in the presence of $CaCl_2$. The carrier such as *E. coli* strain XL1-Blue, is grown until reaching midlog growth. The cells are treated with $CaCl_2$, enhancing their ability to take up DNA. The phage DNA can then efficiently enter the pretreated cells during the period of heat shock.

XL1-Blue competent cells are prepared according to the transformation protocol from Chapter 3. Into each Falcon tube, 0.2 mL of $CaCl_2$ treated cells are added. No DNA is added to the negative control and 1 μL of M13mp19 phage DNA with either ODCase WT or ODCase CysFree inserted, is added to the tube for the positive control.

For each of the experimental mixes, all 30 μL of the mutagenesis reaction is added and the tubes are placed on ice for 5 min. They are shock heated at 42°C for 2 min and cooled to room temperature.

Verification of successful transformation – isolation of phage DNA from candidate plaques: A sterile toothpick containing a plaque from one of the transformation plates is placed into 2 mL LB containing 50 μL of an XL1-Blue/LB culture and incubated overnight at 37°C with shaking. From the overnight culture, 1.5 mL is transferred to a microcentrifuge tube and spun at high speed for 5 min. The supernatant is then transferred to a new tube and spun again. From that supernatant, 1.2 mL is added to a new tube along with 300 μL 30 % PEG and reacted at room temperature for 15 min. After centrifugation, the pellet is resuspended in 200 μL 1x TE. Ethanol precipitation can be preformed to further concentrate the DNA. This is done by adding 200 μL phenol/chloroform/isoamyl alcohol and vortexing for 1 min followed by cooling on ice and repeating. The mix is centrifuged and the top layer of the supernatant is transferred to a new tube. To this tube, 20 μL of sterile 3 M NaOAc and 440 μL of 95 % v/v ethanol are added and mixed. The reaction mix is then stored at -20°C for at least 2 h. To recover the DNA, the mix is centrifuged at a high speed for 10 min. The pellet is vacuum dried and redissolved in 50 μL of 1x TE. Following quantitation of ssDNA by UV spectrophotometry, the ssDNA is used in DNA sequencing to determine the desired mutated sequences.

Results and Discussion

Phage transformation worked successfully with no plaques appearing on the negative control plate and plaques appearing on all experimental plates and the control

M13mp19 plate. To further verify the mutagenesis the phage DNA was then quantified. Table 4.1 contains the concentrations.

Sample	Abs_{260}	μg/μL
C93only-1	0.224	0.83
C93only-2	0.306	1.1
C93only-3	0.581	2.1
C93only-4	0.169	0.62
C93only-5	0.206	0.76
K93C-1	0.400	1.5
K93C-2	0.290	1.1
K93C-3	0.540	2.0
K93C-4	0.220	0.81
K93C-5	0.180	0.67

Table 6: Quantification of Mutagenic Transformants

A 1 % w/v agarose gel run 1 μg of each sample at 100 V until the markers reached 2/3 the length of the gel. Upon viewing under UV light, bands corresponding to the correct molecular weight of the mutagens appeared (gel not provided). To determine if the exact mutations were present, the samples were then sequenced according to Beckman-Coulter protocol on the CEQ 2000XL DNA Analysis Sequencer. For ssDNA, the correct quantity of DNA is between 25-50 fmol for an 8 kbp sized sample. This translates to approximately 130 ng of DNA, between 1-6 μL.

Sample	dH_2O (μL)	ssDNA (μL)	Primer(μL)	DTCS (μL)
C93only-1	2.3	3.7	2.0	12
C93only-2	3.5	2.5	2.0	12
C93only-3	4.5	1.5	2.0	12
C93only-4	1.0	5.0	2.0	12
C93only-5	2.0	4.0	2.0	12
K93C-1	4.0	2.0	2.0	12
K93C-2	3.3	2.7	2.0	12
K93C-3	4.5	1.5	2.0	12
K93C-4	2.4	3.6	2.0	12
K93C-5	1.5	4.5	2.0	12

Table 4.2: Mutagen Sequencing Reaction Mixes

The sequencing results were formatted and transferred into a Word document and reviewed for possible mutations. C93only sequencing samples 1-3 demonstrated the desired sequence: cysteine at position 93, serine at positions 33, 56, and 155 (Figure 4.2). C93only sequencing sample 4 had the same results except position 155 was indeterminable (Figure 4.3). C93only sequencing sample 5 also contained a serine at position 263 (Figure 4.4). K93C samples 1 and 5 contained the mutation with cysteine residue codon at position 93 (Figure 4.5). K93C sequencing samples 2-4 contained no mutations and still had a lysine residue at position 93 (Figure 4.6).

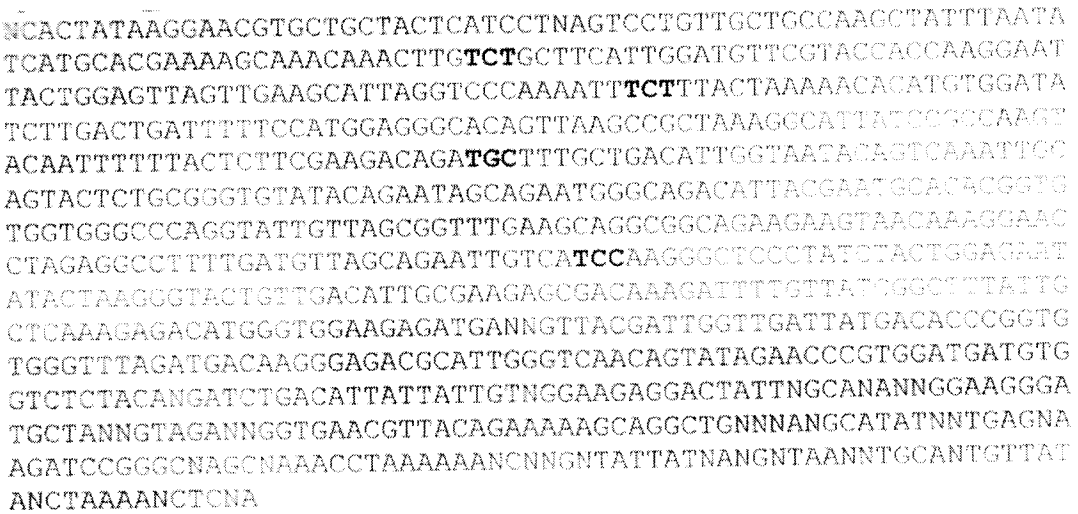

Figure 4.2: C93only Sequencing Sample 1

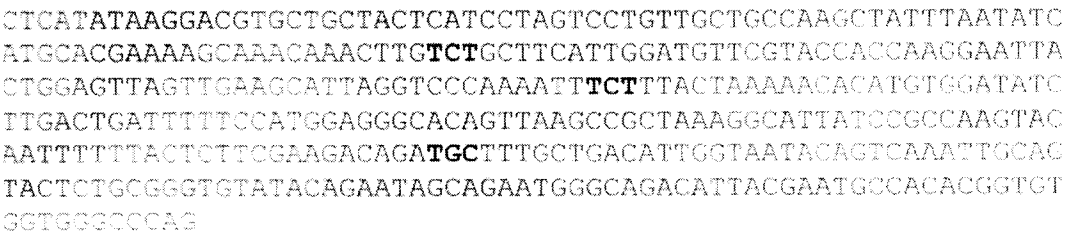

Figure 4.3: C93only Sequencing Sample 4

CCCNANNNNTNGTNNCNATAGAGGAANCTAGNCNNACTCATNCNNTTCCTGTTGCTGCCA
AGCTATTTAATATCATGCACGAAAAGCAAACAAACTTG**TCT**GCTTCATTGGATGTTCGTA
CCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATT**TCT**TTACTAAAAA
CACATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAGGCAT
TATCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGA**TGC**TTTGCTGACATTGGTAATA
CAGTCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGA
ATGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCAGAAGAAG
TAACAAAGGAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCA**TCC**AAGGGCTCCCTAT
CTACTGGAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTA
TCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTA
TGACACCCGGTGTGGGTNTAGATGACAAGGGAGACGCATTGGGTCAACAGTATAGAACCG
TGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTNGGAAGANGACTATTTGCAA
AGGGAAGGGATGCTAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGGAAGCATATN
TGAGAAGA**TNC**GGNCNAGCAAAAC**TAA**AAAAACTGTATTATAAGTAANTGCATGTATACT
AANCTCACAAATTANAGCTNCATTTIAATNATATCAGTNATTANCCCCGGAA

Figure 4.4: C93only Sequencing Sample 5

CCCNATCGNNTNNNNCTATAAGGAACGTGCTGCTACTCATCCTATNTCCTGTTGCTGCCA
AGCTATTTAATATCATGCACGAAAAGCAAACAAACTTGTGTGCTTCATTGGATGTTCGTA
CCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAA
CACATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAGGCAT
TATCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGA**TGC**TTTGCTGACATTGGTAATA
CAGTCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGA
ATGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCAGAAGAAG
TAACAAAGGAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCCTAT
CTACTGGAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTA
TCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATNGGTTGATTA
TGACACCCGGTGTGGGTNTAGATGACAAGGGAGACGCATTGGGTCANCAGTATAGANCCG
TGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTGGGAAGAGGACTATTTGCAA
NNGGAAGGGATGCTANNGTAGAGGGTGAACGTTACAGANNAGCAGGCTGGGAAGCNNNTN
NGANNAGATGCGGGNNGCANNANCTAAAAAACNGNATTTATANGNTNANNTGCANT

Figure 4.5: K93C Sequencing Sample 1

GGNGNATCGANTTNCATATAGAGGAACGTAGCTGCTACTCATCCTNTGTCCTGTTCGCTG
CCAAGCTATTTAATATCATGCACGAAACAGCAAACAAACTTGTGTGCTTCATTGGATGTT
CGTACCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTA
AAAACACATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAG
GCATTATCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGAAAATTTGCTGACATTGGT
AATACAGTCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATT
ACGAATGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCAGAA
GAAGTAACAAAGGAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCC
CTATCTACTGGAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTT
GTTATCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTG
ATTATGACACCCGGTGTGGGTNTAGATGACAAGGGAGACGCATTGGGTCAACAGTATAGA
ACCGTGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTGGGAAGAGGACTATTT
GCANAGGGGAAGGGGATGCTAAGGNTAGANNGGTGANCGNTNACANGANNANGCANNGCN
TGNGNAAGGCATTATTNNNGANAANNGAATGNCNNGNCCCAGCCCAAAAACCTAAAAAAA
ANNCNTGNTATTNNANTANAGNTNAAANTGCCATTGNTATTACNTAAACCCTCNCNNAAT
TTAGAAGCCTTCAANTTNANNTNANNNNCAGNTNNNNANNCCCNNGNANTCCCNNTNAAG
NTNNANCNNGCAGGNCNNTNCNAAGCNNGGNCNNTTANNTC

Figure 4.6: K93C Sequencing Sample 2

The fact that mutations, if present, were not easily determinable at position 263 is not a concern because the primer for PCR for transfer of these genes into expression vectors can contain the codon for serine instead of cysteine, thus ensuring the desired codon at position 263. When reading DNA sequencing results, the beginning and end of the sequence is often replaced with the letter N for any nucleotide. One can view the original data to see if the sequencing was problematic or without catch. A problematic sequence has overlapping bases resulting in the interpretation of the wrong nucleotide at a specific position. The fact that 5 samples for both K93C and C93only were analyzed increases the likelihood that the mutagenesis was successful. From these results, any C93only sample and K93C samples 1 and 5 can be used for PCR and transformation to the new expression system using the vector pCal-n.

CHAPTER V: pCAL EXPRESSION SYSTEM

After limited success using ESP® Yeast Protein Expression and Purification System and ESP® Yeast Protein Expression Vectors by Stratagene, Affinity® Protein Expression and Purification System, also by Stratagene provides new hope for adequate protein expression. This system uses a calmodulin binding peptide (CBP) fused to the cloned gene. A one-step column purification provides near homogeneous protein. CBP has a higher affinity ($K_d = 10^{-9}$) for calmodulin in the presence of calcium. Without calcium, CaM changes conformation and releases the ligand with the addition of EGTA.

Plasmid pCal-n

The cloning vector, pCal, is based on pET-11a and contains the CBP upstream of the MCS. Other features include a T7 operator and terminator with a *lac* operator, a ribosome binding site, thrombin cleavage site, and an ampicillian resistance gene (Figure 5.1)

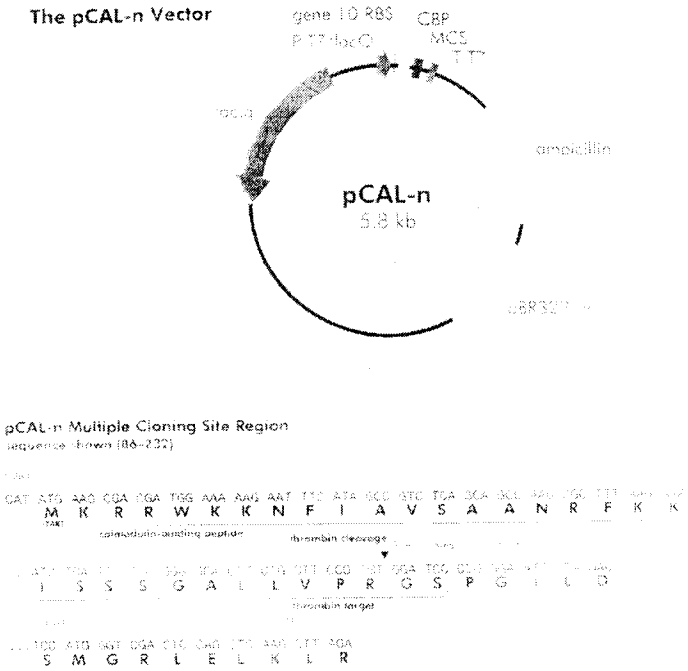

Figure 5.1: Vector pCal-n

E. coli strain BL21 (DE3)

BL21 provides for a high affinity protein expression system. The name is derived from the lamda DE3 lysogen with phage 21 immunity. Transformation efficiency is increased because of endonuclease inactivity seen in BL21. The containment of *lac*1 gene, and *lac* UV5 driven T7 RNA polymerase creates the overexpression of the gene necessary for the overproduction of the protein. Upon induction with isopropyl-β-D-thiogalactopyranside (IPTG) the *lac* UV5 gene is depressed leading to the overexpression of the T7 promoter and cloned gene.

Experimental

Upon first encounter with the new Affinity® system, the ODCase gene was cloned over the CBP and thoughts were to purify the protein using DEAE-Sephadex affinity chromatography. The time consumption and difficulties encountered lead to the cloning of the ODCase WT and mutant genes after the CBP. Much time was spent varying the conditions and protocols to obtain optimal growth and expression.

Oligonucleotide Primer Preparation

Introduction

Oligonucleotide primers are necessary to clone template DNA and introduce restriction sites into DNA. This allows for ligation into a vector to complete an expression system. Care is taken when ordering and preparing primers so that the coding and concentration are correct.

Materials

Oligonucleotides purchased through IDT. NEB provided 10x Thermopol Buffer and Vent® Polymerase. 10x dNTPs obtained through Beckman-Coulter. DNA came from former Qiagen preparations.

Procedure

Oligonucleotides are designed to flank the ODCase start site and introduce restriction sites, specifically NdeI (start) and BamHI (stop). To do this, a 5′-3′ template is created that begins with a few nucleotides followed by the restriction site sequence and additional coding information such as a stop or start site. The oligonucleotides are then prepared by making a 200 μM stock. This is accomplished by resuspending the pellet provided by IDT in variable amounts of sterile dH_2O depending on nmol provided. The 200 μM stock is then diluted 4:1 for the final concentration of 50 μM for use in PCR.

The PCR procedure is run according to protocol listed in chapter 2 on samples of pESP WT, K93C, CysFree, and C93only using 2 μL of each Qiagen preparation. The products were then PCR spin column purified and verified on an agarose gel. The purified PCR products and pCal-n are digested with BamHI and NdeI and verified on an agarose gel. Once verified, they are ligated and then transformed in the same manner as pESP-1 (Chapter 3) into XLI-Blue. Included in the transformation are a negative control with no DNA, a positive control with uncut pCal-n, a ligation control with negative ligation reaction mix, and also another positive control with double digested pCal-n. 300 μL were plated on LB/Amp plates.

Individual colonies were selected from each plate containing pCal WT and mutants and grown in 3 mL LB/Amp overnight at 37°C. The miniprep purified plasmids

were screened on an agarose gel for possible ligations. Any samples appearing to contain an insert were then grown in 50 mL LB/Amp and Qiagen purified. Upon quantification, they were digested with both endonucleases to see if an insert was present. As a enzymatic activity control, 100 ng of pCal 3HAO was also cut with NdeI and BamHI. Those with inserts were again transformed using 5 μL of the Qiagen purified samples and BL21 following standard transformation conditions as previously mentioned. Colonies were grown in 50 mL LB/Amp, Qiagen purified, and quantified for use in sequencing.

Sequencing preparations include template preparation by making 1.6 pmol stock solutions. Either ODCase Start oligonucleotide or ODCase C155S forward oligonucleotide were used. Approximately 250-500 ng of DNA was used in addition to 12 μL DTCS and sterile dH_2O to reach a volume of 20 μL. The samples were prepared as usual and sent to Diana Arnett for DNA sequencing at the YSU Sequencing Center.

Results and Discussion

The ODCase start oligonucleotide sequence is as follows:

5′- GG GAA AGG **CAT ATG** TCG AAA GCT ACA TAT AAG GAA-3′

where the NdeI restriction site is highlighted in bold and the ODCase start site is underlined.

Two stop oligonucleotides were created to code for C263S in the CysFree and C93only genes. BamHI Stop site sequence is as follows:

5′- GG GAA AGG **GGA TCC** TTA GTT TTG CTG GCC GCA TCT TCT- 3′

where the BamHI restriction site is highlighted in bold and the ODCase stop site is underlined.

The BamHI Ser 263 Stop sequence is as follows:

5'- GG GAA AGG **GGA TCC** TTA GTT TTG CTG GCC *GGA* TCT TCT- 3'

where the BamHI restriction site is highlighted in bold, the ODCase stop site is underlined, and the codon for serine is in italics.

PCR spin column purified products verified on a 0.8 % agarose gel using 5 μL, showed bands for ODCase WT, K93C, CysFree, and C93only appearing in between the two lowest bands, 0.5 and 1.0 kb on the DNA ladder. The negative control PCR mix did not appear on the gel indicating no contaminations present (gel not provided).

Digestion of pCal-n with NdeI and BamHI showed enzymatic activity and that pCal-n was cut satisfactorally. Uncut pCal-n was used as a standard and contained two bands. The appearance of only one band in the lane for double-digested pCal-n indicates successful digestion (gel not provided). Prior to digestion, PCR product concentration was estimated by comparing to intensity of the DNA Ladder (Table 5.1).

PCR sample	Concentration in ng/μL
WT	150
K93C	100
CysFree	100
C93only	54

Table 5.1: PCR Product Concentrations

Approximately 500 ng of each PCR mix was digested with BamHI and NdeI using pCal-n as a control in a total volume of 20 μL. They were verified on a 0.8 % w/v agarose gel using 1 μL of each digestion mix. All lanes showed DNA (no data provided).

For each ligation reaction, 4 μL of double digested pCal-n (0.186 μg/μL) was used in addition to 3 μL of each digestion mix. A negative ligation was also performed

for verification as seen in the transformation. Proper molar ratio was determined by binding the molar ratio of pCal-n to digestion mix.

PCal-n 8000 bp x 660 g/mol/bp = 3.8×10^6 g/mol

100 ng / 3.8×10^6 ng/mol = 2.7×10^{-5} nmol

Insert 800 bp x 660 g/mol/bp = 5.3×10^5 g/mol

Ratio 5.3×10^5 / 2.7×10^{-5} = 14 x as much (only 2:1 minimum).

For the transformation into XLI-Blue, half of each ligation reaction was used along with a negative control with no DNA, and two positive controls of 3.5 μL of double digested pCal-n, and 10 ng uncut pCal-n. Colonies appeared on all plates except for the negative control plate. Ten individual colonies were selected from each transformation plate to screen for proper ligation on a 0.8 % agarose gel. The gel containing pCal WT showed bands in lanes 4 and 10 appearing raised compared to lane 2 containing pCal-n (Figure 5.2). For the gel containing pCal K93C, lane 8 appeared to contain insert as compared to pCal in lane 2 and pCal 3HAO in lane 3 (Figure 5.3). The pCal CysFree agarose gel contained several lanes with bands raised when compared to pCal-n in lane 2 and pCal 3HAO in lane 3 (Figure 5.4). Lanes 7 and 8 were identified for further purification. The miniprep gel for pCal C93only showed lanes 9 and 11 being raised (Figure 5.5) compared to lane 2 (pCal) and in accordance with the size of lane 3 (pCal 3HAO).

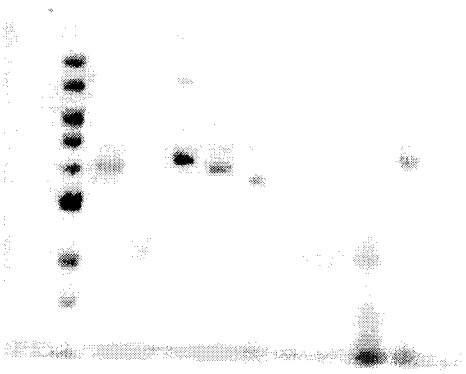

Figure 5.2: pCal WT Insert Screening

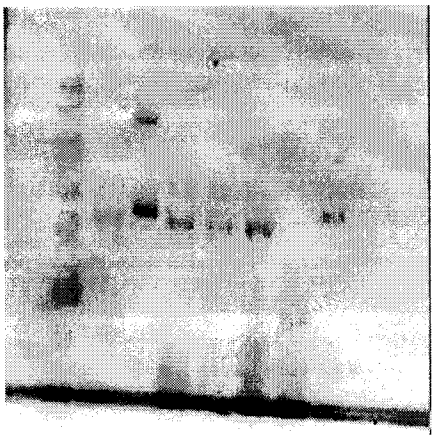

Figure 5.3: pCal K93C Insert Screening

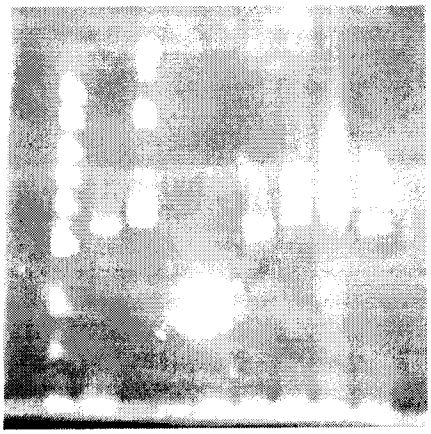

Figure 5.4: pCal CysFree Insert Screening

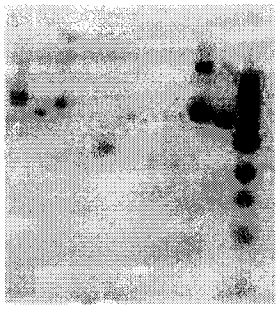

Figure 5.5: pCal C93only Insert Screening

A 50 mL culture of LB/Amp was grown overnight containing samples of possible inserts. Qiagen purification enabled quantification in which enabled double digestion to verify the presence of the inserted DNA into pCal-n. For the digestion, 1 μg of each pCal construct was used and reacted overnight at 37°C including pCal 3HAO as a control. Each mixture (pCal C93only not digested at this time) was run on a 0.8 % agarose gel and inserts were present for each (Figure 5.6). C93only insert verified at a later time on an agarose gel (Figure 5.7).

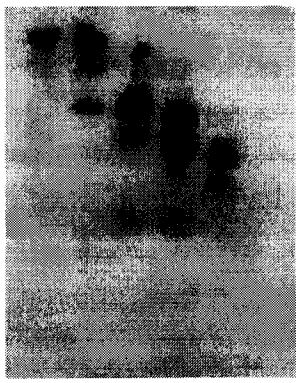

Figure 5.6: pCal WT, pCal K93C, and pCal CysFree Insert Verification

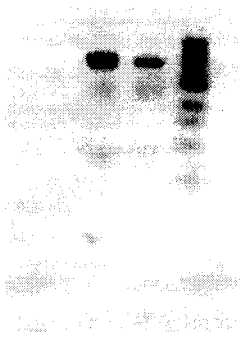

Figure 5.7: pCal C93only Samples A and B Insert Verification

For the transformation into BL21 from XLI-Blue, all plates contained colonies with the exception of the negative control indicating successful transformation. Colonies from these plates were then grown in 50 mL LB/Amp, Qiagen purfied, and quantified for sequencing.

There were samples of each pCal construct and one control sample for DNA sequencing. The DNA sequencing results for pCal WT and pCal K93C are listed below with lysine and cysteine residues at position 93 in bold. An ODCase start primer was used in sequencing both samples.

```
TGNCTAGTCCTGTTGCTGCCAAGCTATTTAATATCATGCACGAAAAGCA
AACAAACTTTGTGCTTCATTGGATGTTCGTACCACCAAGGAATTACTGG
AGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAACACATGT
GGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAG
GCATTATCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGAAAATTTG Lys 93
CTGACATTGGTAATACAGTCAAATTGCAGTACTCTGCGGGTGTATACAG
AATAGCAGAATGGGCAGACATTACGAATGCACACGGTGTGGTGGGCCCA
GTGTATTGTTAGCGGTTTGAAGCAGGCGGCAGAAGAAGTAACAAAGGAA
CCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCCTAT
CTACTGGAGAATATACTAAGGGTACTGTTNGACATNGCGAAGAGCGACA
AAGATTTTGTTATCGGCTTTATTGCTCCAAAGAGACATNGGGTGGAAAG
AGATGAANGGTTACGATTNGGTTGGATTTATGACCACCCCGGGTGNTGN
GGNTTTTANAATGNACCAAGGGGNNNAACNGCCATTTGGGGGGTTCAAA
CCANGTTATTANGAAAACCCCG
```

Sequencing pCal WT

TCATCCTAGTCCTGTTGCTGCCAAGCTTATTTAATATCATGCACGAAAA
GCAAACAAACTTGTGTGCTTCATTGGATGTTCGTACCACCAAGGAATTA
CTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAACAC
ATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCT
AAAGGCATTATCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGA**TGC** Cys 93
TTTGCTGACATTGGTAATACAGTCAAATTGCAGTACTCTGCGGGTGTAT
ACAGAATAGCAGAATGGGCAGACATTACGAATGCACACGGTGTGGTGGG
CCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCAGAAGAAGTAACAAAG
GAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCC
TATCTACTGGAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGA
CAAAGANNTGTTATCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAG
ATGANNGTNACGATTGGTTGATNATGACACCCGGTGTGGGTTTAGATGA
CAAGGGAGACGCATTGGGTCNACAGTATAGAACCGTGGATGATGTGGTC
TCTACAGGATCTGACATNATNATNGTNGGANNANGACTATNTGCAAGGG
GAAGGNATGCTAAGGTANAGGNTGAACGTACANAAAAGCNNGCNTGGAA
GCATNTNNGANANGATGCGGCCANCAAACTANNGATCCNNGNANTCNAA
CTCATGGGTTACTCAACCAGCTNNTCGCGCACAGNCCAAGACTANTGGC
GCNCNNACATANCNTACCTGCTNNGTTNNNNGCGANGACCACGATCCA

Sequencing pCal K93C

Sequencing for pCal CysFree and pCal C93only was completed using two different primers. A second primer, ODCase C155S, was used in case the sequencing truncated before reaching C263S. Data from C155S is not included because it is evident from sequencing with the ODCase start primer that all cysteine to serine mutations were present. Unfortunately, the sequencing results using the C155S primer for CysFree did not produce sufficient data. Serine residues are highlighted in bold.

TGCTAGTCCTAGTNTGCTGCCAAGCTATTTAATATCATGCACGAAAAGC
AAACAAACTTG**TCT**GCTTCATTGGATGTTCGTACCACCAAGGAATTACT Ser 33
GGAGTTAGTTGAAGCATTAGGTCCCAAAATT**TCT**TTACTAAAAACACAT Ser 56
GTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAA
AGGCATTATCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGAAAATT
TGCTGACATTGGTAATACAGTCAAATTGCAGTACTCTGCGGGTGTATAC
AGAATAGCAGAATGGGCAGACATTACGAATGCACACGGTGTGGTGGGCC
CAGTGTATTGTTAGCGGTTTGAAGCAGGCGGCAGAAGAAGTAACAAAGG
AACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCA**TCC**AAGGGCTCCCT Ser 155
ATCTACTGGAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGAC
AAAGATTTTGTTATCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAG
ATGAAGGTTACGATTGGTTGATTATGACACCCGGTGTGGGTTTAGATG
CAAGGGAGACGCATTGGGTCAACAGTATAGAACCGTGGATGATGTGGTC
TCTACAGGATCTGACATTATTATTGTTNGNAAGAGGACTATTTGCCAAA
GGGAAGGGGATGCTAAGGGTAGAGGGGTGAACGTTTACAGAAAANCCAG
GCNNGGNNNNNCATANTNNGAANAAGA**TCC**GGCCCAGCCAAAACTAAAG Ser 263?
GANTCNGGCNGNCTANCAANNCCCCGAAANGNAAGCCTGAGTTGGGCTG
CNTGNCNNCCNCTTGANCCATTANCTANNCNATACNCCNCNTGGNNGNC

Sequencing pCal CysFree

GNNCCCCCNNTTTCCCCNTATCATCGCTANTCCTAGTATGCTGCCAAGC
TATTTAATATCATGCACGAAAAGCAAACAAACTTG**TCT**GCTTCATTGGA Ser 33
TGTTCGTACCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCC
AAAATT**TCT**TTACTAAAAACACATGTGGATATCTTGACTGATTTTTCCA Ser 56
TGGAGGGCACAGTTAAGCCGCTAAAGGCATTATCCGCCAAGTACAATTT
TTTACTCTTCGAAGACAGATNANNTTTGCTGACATTGGTAATACAGTCA
AATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACAT
TACGAATGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAG
CAGGCGGCAGAAGAAGTAACAAAGGAACCTAGAGGCCTTTTGATGTTAG
CAGAATTGTCA**TCC**AAGGGCTCCCTATCTACTGGAGAATATACTAAGGG Ser 155
TACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTATCGGCTTTATT
GCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTA
TGACACCCGGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACA
GTATAGAACCGTGGATGATGTGGTCTCTACAGGATCTGACATNATTATT
GTTGNNAGAGGACTATTTGCAAAGGGGAAGGGATGCTAAGGTAGAGNGG
TGACCGTTACAGAAAAGGCAGGCNTGGGGAAGCATATTTGAGNAAGAN**T** Ser 263
CCGGGCCAGCNAAAANCTAANNGNATNCCCGNGGGGAATTCTTAGAACN
TCCCATTGGGNTCNNACNTCGAAGCCCTCCAAGCNNTNANANTNCNNGG
CNTGCNTAACAAAAGCCNNGNANNANGNANNCCTGAAGTTGGGCNNGCN
NGNCNNCCGCNNGANNCNANTACCNTANCNNTNNNCCCNNTGNNGNNCC
NCTNAACGGGGTTTTTGANNGNNTTTTTTGCCNG

Sequencing pCal C93only

Protein Expression

Introduction

When protein is grown in media, select conditions are necessary to obtain high levels of expression and production. It is best to determine midlog phase of growth experimentally, since individualized pCal constructs may behave slightly different than those mentioned in the protocol by Stratagene. Once midlog phase of growth is determined, it is reasonable to assume that the gene has been overproduced and now the protein can be overexpressed. Too little or too high a concentration of the inducer, IPTG, can cause less than desired protein expression. For this reason, 0.4 mM IPTG was used based on previous works with this expression system.[28] The proper amount of lysis using bead beatings is also necessary to determine experimentally so as to not destroy the protein but to lyse enough cells to obtain the highest possible amount of protein.

Materials

Most reagents aforementioned in use of LB, LB/Amp and PBS. IPTG was purchased from Acros. 0.1mm glass beads purchased from Biospec. Tris-HCl pH 8.2 buffer includes 50 mM NaCl, 20 mM Tris-HCl.

Procedure

Midlog phase determination: A Klett flask with 50 mL LB/Amp was inoculated with a colony from a LB/Amp plate containing pCal WT. The flask was incubated at 37°C and a Klett reading was taken on a Klett Summerson Photoelectric Colorimeter and numbers were recorded until growth ceased.

IPTG Induction Determination: A test tube containing 5 mL LB/Amp was inoculated overnight with a colony from a pCal WT agar plate of LB/Amp. 0.5 mL are

then transferred to a secondary culture of 50 mL LB/Amp and grown at 37°C with shaking for 3 h. The culture is induced with 0.4 mM IPTG and 1 mL fractions are collected every 2 h beginning at time zero. The fractions are then centrifuged at high speed for 5 min and resuspended in 50 μL 2x SDS, boiled for 5 min and 15 μL is run on an SDS-PAGE gel using a Biorad Power Pak 300 at 120 V. Also on the gel is 10 μL molecular weight markers and 15 μL of a 25:15 μL mix of 2x SDS buffer and purified ODCase as a standard. The gel is run at 120 V and is completed when the orange marker appears halfway down the separating part of the gel. The gel is then stained for 5 min and destained overnight. The same procedure is completed for pCal CysFree, pCal C93only, and pCal K93C.

Bead beating: Once cells reach sufficient growth and protein is adequately expressed, the culture is centrifuged at 3000 x g for 20 min at 4°C. The pellet is washed with 20 mL cold PBS and resuspended in 10 mL Tris Buffer pH 8.2. The solution is applied to the bead beating apparatus and 0.1 mm glass beads are added along with lysis buffer. The cells are beaten in 1 min intervals, removing 100 μL after each pulse. The 100 μL is centrifuged to pellet the debris and the lysate is transferred to a new tube and protein concentration is determined using a Bradford assay.

Results and Discussion

Midlog Growth Determination: The following Klett readings and times are as follows:

Time (h)	Klett number
0	0
11.22	134
13.25	154
14.67	132

Table 5.2 Growth Determination pCal

Taking the log of the Klett readings and using 140 as the time for complete growth, midlog would correspond to a Klett reading of 70, which occurs in three hours of growth.

Induction: Collections of cells for pCal WT occurred at 0 h, 1.85 h, 3.72 h, 6.1 h and 8.1 h. The first gel contains times 0-3.72 h induced and uninduced. The second gel contains times 0, 6.1, and 8.1 both induced and uninduced. ODCase bands appeared on the gels at the 3.72 h time mark and were comparable in intensity to the purified ODCase standard. Using simple ratios, there was about 28 mg/ L of protein. The difference between 3.72 h and 6.1 and 8.1 were indistinguishable leading to the conclusion that four hours is sufficient enough time for proper induction of the ODCase gene. There were no immediate findings for induced vs. uninduced at this time (Figure 5.8).

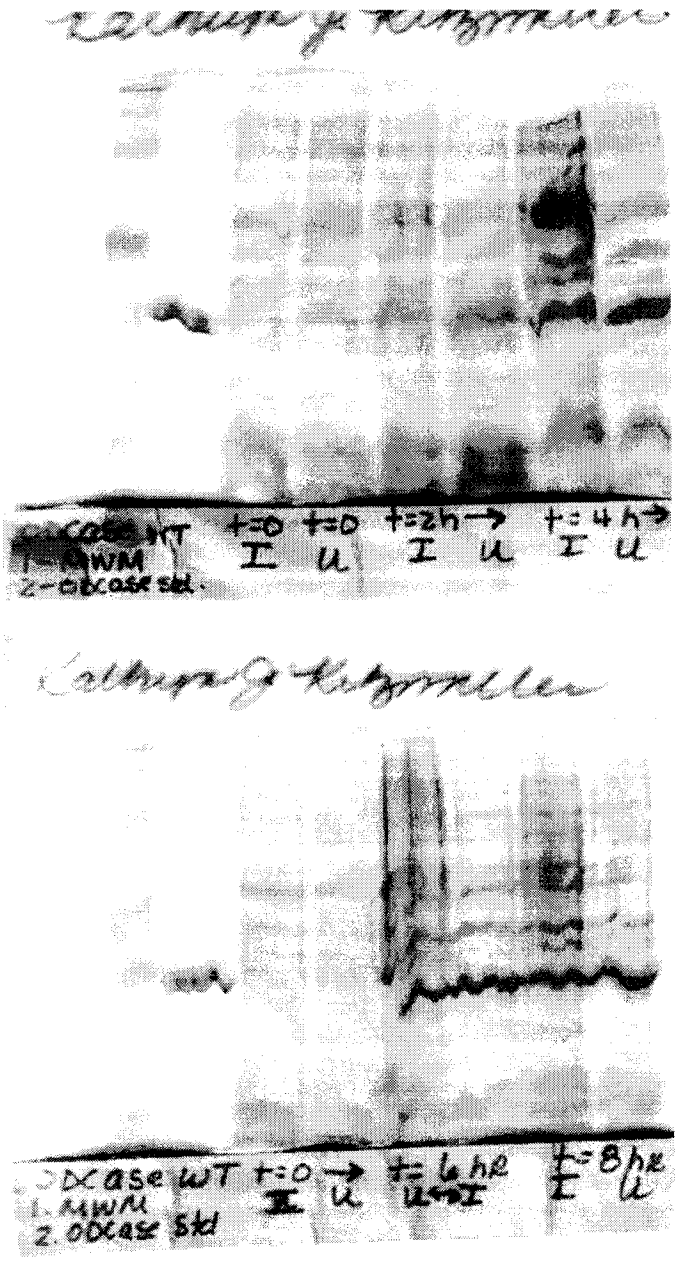

Figure 5.8: SDS Page Gels for Induction

Collections of cells for pCal CysFree and pCal C93only were collected at 0 h, 2 h, 4 h, and 6 h. The gels revealed that no more total protein occurs after 4 h of induction (gel not provided). The collection of cells for pCal K93C was taken at 0 h, 2 h, and 4 h. Again it was shown that protein induction is complete at 4 h (gel not provided).

Activity and Purification

Introduction

The purification of proteins is a subject many have spent much time devising and improving. Obtaining pure protein is often dubious work. Separation of protein components can be done based on size, ionic charges, binding specificities, polarity, and molecular size. One particular method is salting in which precipitates proteins allowing others to remain in solution. Ammonium sulfate concentrations are increased steadily until unwanted proteins precipitate out and the desired protein remains in solution. Dialysis is then performed to remove excess salt and the protein continues through purification until completed. Often an ion exchange column or specific binding column is used and fractions are collected. It is then determined which eluted fraction contains the desired protein and its purification again through SDS-PAGE. Ion exchange chromatography separates proteins by size and charge. In the case of the ODCase constructs, an anion exchange column is used because the protein has an overall negative charge. ODCase remains bound to the column until a molecule with a higher affinity for the column is washed through. ODCase protein elutes and is verified through SDS PAGE electrophoresis.

Materials

DEAE resin, ammonium sulfate, with column and equipment from Biorad.

Procedure

Ammonium sulfate precipitation: For these experiments, pCal CysFree was used to also determine if activity was present. Precipitation with ammonium sulfate was completed in two steps. The first step was a 60 % addition and the second step brought

the ammonium sulfate concentration up to 90 %. For 100 mL of lysate, 36.19 g are necessary. The salt was added slowly over the course of 10 min. The solution was then stirred for 30 min at 4°C. The solution was centrifuged at 3000 x g for 20 min. The pellet was discarded and contained unwanted proteins. The supernatant was kept and for 90 %, 20.1 g were added in the same manner as before. Following centrifugation, the pellet was kept this time. The pellet was dissolved in Tris Buffer and dialyzes overnight. Verification on an acrylamide gel demonstrated at what concentration of salt, the protein falls out of solution.

Ion Exchange Chromatography: To prepare the column, DEAE resin slurry was added to the column provided by Biorad and allowed to settle. The column was washed several times with Tris Buffer, adding more resin until the bed volume is 2/3 the length of the column. The column was then connected to a pump and a fraction collector. The flow rate was adjusted to 4 mL/min with 2.1 min/fraction. The lysate from the overnight digestion was applied to the column. Buffer was added with decreasing pH starting at pH of 8.8 and dropping to a pH of 7.5. Fractions are tested quantitatively using Bradford reagent as a color indicator. In a microfuge tube, 450 μL of Bradford reagent was added with 50 μL of each fraction. The tubes that changed color from brown to blue, were saved to be verified as containing protein. Once quantified using a standard Bradford assay, the fractions are run on an SDS PAGE gel. Upon the presence of a band for ODCase, activity is measured as described previously in Chapter 3.

Results and Discussion

Ammonium Sulfate Precipitation: A simple ratio was used in determining the amount of ammonium sulfate to add due to varying amounts of lysate. For a sample

containing 61 mL, 22 g of ammonium sulfate was added to complete 60 % concentration. To reach a concentration of 90 %, 14 g of ammonium sulfate was added to a 70 mL culture. Samples were kept from the bead beating, 60 % supernatant, 60 % pellet, 90 % supernatant, and 90 % pellet. The concentrations were determined using the Bradford assay and equal amounts of protein were run on an SDS gel (gel not included). Molecular weight markers were run in lane 1, ODCase standard in lane 2, bead beating in lane 4, 60 % pellet in lane 6, 90 % pellet in lane 8, and 90 % supernatant in lane 10. A band corresponding to ODCase appeared in experimental lanes 4, 6, 8, and 10. To account for protein in all the samples, the ammonium concentration was adjusted to 50 % from 60 % still ending at 90 %. The polyacrylaminde gel (gel not included) showed similar results and it was decided to use the pellet from the 90 % precipitation for column purification.

Ion Exchange column: The first attempt at column purification resulted in no fractions containing protein in a high enough concentration to respond to the color indicator. Column flow and fraction collection were then adjusted and the pH dropped to 7.5. This resulted in fractions 5 and 6 having positive indicators. The protein was quantified and ran on a polyacrylamide gel. The gel showed bands corresponding to the presence of ODCase along with some other proteins in the lanes 4 and 6 respectively, representing fractions 5 and 6. Again ODCase purified was used as a standard in lane 2, and lane 3 contained crude protein before column purification.

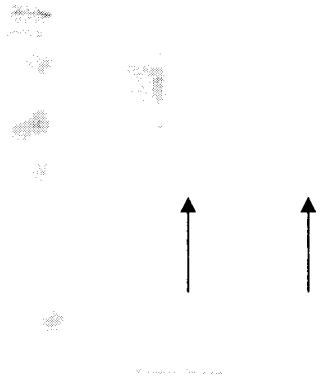

Figure 5.9: SDS Gel Ion Exchange Column Purified pCal CysFree

Specific Activity: Activity was tested using 30, 50, and 100 μL of fraction 5 and 30 μL of fraction 6. Plots were constructed for each various level of protein added. Table 5.3 contains the collected data.

Sample	Slope	μg used	Specific Activity
30 μL Fraction 6	0.0006	2.01	0.129 nmol μg^{-1} min^{-1}
30 μL Fraction 5	0.0017	1.98	0.378 nmol μg^{-1} min^{-1}
50 μL Fraction 5	0.0001	3.30	0.013 nmol μg^{-1} min^{-1}
100 μL Fraction 5	0.001	6.60	0.067 nmol μg^{-1} min^{-1}

Table 5.3: Specific Activity pCal CysFree

Activity was also tested using crude pCal WT. The activity was found to be 0.15 nmol μg^{-1} min^{-1}. Based on the poor levels of activity and difficulties in purifying the protein, a new pCal construct was devised which included the CBP fusion peptide.

Expression and Purification with pCal Fusion Protein, $pCal_f$

Introduction

The MCS was investigated for the presence of cysteine residues and it was found to contain none. This allows for future experimentation by chemically modifying

cysteine residues. The CBP construct showed promise in ease of purification by use of affinity chromatography. New oligonucleotide primers were ordered and ligation and transformation was redone to include the CBP.

Materials

New materials not previously mentioned for pCal vector construction and transfer into BL21 include HindIII as purchased from New England Biolabs and oligonucleotides for BamHI Start and HindIII Stop purchased from IDT. Calmodulin affinity resin provided by Stratagene. $CaCl_2$ binding buffer: 50 mM Tris HCl pH 8.0, 150 mM NaCl, 10 mM DTT, 2 mM $CaCl_2$. Elution buffer: 50 mM Tris HCl pH 8.0, 10 mM DTT, 2 mM EGTA, 150 mM NaCl.

Procedure

Procedure followed same as before with most experimentation performed by other lab members. Once the cells were transformed into BL21 and verified by the presence on an agarose gel, protein expression was continued as before under the assumption that no major changes occurred.

A 1 L culture of BL21 $pCal_f$ CysFree was centrifuged at 3000 x g for 30 min at 4°C and resuspended in $CaCl_2$ lysis buffer. The cells were then lysed and reacted with CBP resin on a shaking bed overnight at 4°C for Batch Binding Method of purification. The resin was prepared according to Stratagene. Ethanol was decanted and the resulting resin washed twice in 5 bed volumes of $CaCl_2$ binding buffer. After the second wash, the resin is combined with an equal volume of binding buffer and ready for use.

The resin slurry was then applied to the column and allowed to flow through. The flow through was collected to monitor the presence of protein. The column was washed

with 25 mL of binding buffer until no more protein eluted. This was again checked through a color indicator using Bradford reagent. Elution buffer was then applied and fractions collected and tested for protein. Any fractions containing protein were then subject to quantification using a Bradford assay, SDS PAGE electrophoresis for verification and specific activity. The column was regenerated by washing with 3 volumes of 0.1 M $NaHCO_3$ pH 8.6 containing 2 mM EGTA followed by 3 volumes of 1 M NaCl containing 2 mM $CaCl_2$. After which it was washed with 3 volumes of 0.1 M acetate buffer pH 4.4 with 2 mM $CaCl_2$. The final wash includes binding buffer followed by storage in 20 % v/v ethanol at 4°C.

Poor results from the column caused induction and protocols to be reevaluated and adjusted as necessary. Induction determination was again tested using various amounts of IPTG, times, temperature conditions, and reagents in the media. The solubility of the protein was called into question due to low levels of activity. Therefore, two 50 mL flasks of LB/Amp were inoculated with a colony from $pCal_f$ CysFree plate. One flask incubated at room temperature overnight and the other flask incubated at 37°C overnight. Into 4 flasks containing 250 mL of LB/Amp, 250 μL of the primary cultures were added for both the room temperature and 37°C samples. To each of these flasks various levels of IPTG were added. Flask 1 contained no IPTG and therefore the cells would not be induced. Flask 2 contained 535 μL of 20 mg/mL IPTG. Flasks 3 and 4 contained 1.07 and 2.14 mL of IPTG respectively. Every 2 h, 50 mL was removed from each of the 8 flasks and centrifuged to pellet the cells. After resuspending the cells in $CaCl_2$ binding buffer, 1 mL was removed and again centrifuged at a high speed and the pellet kept and resuspended in 50 μL 2x SDS Buffer. The rest of the cells are lysed and

mixed in a 1:1 ratio with 2x SDS Buffer for use on a polyacrylamide gel. Each gel features molecular weight markers in lane 1, purified standard ODCase in lane 2 followed by samples in increasing time with whole cells in the first lane and lysed cells in the following lane.

Another test to determine what would solubilize the protein was including 10 % glycerol in the media and or the use of 0.1 % triton in the lysis buffer. This was tested by inoculating a 50 mL flask of LB/Amp with a colony from $pCal_f$ WT and growing overnight at 37°C. A second inoculation containing 10 mL from the primary culture in LB/Amp plus 10 % glycerol was grown for 3 h at 37°C and induced with 4.2 mL IPTG (20 mg/mL). After 5 h, 1 mL was removed and two 50 mL samples of cells were collected by centrifugation. The pellets were resuspended in lysis buffer, one containing 0.1 % triton. They were then lysed and quantified using a Bradford Assay. The 1 mL collection was centrifuged and resuspended in 1 mL lysis buffer. The samples were prepared for an SDS gel by mixing equal volumes of sample with 2x SDS buffer.

Results and Discussion

Induction Time Determination: Results from monitoring the amount of protein induced for the $pCal_f$ constructs demonstrated that 4-6 h are ample time to induce maximal amount of protein. The whole cells were run on a polyacrylamide gel using molecular weight markers in lane 1 and purified ODCase in lane 2. The first lane of protein occurred at time = 0, followed by time at 2, 4, 6, and 8 h (Figure 5.10) From the gel, no marked increase in induction occurs after 4 h. This coincides with previous efforts in determining induction time.

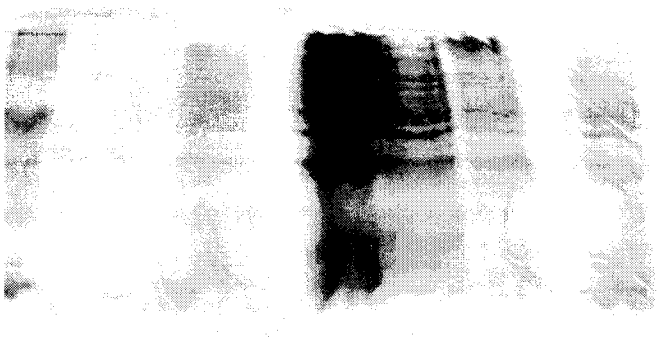

Figure 5.10: Induction Determination Using $pCal_f$ K93C

Induction Concentration Determination: No gels were run using various amounts of IPTG at 37°C. Instead, only the samples from room temperature were run on polyacrylamide gels. The three gels included 0 IPTG, 535 μL IPTG, and 1.07 mL IPTG. From the gels, it was evident that the best protein expression occurred on the gel containing 1.07 mL IPTG (gel not provided). This translates to an amount of IPTG that is 4.2 mL in a 1 L culture. Previous induction had been using 9.6 mL of IPTG in 1 L which may have been too much to induce the protein effectively.

Media Conditions Determination: The last effort to express the protein was using 10 % glycerol in the media and 0.1 % triton in the lysis buffer. For the polyacrylamide gel, 10 μL of molecular weight marker was run in lane 1, 20 μL of purified ODCase in lane 2, followed by 20 μL of each sample with unlysed cell in lane 3, lysed protein with triton in lane 4 and lysed protein with no triton in lane 5 (Figure 5.11). It is evident from the gel that the protein is now soluble in solution and has better expression in the presence of triton.

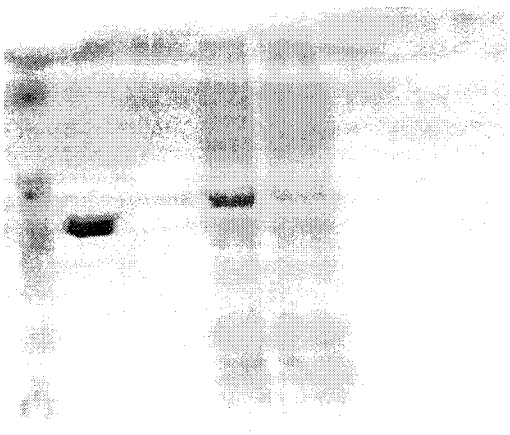

Figure 5.11 Solubility Determination $pCal_f$ WT

CHAPTER VI: CONCLUSIONS

The process of cloning, mutating, expressing and purifying the enzyme ODCase and mutants has been an involved learning experience. Numerous techniques were used for each part of the total project, all aimed at providing an adequate expression system. The overall experiments were successful with arriving at an expression system that overexpression of the gene leading to overproduction of the protein. This expression system also provides an easy one-step batch binding purification and is the basis for future investigations into the mechanistic study of ODCase.

The first part of the experiment involved cloning the ODCase WT, ODCase K93C, and ODCase CysFree genes into pESP and then transferring them to *S. pombe*. It was achieved and verified through both agarose gel electrophoresis and DNA sequencing. The expression system, regardless of cloning success, did not provide adequate expression and purification of the protein. One beneficial part of using the pESP expression system was when it was found that pESP K93C was really just pESP WT. At that time thoughts had been given to making a C93only mutant and this provided just the opportunity to do so.

The mutagenesis experiments creating K93C mutations worked and allowed for increased understanding of the properties of DNA synthesis. Without the created mutants, the project may never reach a satisfactory conclusion regarding the mechanism of ODCase.

The last major part of the thesis, cloning and expression in pCal provided much experience using old techniques and applying new techniques to create a workable system. The system now allows for increased protein expression when induced with mM

IPTG (4.2 mL/1 L) for growth at 37°C for 5 h. the expression system supports pCal WT, pCal CysFree, and pCal C93only. Future work includes the purification of pCal CysFree and determining whether or not activity is present. Once that is determined, pCal C93only will also be purified, but will have no activity due to the presence of a cysteine residue at the active site. The purified protein will all undergo chemical modification with ^{15}N bromoethylamine, converting the cysteine residues into analogs with restored activity. The binding of the isoteric inhibitor, UMP-6-thiocarboxamide, and subsequent NMR spectroscopy should provide information into the mechanism in determining whether OMP binds with C6 or O2 facing Lys 93.

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