Iso-orotate Decarboxylase:

Screening for the Gene from *Rhodotorula glutinis* cDNA library and

Properties of a Synthetic Gene

Ragini Kankanala

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Chemistry

Program

Youngstown State University

May, 2006

Iso-orotate Decarboxylase:

Screening for the Gene from *Rhodotorula glutinis* cDNA library,

Properties of Synthetic Gene

Ragini Kankanala

I hereby release this thesis to the public. I understand this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature: _____ 5/4/06

Ragini Kankanala Date

Approvals: _____ 5/4/06

Jeffrey A. Smiley, Ph.D., Thesis Advisor Date

_____ 5/3/06

Thomas Kim, Ph.D., Committee Member Date

_____ 5/4/06

John A. Jackson, Ph.D., Committee Member Date

_____ 5/5/06

Peter J. Kasvinsky, Ph.D., Dean of Graduate Studies Date

Thesis Abstract

Iso-orotate decarboxylase (IDCase) is the final enzyme in the thymine salvage pathway, which is possessed by few organisms. The reaction catalyzed by IDCase is unusual among enzymatic decarboxylases. The IDCase gene from *Neurospora crassa* has been isolated and transferred to *E. coli* in the past. But the protein from the *E. coli* was not produced in large amounts. The IDCase sequence from a *Rhodotorula glutinis* cDNA library was sought, using gene sequence information from *N. crassa* compared with the gene of *Aspergillus nidulans*, to determine the sequence of amino acids that might also be in the IDCase gene sequence of *R. glutinis*. An IDCase amino acid sequence of 12 amino acids was found to match in the sequence from *N. crassa* compared with *A. nidulans* and was used to design an IDCase gene probe. Screening was done for the IDCase gene using hybridization technique with the *R. glutinis* cDNA library. However, the sequencing data was not promising.

The project shifted to the use of the IDCase gene from *Magneportha grisea.* Protein isolated from BL21 codon plus cells containing pCal-*M. grisea* IDCase plasmid was assayed by decarboxylation of the labelled [*carboxy*-^{14}C] iso-orotate to $^{14}CO_2$. The assays did not show high activity of the IDCase protein. There is likely a codon bias preventing over-production of the heterologous protein in *E. coli*, which led to design of a Synthetic IDCase *M. grisea* gene. On successful cloning, isolation of the protein from BL21 codon plus cells containing the optimized Synthetic pcal-IDCase *M. grisea* plasmid was done, which showed high activity with [*carboxy*-^{14}C] iso-orotate radioactivity assays. The SDS-PAGE gels showed over-expressed IDCase bands at 42.9 kDa. Partial purification of the IDCase protein was done by DEAE Sephacel column chromatography

with the protein coming off from the column with salt gradient of 20 mM NaCl to 200 mM NaCl with MOPS lysis buffer, pH 6.5. Purification of the IDCase protein would help to carry on further research on the catalytic mechanism of the IDCase enzyme and the cause of rice blast disease by *M. grisea*.

Acknowledgements

I would like to acknowledge the Department of Chemistry and the School of Graduate Studies at YSU for giving me an opportunity to do my Masters. I am thankful to Dr. Jeffery A. Smiley for his guidance and support throughout my research.

Finally, I would like to express my gratitude to my father K Siva Rama Krishnaiah, my mother K. Sambrajyam, husband, brother, sister-in-law, sister and friends who have always been with me in my good and bad moments.

Table of Contents

Figures in appendix are marked with an *

List of Figures

List of Tables

List of Symbols and Abbreviation

BSA	Bovine serum albumin
IPTG	Iso-propyl-β-D-thiogalactopyranoside
MOPS	Morpholinepropanesulfonic acid
ppt	precipitation
PMSF	Phenyl methyl sulfonyl fluoride
M. grisea	*Magnaporthe grisea*
N. crassa	*Neurospora crassa*
R. glutinis	*Rhodotorula glutinis*

Chapter 1

Introduction

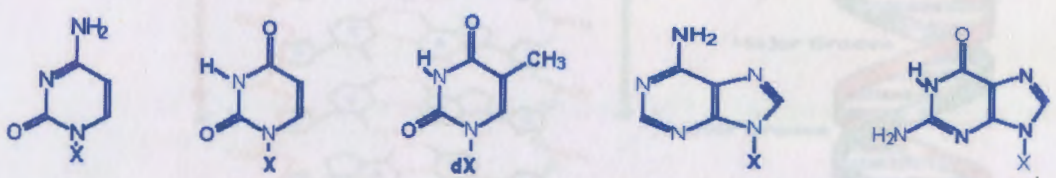
Hundreds and thousands of proteins, in the complex system of living organisms, help to maintain the cellular functions. The proteins are made up of amino acids assembled piece by piece to exact specification. To manage the complex system, a large amount of information is required which is stored in nucleic acids. (1)

Nucleic acids

Nucleic acids are large molecules with alternating sugar and phosphate molecules bonded together as long chains with the nucleotide base attached to the sugar. The order of arrangement of nucleotide bases in nucleic acids codes for the genetic information. Nucleic acids are made up of DNA and RNA. DNA contains the genetic information that is transcribed into RNA, which leads to the production of proteins. In some viruses RNA is the main genetic material.

Nucleotide bases

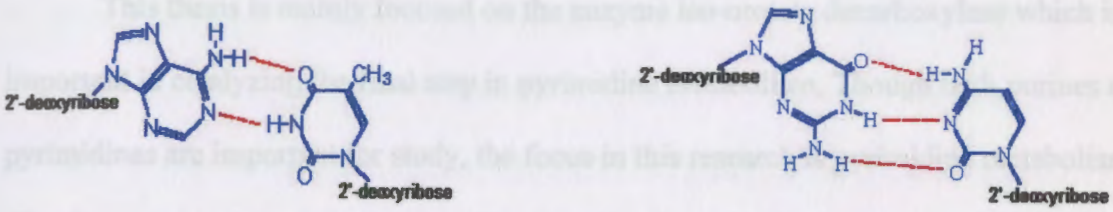
Nucleotide bases are purines and pyrimidines. Purines are adenine and guanine, pyrimidines being thymine, cytosine and uracil.



1 2 3 4 5

Figure 1 - 1: PYRIMIDINES: 1) Cytosine, C. 2) Uracil, U. 3) Thymine.
PURINES: 4) Adenine, A, 5) Guanine.
Nucleoside: X = ribose or deoxyribose.
Nucleotide: X = ribose phosphate.
Source: http://www.indstate.edu/thcme/mwking/nucleic-acids.html

In nucleic acid base pairing, adenine binds to thymine with two hydrogen bonds while guanine binds to cytosine in DNA and with uracil in RNA with three hydrogen bonds making the bonding more stable and strong.



A-T Base Pair **G-C Base Pair**

Figure 1 - 2: Adenine (A) binds to Thymine (T) with 2 hydrogen bonds. Guanine (G) binds to Cytosine(C) with 3 hydrogen bonds. Bonding between G-C is stronger than between A-T.
Source: http://www.indstate.edu/thcme/mwking/nucleic-acids.html

DNA contains deoxyribose as sugar moiety with adenine, guanine, thymine and cytosine as nucleotide base.

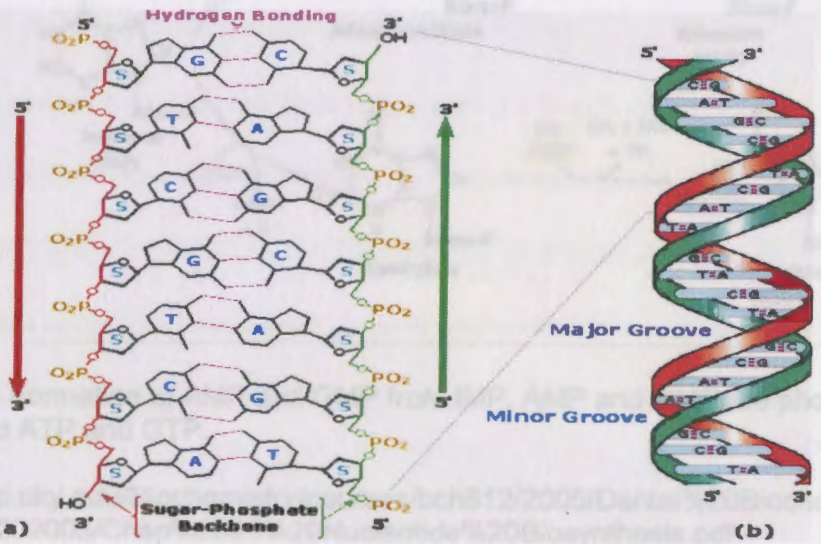


Figure 1- 3: Double helix structure
Source: http://www.cem.msu.edu/~reusch/VirtualText/nucacids.htm

RNA contains ribose as sugar moiety with adenine, guanine, thymine and uracil as nucleotide bases.

Many nucleotide analogues can be chemically synthesized; some of these have a therapeutic potential. These analogues are used as anti-tumor agents, antiviral agents, to treat gout, and to suppress immune system after organ transplantation. (2)

This thesis is mainly focused on the enzyme iso-orotate decarboxylase which is important in catalyzing the final step in pyrimidine metabolism. Though both purines and pyrimidines are important for study, the focus in this research is pyrimidine metabolism. The *de novo* biosynthesis of inosine 5'-monophosphate (IMP) shows the synthesis of purines. Adenosine 5'-monophosphate (AMP) and guanosine 5'- monophosphate (GMP) are synthesized from purine monophosphate for which the precursor is IMP.

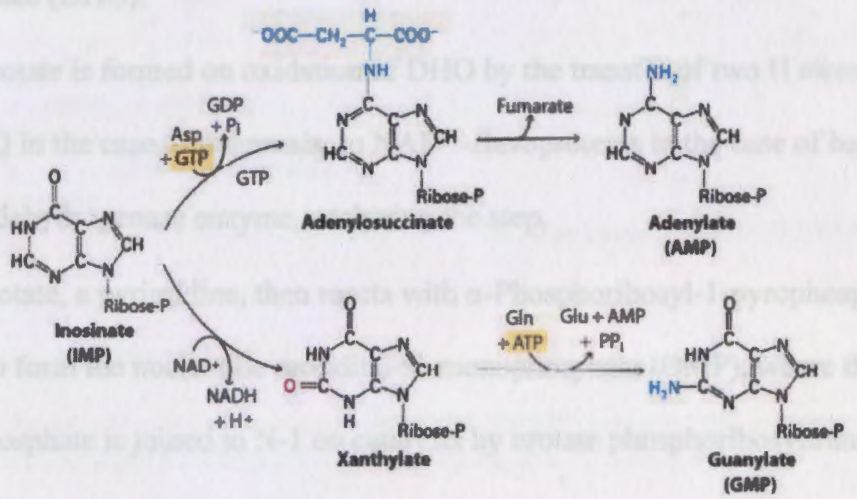


Figure 1 - 4: Formation of AMP and GMP from IMP. AMP and GMP are phosphorylated further to form ATP and GTP.
Source:
http://www.mc.uky.edu/Biochemistry/courses/bch812/2005/Dental%20Biochem%20Lecture%20CD%202005/Chap%2025%20Nucleotide%20Biosynthesis.pdf

The metabolism of pyrimidines is similar to the biosynthesis of purines with differences in the enzyme steps involved and the product in the *de novo* pyrimidine synthesis being uridine 5′-monophosphate (UMP). (3) Mary Ellen Jones first discovered

the *de novo* pyrimidine biosynthesis pathway in *N. crassa*. Strains of fungi grew in orotic acid as the only pyrimidine source. [5] The precursors for UMP are carbamoyl phosphate and aspartate. The following are the steps in *de novo* pyrimidine biosynthesis:

- Step I: carbamoyl phosphate is formed from bicarbonate, glutamine, 2 ATP molecules and H_2O, carbamoyl phosphate synthetase II (CPS II) catalyzing the step I.
- Step II: Asparatate transcarbamoylase (ATCase) catalyses the condensation of carbomyl phosphate and asparatate to yield N- carbamoylaspartate.
- Step III: Dihydroorotase catalyses the ring closure and dehydration between the $-NH_2$ group of carbomyl phosphate and the former β-COO^- of aspartate to form dihydroorotate (DHO).
- Step IV: Orotate is formed on oxidation of DHO by the transfer of two H atoms – to coenzyme Q in the case of mammals, to NAD^+ -flavoproteins in the case of bacteria – with DHO dehydrogenase enzyme catalyzing the step.
- Step V: Orotate, a pyrimidine, then reacts with α-Phosphoribosyl-1-pyrophosphate (α-PRPP) to form the nucleotide orotidine-5'-monophosphate (OMP), where the ribose-5-phosphate is joined to N-1 on catalysis by orotate phosphoribosyltransferase.
- Step VI: this is the final step in the *de novo* pyrimidine biosynthesis which yields UMP. OMP decarboxylase catalyzes the decarboxylation of OMP to yield UMP

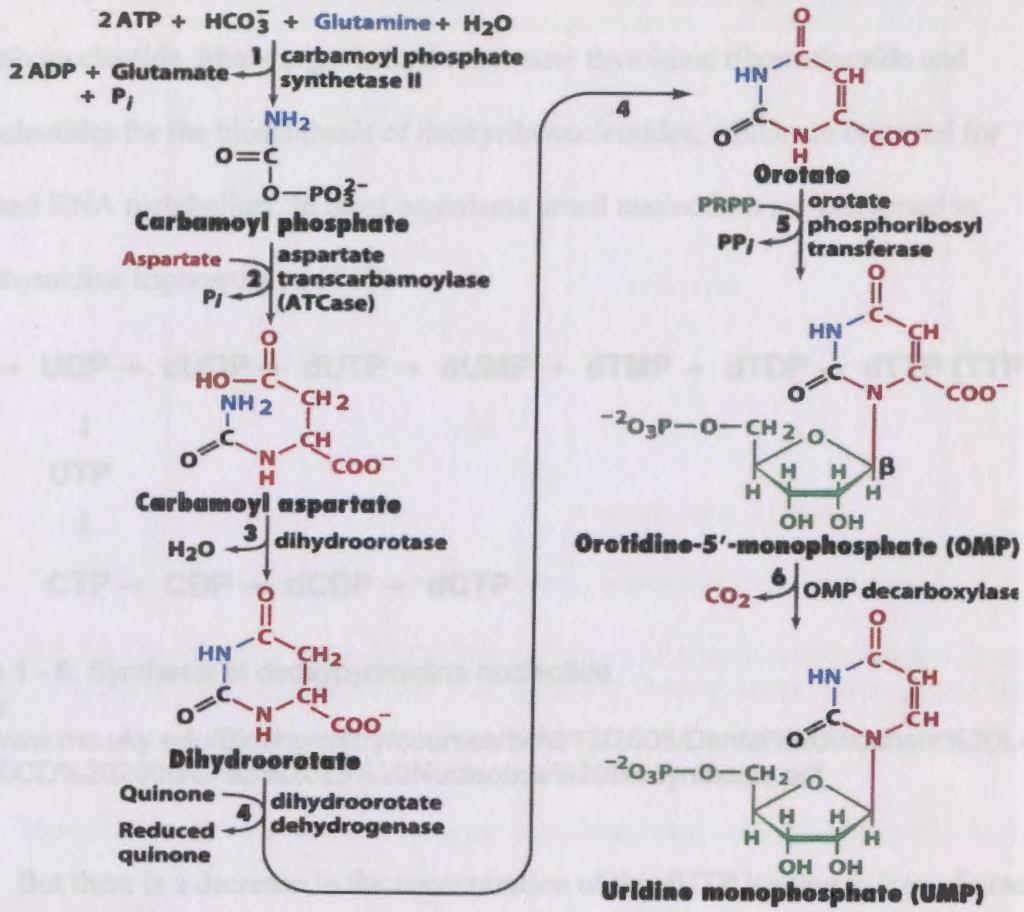


Figure 1 - 5: De novo biosynthesis of pyrimidine.
Source:
http://www.mc.uky.edu/Biochemistry/courses/bch812/2005/Dental%20Biochem%20Lecture%20CD%202005/Chap%2025%20Nucleotide%20Biosynthesis.pdf

In bacteria, regulation of the *de novo* pyrimidine biosynthesis occurs at step II of the above stated mechanism where ATCase enzyme is activated by ATP and inhibited by CTP or UTP, while in animals the activation occurs at step 1 with ATP or PRPP activating the CPS II enzyme and UDP or UTP inhibiting it. On formation UMP converts into UDP. This is converted to a dNTP by a ribonucleotide reductase.

Incorporation of the ribonucleotides into DNA occurs only on reduction to deoxyribonucleotide. Many organisms do not reuse thymidine ribonucleoside and ribonucleotides for the biosynthesis of deoxyribonucleotides, which are essential for DNA and RNA metabolism. In most organisms uracil nucleotides are converted to deoxythymidine triphosphate (dTTP)

UMP → UDP → dUDP → dUTP → dUMP → dTMP → dTDP → dTTP (TTP)

↓

UTP

↓

CTP → CDP → dCDP → dCTP

Figure 1 - 6: Synthesis of deoxypyrimidine nucleotide
Source:
http://www.mc.uky.edu/Biochemistry/courses/bch812/2005/Dental%20Biochem%20Lecture%20CD%202005/Chap%2025%20Nucleotide%20Biosynthesis.pdf

But there is a decrease in the concentration of the dUTP leading to loss of uracil without being incorporated into DNA.

Thymidine Salvage Pathway

Through thymidine salvage pathway, a few organisms reuse the thymidine ribonucleotides and ribonucleosides. The pathway was first discovered by Palmatier *et al.* Thymine is converted to uracil, which is reincorporated into the total pyrimidine pool.

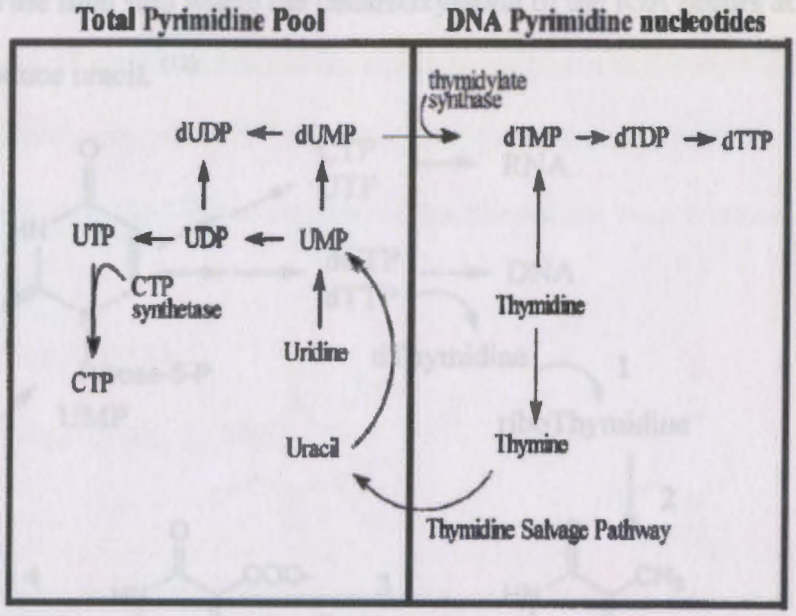


Figure 1-7: Total pyrimidine pool. Thymidine salvage pathway allows thymidine to enter the total pyrimidine pool Source: Danny Yun, Master's Thesis. 1999, Youngstown State University.

The ring-labeled thymidine was taken into the system with the incorporation into both DNA and RNA.

Iso-orotate decarboxylase (IDCase)

Iso-orotate decarboxylase (IDCase) is the enzyme which catalyses the conversion of isoorotate to uracil which is a deoxyribonucleotide base completing the conversion of thymidine to uracil. The 4 steps in the thymidine salvage pathway are as follows:

- Step 1: oxidation of thymidine to thymine ribonucleoside.
- Step 2: Thymine nucleoside cleaves at the C1 and N1 hydrolytically to produce thymine and ribose.
- Step 3: Oxidation of thymine to 5-carboxyuracil (iso-orotate, IOA).

- Step 4: This is the final step where the decarboxylation of the IOA occurs at the 5th position to produce uracil. [12]

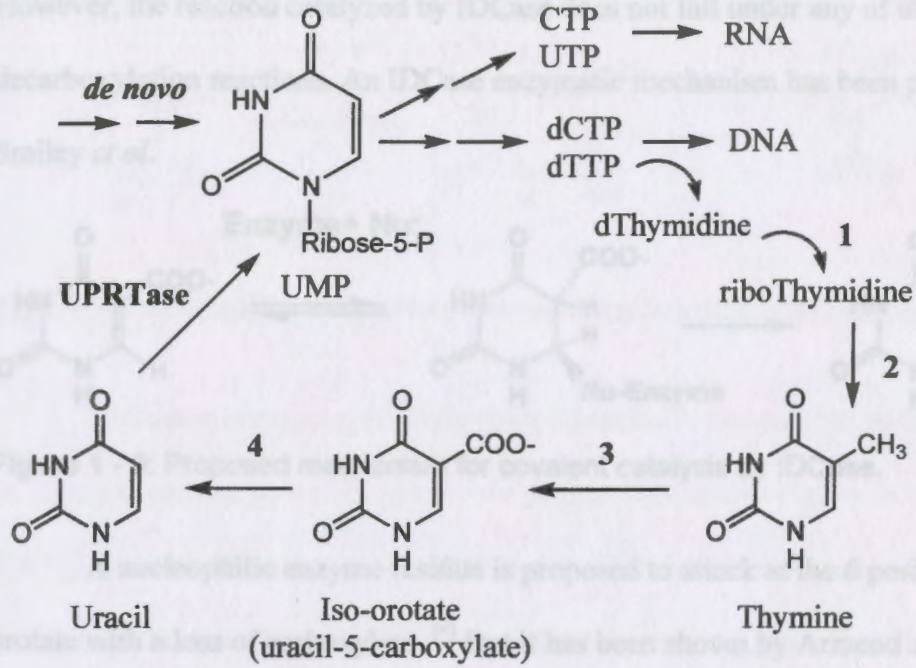


Figure 1 - 8: Thymidine Salvage Pathway. The 4th step is catalyzed by IDCase. IDCase is identified only in certain fungi.

The first research on IDCase enzyme published by Abbott and co-workers states that mammals are capable of producing iso-orotate but lack the capacity to decarboxylate it to produce uracil. A [^{14}C] labeled study on thymidine salvage pathway in fungi showed incorporation of [^{14}C] from thymidine into RNA and not DNA. This lead to the belief that thymidine a precursor for DNA thymine nucleotides, undergoes demethylation to uracil which is a precursor to RNA nucleotides. The research of Abbott *et al.* also showed that the enzyme and the pathway are present in *N. crassa*. [6]

Proposed mechanisms for IDCase catalysis

IDCase is interesting to study as the reaction from iso-orotate to uracil is an unusual decarboxylation reaction. Decarboxylation reactions commonly use the

following types of substrates in the 4 main types of enzymatic mechanisms: amino acid, α -ketoacid, β –ketoacid and β-hydroxy acids which are oxidized in the reactions. However, the reaction catalyzed by IDCase does not fall under any of the above decarboxylation reactions. An IDCase enzymatic mechanism has been proposed by Smiley *et al.*

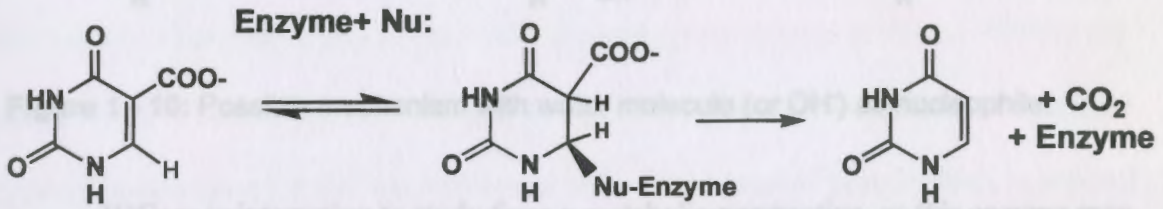


Figure 1 - 9: Proposed mechanism for covalent catalysis by IDCase.

A nucleophilic enzyme residue is proposed to attack at the 6 position of the iso-orotate with a loss of carboxylate. [4] But it has been shown by Armend A. Axhemi that on site directed mutagenesis of proposed nucleophilic cysteine 272 of *N. crassa* IDCase mutated into an alanine residue, the activity of the mutant protein was not noticeable with a conclusion that the original Cys272 did not play a catalytic role. (Cys272 of *N. crassa* IDCase is the only cysteine residue within conserved regions of sequences from several fungi.)

The IDCase gene sequence has a very high similarity with the gene sequence of certain amidohydrolase enzymes. The catalytic site in the amidohydrolase is a group of histidine and carboxylate residues that activate a water molecule, making it a nucleophile. The chemical reactions catalyzed by the enzymes cytosine deaminase and dihydroorotase, which belong to the amidohydrolase superfamily, show similarity in structure to the substrate/product of the IDCase catalyzed reaction. [19] There is a possibility that IDCase may be an amidohydrolase enzyme with a divalent metal center.

The possible mechanism for IDCase with the water molecule as nucleophile attacking iso-orotate at C6 with the loss of carboxylate is shown.

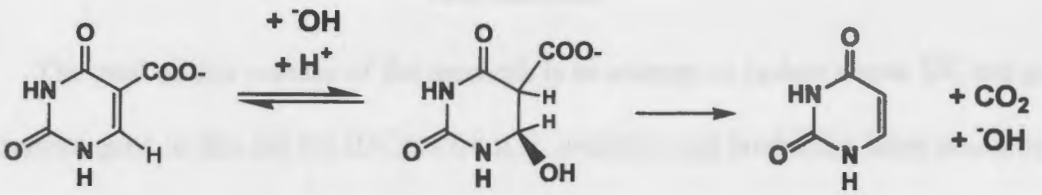


Figure 1 - 10: Possible mechanism with water molecule (or OH^-) as nucleophile.

IDCase is interesting to study from a metabolic perspective, as this enzyme may be an important enzyme in *Magnaporthe grisea*, the causative agent in rice blast disease. IDCase gene has been isolated from *N. crassa*. This was transferred to *E. coli*, and the IDCase has been produced. But the protein production was low. There is a need to isolate a new IDCase gene which might increase the production of the protein and can be easily purified. Protein purification will be necessary for enzymological experiments designed to provide evidence for a catalytic reaction mechanism. The new IDCase gene can be used to compare with other IDCase genes of other organisms to determine the essential amino acids required for enzyme activity.

The goals of the experiments in this thesis are as follows: 1) Attempt to isolate an IDCase gene from a cDNA library of *R. glutinis* with a hope to convert the gene into a plasmid, transform the plasmid into BL21 codon plus bacterial strain, isolate the plasmid and sequence it. 2) Isolate the IDCase gene from *M. grisea*, for which the sequence is known. 3) Construct a synthetic version of the *M. grisea* gene, and isolate and purify the protein from a plasmid carrying this synthetic gene.

Chapter 2

Screening for the IDCase Gene in a cDNA Library from *Rhodotorula glutinis*

Introduction

The goal of this portion of the research is to attempt to isolate a new IDCase gene. The current gene in this lab for IDCase from *N. crassa* is not producing large amounts of IDCase when introduced into *E. coli* cells. Several genes isolated in this lab showed the desired protein band on SDS-PAGE gels in significant abundance even in the unpurified lysate. If a new gene for IDCase, which can give over-expressed protein band, is isolated then purification of IDCase will be easier.

IDCase is present in the strain of yeast called *R. glutinis*. Enzyme assays have been done in this lab to see for the presence of IDCase in *R. glutinis*. So it should be possible to confirm the IDCase gene from *R. glutinis*, if a candidate gene is isolated. A cDNA library from *R. glutinis* has been used in our lab for the isolation of the thymine-7-hydroxylase (THase) gene, [12] so this should be usable to isolate the IDCase gene as well. THase precedes IDCase in the thymidine salvage pathway. Previous research has also shown that THase has been purified from *R. glutinis* cultures, grown on thymine as the only nitrogen and pyrimidine source which is converted to IOA by the enzyme thymine 7-hydroxylase in *R. glutinis*. If the IDCase gene and enzyme were not to be present, accumulation of IOA would occur. [12]

Individual genes can be identified and isolated from a cDNA library using an oligonucleotide that is complementary to the desired sequence. We can design an oligonucleotide if we know the amino acid sequence in the IDCase gene. In order to determine the possible amino acid sequence that might be present in the IDCase gene of

R. glutinis, comparison the amino acid sequence of *N. crassa* with that of *A. nidulans* was done.

```
A. nidulans   233 TTIAVTRMLLSGVFDRFPRLKILLAHSGGTLPFLAGRIESCILHERKFISGGGDVQGPQR
N. crassa     232 TTIAVTRMYLAGVFDQVPKLNMLLAHSGGTLPFLAGRIESCILHDGHLHSAAGT--KPKK
                  ******** * ****  * *  **********************     * *    *

A. nidulans   293 SVWDVLKTNIYLDAVVYGKPGLEAAMTASGSD---RLLFGTDHPFFPPLDS-KDNSWPSV
N. crassa     290 TIWEVLSSQIYLDAVVYSDVGLKAAVQASGPEGHERLMFGTDHPFFPPLGSDEEGEWESV
                   * **   ********   ** **  ***     ** ********** *     * **

A. nidulans   349 TTNYQAIHATFDTNS-------KTVADVLGGNAARILNL
N. crassa     350 TWNGAAVRKAFGAEDGEDSEEGKKVRGVMGANAVRVLNLRRDGCR
                  * *  *    *           * *  * * ** * ***
```

Figure 2-1: Comparison of *A. nidulans* IDCase amino acid sequence with *N. crassa*. Asterisks are written below the portions of sequences that are identical when aligned.

The two amino acid sequences had only partial identities. The IDCase sequence of 12 amino acids was taken that is identical in *A. nidulans* and *N. crassa* IDCase amino acid sequences. This is the start point for designing the oligonucleotide and attempting to identify the IDCase sequence in the cDNA library from *R. glutinis*. For screening of new IDCase, a hybridization technique has been used.

Sequencing for IDCase gene using hybridization technique

The library of genes is composed of a collection of phages, each with a potentially different insert. The phages are introduced into host *E. coli* cells, and the infected cells are plated onto agar plates so that about 1000 plaques will form on a 15-cm diameter plate. Nylon filter papers are placed onto the plaque-covered plates, and a replica pattern is produced on the paper. If the paper is then immersed in a solution containing the oligonucleotide, the probe will anneal to the paper on the spots corresponding to the original plaques, if that plaque contains DNA complementary to the probe. A visualization technique is necessary to identify the plaque where the annealing of the

probe to the DNA complementary strand occurred, for which the probe is bound with digoxygenin (DIG) attached covalently.

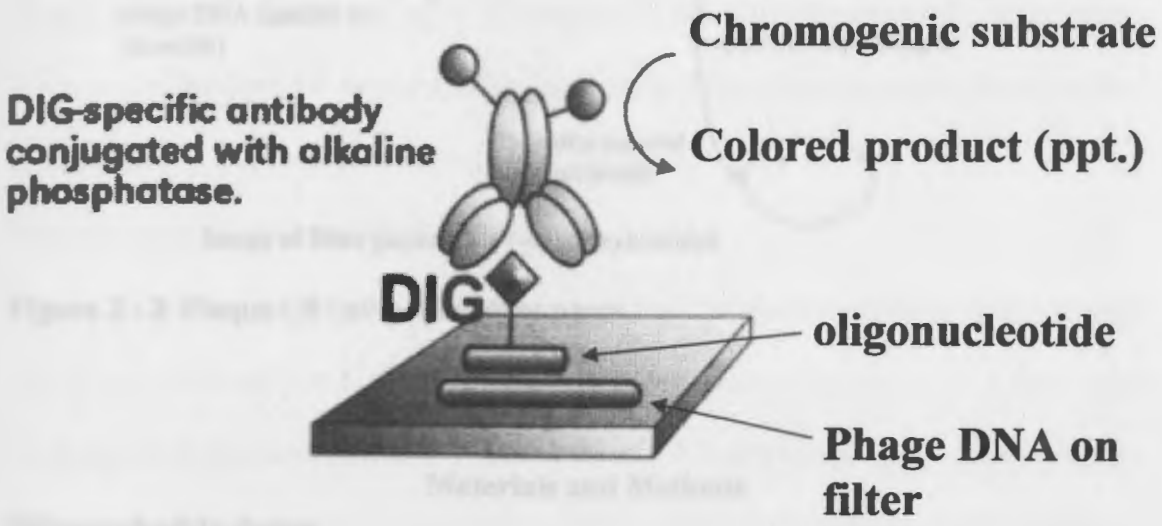


Figure 2- 2: Schematic representation of Hybridization technique. The representation shows the concept of probing for the gene sequence in a cDNA library using a complimentary oligonucleotide that is attached to digoxygenin (DIG). DIG binds specifically with the antibody - alkaline phosphatase conjugate.

DIG is specific for antibody that is conjugated with alkaline phosphatase which appears as a colored spot on the nylon filter paper and the location of the corresponding plaque is determined on the Petri dish.

The isolated plaque which contains the annealing portion should be then converted to the plasmid phage so that the plasmid can be used for transformation into the desired strain of bacteria. In this chapter, plaques from an *R. glutinis* cDNA library will be screened by using the hybridization technique in order to get a positive plaque, which might contain the IDCase gene.

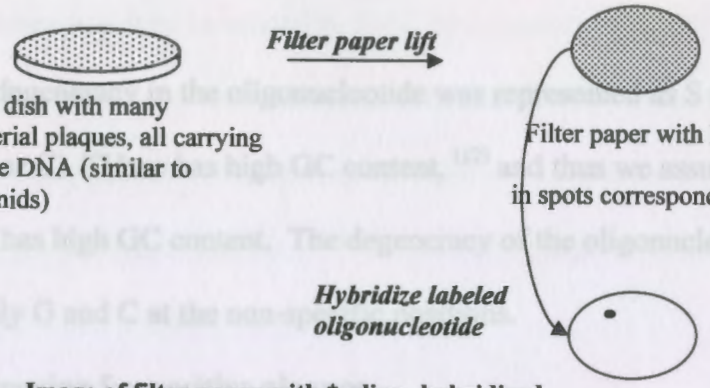


Figure 2 - 3: Plaque Lift using nylon filter paper.

Materials and Methods

Oligonucleotide design

The isolation will be based on hybridization of a synthetic DNA oligonucleotide, labeled for visualization with digoxygenin (DIG) attached covalently. A 36-base HPLC purified oligonucleotide was ordered from Integrated DNA Technologies, In. (IDT). The following is the sequence of degenerate oligonucleotide.

5'- /5DigN/GCS CAC TCB GGC GGC ACS CTS CCS TTY CTS GCS GGC -3'.

The 12 amino acids are Ala256 through Gly267 of the *N. crassa* sequence.

Protein:	**Ala**	**His**	**Ser**	**Gly**	**Gly**	**Thr**	**Leu**	**Pro**	**Phe**	**Leu**	**Ala**	**Gly**
Possible	**GCT**	**CAT**	**TCT**	**GGT**	**GGT**	**ACT**	**CTT**	**CCT**	**TTT**	**CTT**	**GCT**	**GGT**
Codons	**GCC**	**CAC**	**TCC**	**GGC**	**GGC**	**ACC**	**CTC**	**CCC**	**TTC**	**CTC**	**GCC**	**GGC**
	GCA		**TCA**	**GGA**	**GGA**	**ACA**	**CTA**	**CCA**		**CTA**	**GCA**	**GGA**
	GCG		**TCG**	**GGG**	**GGG**	**ACG**	**CTG**	**CCG**		**CTG**	**GCG**	**GGG**
			AGT				**TTG**			**TTG**		
			AGC				**TTA**			**TTA**		
Probe:	**GCS**	**CAC**	**TCB**	**GGC**	**GGC**	**ACS**	**CTS**	**CCS**	**TTY**	**CTS**	**GCS**	**GGC**

Table 2 - 1: Designing of probe for isolation of IDCase gene sequence. Gene sequence from protein sequence for the amino acids used for designing the probe. The possible codons for each amino acid can be seen where the last nucleotide base is different leading to degeneracy.

The degeneracy in the oligonucleotide was represented as S (G or C), B (G, C, or T) and Y (T or C). THase has high GC content, [12] and thus we assume that *R. glutinis* IDCase also has high GC content. The degeneracy of the oligonucleotide therefore has predominantly G and C at the non-specific positions.

Primary screening for positive plaques

XL1 Blue strain of *E. coli* is mixed with lambda phage containing cDNA library constructed from mRNA of the *R. glutinis* genes. An isolated colony of XL1 Blue from a working stock plate was inoculated into 50 ml of LB (basic composition of LB: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.4) with 1/1000 volume of 1 M $MgSO_4$ and 1/100 volume of 20% maltose added for optimal adsorption of the phage to bacteria during transduction or plaque titering. The culture was kept for growth with shaking at 140 rpm in incubator at 37 °C until the cells were dense. The culture was removed and centrifuged at 5000 rpm for 5 minutes. The cell pellet was resuspended in 25 mL of 10 mM $MgSO_4$ and stored at 4 °C. Into ten 15-ml Falcon tubes, 50 µL of the resuspended cell culture and 1.2 µL of the lambda phage carrying the cDNA library from *R. glutinis*, were added and mixed. The tubes are then incubated at 37 °C without shaking. After removing the tubes, 7.5 mL of LB soft top agarose (LB plus 0.75% agarose) maintained at 45 °C was added to each tube. The contents of the tube are mixed quickly and poured onto large Petri dishes containing LB + 1.5% agar + 10 mM $MgSO_4$ with gentle swirling of the Petri dish so that the LB top soft agarose spread uniformly throughout the plate.

Ten large Petri dish plates were prepared according to the previous stated procedure. The plates are allowed to cool at room temperature, to allow the top soft

agarose to harden and then incubated at 37 °C by inverting the plates until the plaques are distinctly visible. [9] Usually the incubation time was up to 7 h. Plaque lifting and hybridization using positively charged nylon filter papers was done in accordance with the protocol from the manufacturer. [9] The nylon filter papers are aligned with the respective agar plates to locate the positive plaque.

Phage amplification

Suspected positive isolated plaques were picked and each agar plug was placed in 500 µL of 1X lambda dilution buffer (Roche) with gelatin added to a final concentration of 0.01% to stabilize the phage for long-term storage. The contents are vortexed, incubated at 37 °C for 3 h and stored in the freezer. A secondary screening was done using each isolated positive plaque phage following the same procedure as for primary screening.

Conversion of lambda phage into plasmid phage

The secondary phage was used to convert into plasmid. Single plaque conversion was done in accordance to the manufacturer's protocol. [9] Isolated colonies of BM25.8 *E. coli* cells from the LB/agar plates (+ ampicillin) were used to prepare plasmid mini-prep separately.

Plasmid mini-prep

Individual 5-mL culture tubes of LB plus 50 µg/mL ampicillin were inoculated using sterile technique. The tubes are allowed to grow overnight at 37 °C in incubator with shaking at 200 rpm. A volume of 1.5 mL of each overnight culture is taken in microcentrifuge tubes and centrifuged for 1 minutes at 13000 rpm. The cell pellet was recovered by decanting the supernatant. The cell pellet was resuspended in 200 µL of

Solution A (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA). 300 µL of Solution B (0.2 M NaOH, 1 % sodium dodecyl sulfate) was added to the resuspended solution. The microcentrifuge tube was inverted 4 - 6 times to mix. 300 µL of cold Solution C (3 M potassium acetate, 2 M acetic acid, which was adjusted to a pH 4.8) was added and allowed to sit for 5 minutes on ice. The microcentrifuge tubes are again centrifuged for 5 minutes at 13000 rpm. From this 750 µL of the supernatant was transferred to a new microfuge tube, and 450 µL of isopropanol was added. The tube was allowed to sit for 5 minutes at room temperature. The tube was set to centrifugation for 5 minutes at 13000 rpm. The supernatant was removed, and the pellet was resuspended in 50 µL TE buffer. The presence of plasmid was tested by running a 0.8% agarose gel electrophoresis. Since the quality of mini-prep DNA from BM25.8 is poor, the prepared plasmid was used to transform into *E. coli* XL1 Blue cells.

***E. coli* transformation**

The plasmid mini-prep was used to transform XL1-Blue strain by taking 0.2 mL of $CaCl_2$ treated competent cells and adding 100 ng of the prepared plasmid mini-prep in chilled Falcon tube. A negative control tube is also prepared with only the $CaCl_2$ cells and no DNA added. The tubes are set to sit in ice for 5 minutes. and transferred to 42 °C water bath for 2 minutes. The tubes from water bath are removed and rapidly set on ice. 1 mL of sterile LB media is now added to each tube. The tubes are then incubated at 37 °C with shaking for 60 minutes. 100 µL of each sample of cells are transferred onto small LB/Agar/Ampicillin plates and spread with a flame sterilized spreader. The plates are incubated overnight at 37 °C.

The isolated colonies from the XL1 Blue transformation are kept for overnight cultures of 5 mL using LB+ampicillin culture tubes to do new plasmid mini-prep on the cultures using the previously stated procedure. Electrophoresis on a 0.8 % agarose gel was run.

Using the 5-mL culture of plasmid mini-prep, a 50-mL secondary culture was started by incubating at 37 °C with shaking at 200 rpm overnight. The culture was centrifuged and the wet cell mass was used to purify the DNA using QIAGEN HiSpeed Plasmid Midi Kit. The presence of plasmid was determined by running a 0.8% agarose gel and quantified by UV/Visible spectrophotometry at 260 nm.

Sequence analysis of the DNA was done using the following designed primers:

Triplex 5'sequencing:

5' – CTC CGA GAT CTG GAC GAG – 3'

Triplex 3' sequencing:

5' – TAA TAC GAC TCA CTA TAG GG – 3'

The sequencing reaction conditions were set up and sample preparation for sequencing was done as specified in the Beckman Coulter manual. [10]

Results and Discussion

In the primary screening for positive plaques, approximately 1000 plaques arose on all large plates. A total of 50 plates were screened, representing approximately 50,000 members of the library. Six suspected positive plaques numbered as 2, 4, 7, 8, 1P1 and 1P2 were picked and the phage amplification was done separately. The replating for secondary screening gave around 100 plaques for plates 1P1 and 7, 75 plaques for plate 1P2, 20 plaques for plates 4 and 8 and 15 plaques for plate numbered 2 with the amplified

phage. The secondary screening gave 5 positive plaques which were numbered as 7-1, 7-2, 7-3, 1P1 and 1P2. The amplified phage supernatant was taken for phage conversion into plasmid in BM25.8 cells. Isolated colonies were obtained for all the phages except for 7-2. 0.8% agarose gel electrophoresis showed bands from plasmid mini-prep done on the isolated colonies of BM25.8.

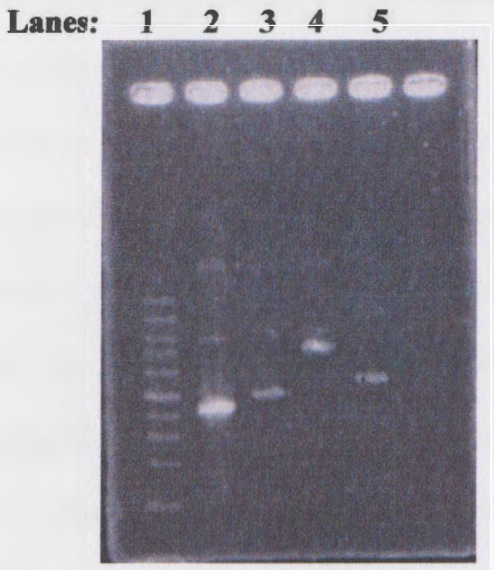


Figure 2 - 4: 0.8% Agarose gel showing plasmids 7 - 1, 7 - 3, 1P1 and 1P2 in lanes 2, 3, 4 and 5 respectively with 1 kbp DNA ladder in lane 1.

The four plasmids were used to transform XL1 Blue cells separately. Well isolated colonies were obtained for all the four plasmids. Successful purification of DNA for the four plasmids was done with QIAGEN HiSpeed Plasmid Midi Kit. Purification of the four plasmids gave DNA that was used as template in sequencing samples. The sequencing data was obtained only for 7-2 and 1P1 (Sequencing data for 1P1, Appendix). The sequences of *A. nidulans* and *N. crassa* IDCase showed two histidine residues separated by an amino acid at the beginning of the sequence. The histidine residues should also be present at the beginning of the IDCase gene sequence in *R. glutinis*, which we did not see in the sequencing data of both the plasmids. The small sizes of plasmids 7-

1, 7-3 and 1P2 (Figure 2-4) indicates that only a small insert is present within the plasmid, probably not large enough to contain the entire IDCase gene. Thus, the screening procedure yielded no sequences that appeared to be possible IDCase sequences.

Chapter 3

Attempted PCR Amplification of a DNA fragment containing a portion of both THase and IDCase genes.

Introduction

PCR is an *in vitro* technique to amplify a specific DNA fragment. In this portion of the project, the goal was to amplify a DNA fragment from genomic DNA that may contain a portion of the *R. glutinis* IDCase gene.

In *A. nidulans* and *N. crassa*, the IDCase gene and the THase gene are adjacent to each other from the genomic sequencing. We hypothesized that *R. glutinis* may also have the same sequence arrangement. [11] Amplification of the DNA fragment containing portions of the THase gene may extend to produce a portion of IDCase gene. The primers for PCR are designed with a portion of THase gene as start site so that the extension of the DNA strand occurs towards the IDCase gene in PCR amplification. The second fragment is designed complementary to the oligonucleotide sequence in the previous chapter. In the PCR reaction we used the genomic DNA of *R. glutinis* as template, with primers were designed so that they are complementary to the two possibly adjacent genes.

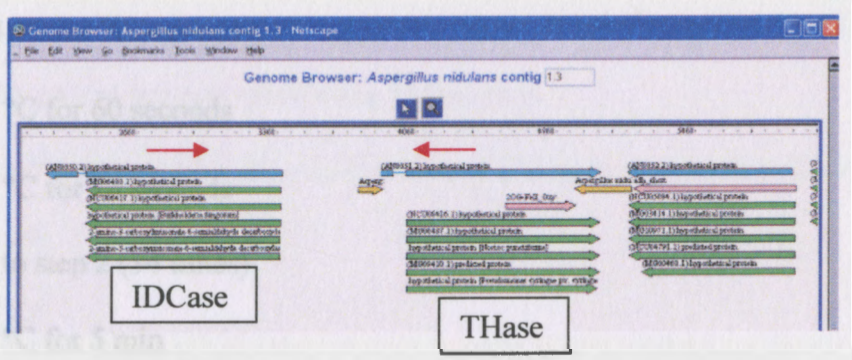


Figure 3-1: A portion of the *A. nidulans* genome. The genome is represented schematically in the web site at http://www.broad.mit.edu. PCR amplification could be carried out using *R. glutinis* DNA, using primers shown schematically as red arrows, if the two genes are situated similarly in *R. glutinis*.

Materials and Methods

The primers used are: "*R. glutinis* IDCase fragment NdeI" with 38 bases:

5'-ATG TTC ATA TGG CCS GCS AGR AAS GGS AGS GTG CCG CC -3'.

'S' is for G or C nucleotide base. This is a reverse complementary primer intended to anneal to the IDCase gene about 750 bp from the start site. The second primer is "*R. glutinis* IDCase fragment HindIII" with 38 bases is complementary to the start point of THase gene sequence.

5'- ATG TTA AGC TTG ACG GGG ACG ATG CCA GAC GAG ACC AT -3'

Primers were obtained from Integrated DNA Technologies, Inc. (IDT). If the PCR gives the amplified DNA, sequencing of the amplified DNA gives the portion where the annealing of the primers occurred. The desired PCR product would be about 1000 bp, since there is an intervening sequence between the IDCase and THase genes in the known genomes.

The first PCR reaction was done using 1 μL of undiluted *R. glutinis* genomic DNA as template. The following is the program:

Step 1: 94 °C for 5 minutes

Step 2: 94 °C for 30 seconds

Step 3: 58 °C for 60 seconds

Step 4: 72 °C for 75 seconds

Step 5: go to step 2 (34 times)

Step 6: 72 °C for 5 min

Step 7: 4 °C for ever

The 2nd, 3rd 4th PCR attempts were done with 3 µL of 1:10 dilution, 1 µL of 1:10 dilution, 3 µL of 1:100 dilution of *R. glutinis* genomic DNA as template. The 5th PCR attempt was done using 1 µL of 1:100 dilution of the *R. glutinis* genomic DNA with THase primers. The 5th PCR was a control reaction. For 2nd, 3rd, 4th and 5th PCR attempts the gradient was used and 12 PCR tubes were kept. The gradient PCR program is as follows:

Step 1: 94 °C for 5 minutes

Step 2: 94 °C for 1 minute

Step 3: 52 °C – 70 °C for 1 minute

Step 4: 72 °C for 1 minute 15 seconds

Step 5: go to step 2 (34 times)

Step 6: 72 °C for 5 minutes

Step 7: 4 °C for ever

Step 8: end

The temperatures in the 12 PCR tubes holdings are 52 °C, 52.5 °C, 53.4 °C, 54.9 °C, 57 °C, 59.6 °C, 62.6 °C, 65.2 °C, 67.2 °C, 68.6 °C, 69.6 °C and 70 °C.

A necessary control reaction was carried out to see if the *R. glutinis* DNA can be amplified by pair of primers specific for THase gene.

The primers used for the amplification of THase in the genomic DNA of *R. glutinis* are:

THase 5' PCR

5' TCC GTG AAG TCA TAT GGT CTC GTC TG 3'

and THase 3' PCR

5' TCC GTG AAA TAA GCT TTT CAC TTC TTG C 3'

Results and Discussion

The amplification of the IDCase-THase fragment did not occur. There was no amplified DNA present when 1.2% agarose gel was run. Several different PCR conditions were used, but none with success. There was a band seen for the amplified THase segment on the agarose gel (data not shown).

Several explanations are possible for the lack of a positive result. The two genes may be situated differently in the *R. glutinis* genome. There might be an intron present in either the IDCase or the THase genes in the genome of *R. glutinis* resulting in the lack of a result for the IDCase-THase fragment amplification.

Chapter 4

Optimization of IDCase Activity Produced from a Plasmid Carrying a Synthetic Version of the Magnaporthe grisea Gene

Introduction

With the sequencing results of screening for IDCase gene from cDNA library of *R. glutinis* and the PCR amplification of the IDCase-THase fragment not successful, the *Magnaporthe grisea* gene was targeted to look for high activity of the IDCase protein using [*carboxy*-^{14}C] iso-orotate radioactivity assays. The *N. crassa* IDCase gene in our lab did not produce IDCase in large amounts. We hoped that the IDCase *M. grisea* would yield large amounts of the protein.

The gene sequence for IDCase *M. grisea* was known from previous research on this fungal species and is available online at http://www.broad.mit.edu. *M. grisea* is the causative organism for rice blast disease, and IDCase might be an important enzyme in the microorganism. We intend to produce an *E. coli* system with IDCase *M. grisea* that over-expresses the IDCase, making it easy to purify the IDCase from *E. coli* cells.

A synthetic IDCase *M. grisea* gene was used to optimize the IDCase activity. The reason for making a synthetic IDCase gene is the codon usage in *E. coli* cells. A rare codon bias exists in *E. coli*. There are 61 amino acid codons found for the production of mRNA molecules of *E. coli*. The different tRNA present depends on the frequency of the codon usage, which leads to a situation where the mRNA is abundant and the tRNA is low. If the tRNA is not present for the mRNA, there will be a slower rate of protein production. This situation is more pronounced when the cloned plasmid with a heterologous gene insert is transformed to *E. coli* for over-expression of the protein.

There may be rare codons in the gene insert which leads to mistranslation and the resulting protein synthesized is of low quantity and quality. [15, 16, 17, 18] We found that the gene sequence of the insert DNA fragment in the IDCase *M. grisea* wild type had rare codons for amino acids. Four of the amino acids codon usage in IDCase *M. grisea* and the Synthetic IDCase *M. grisea* are shown below. The manufacturer changed the rare codons of amino acids in the Synthetic IDCase *M. grisea* gene. The codon usage for the other 15 amino acids is shown in the Appendix of this thesis.

Codon usage:

Arginine codons

	CGT	CGC	CGA	CGG	AGA	AGG
IDCase *M. grisea*	0	3	6	0	5	2
Synthetic IDCase *M. grisea*	10	9				

Leucine codons

	CTT	CTC	CTA	CTG	TTA	TTG
IDCase *M. grisea*	8	12	2	15	3	6
Synthetic IDCase *M. grisea*	0	0	0	46	0	0

Isoleucine codons

	ATT	ATC	ATA
IDCase *M. grisea*	4	7	11
Synthetic IDCase *M. grisea*	11	11	0

	Proline codons			
	CCT	CCC	CCA	CCG
IDCase *M. grisea*	8	9	6	4
Synthetic IDCase *M. grisea*	0	0	0	27

The synthetic IDCase *M. grisea* gene was used with the hope that the yield of IDCase would be in large amounts in *E. coli* cells.

Molecular cloning of the synthetic IDCase *M. grisea* DNA fragment was done by inserting it into a plasmid, which is an autonomous replicating DNA molecule. The DNA fragment is replicated in the host organism using the recombinant plasmid producing large amount of the protein in the host cells. [13] The plasmid used was pCAL-n.

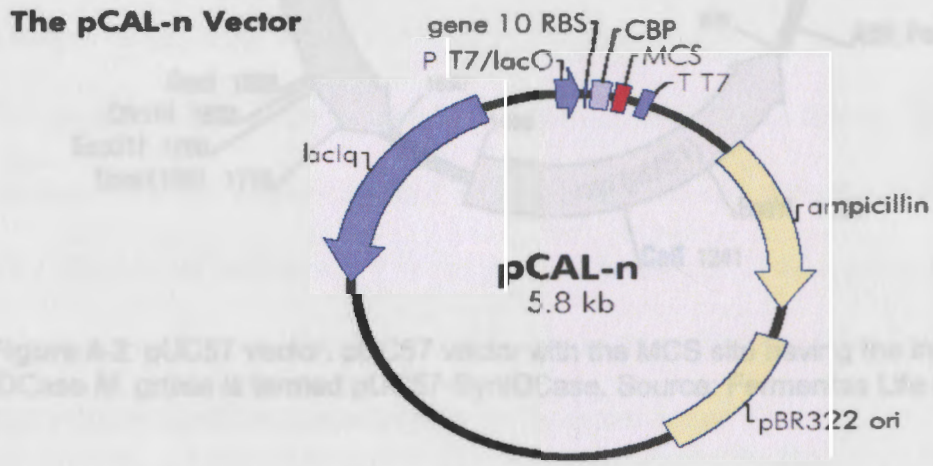


Figure 4 - 1: pCAL-n. pCAL-n plasmid vector with ampicillin resistant gene and multiple cloning sites (MCS). Source: Stratagene.

pCAL-n has the antibiotic resistance, thus enabling growth of only those cells with the recombinant plasmid when grown in certain antibiotics and the host organism is *E. coli* for the molecular cloning.

Materials and Methods

The optimized IDCase *M. grisea* was ordered from GenScript and was provided in the plasmid pUC57-synIDCase.

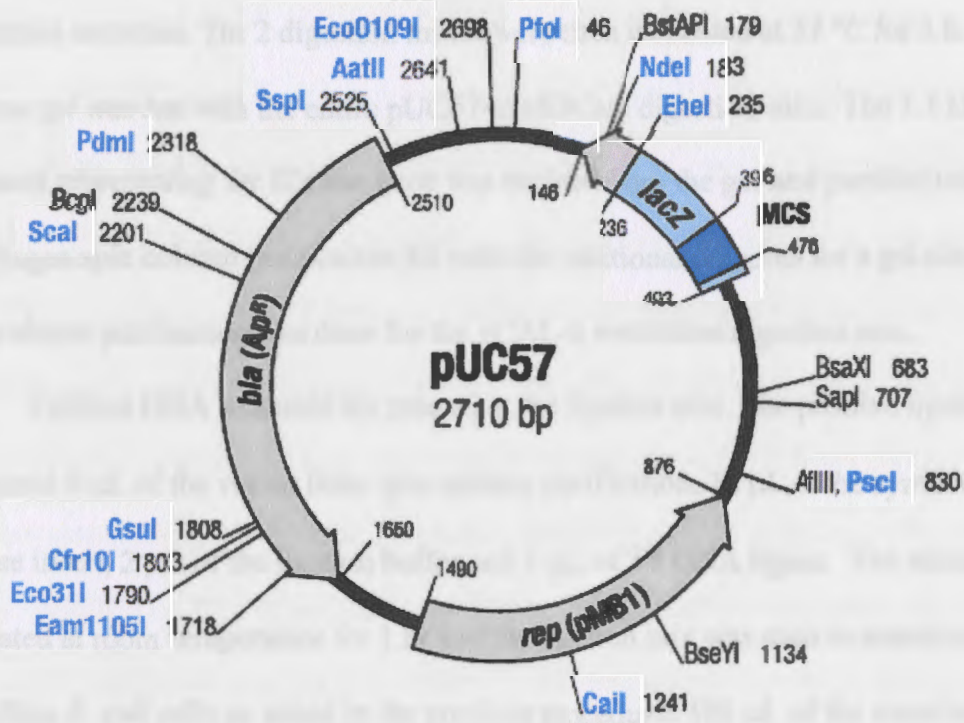


Figure 4-2: pUC57 vector. pUC57 vector with the MCS site having the insert synthetic IDCase *M. grisea* is termed pUC57-SynIDCase. Source: Fermentas Life Sciences.

The manufacturer changed the rare codons in the IDCase *M. grisea* wild type so that the synthetic IDCase gene insert was suitable for the *E. coli*. We designed the gene further with restriction sites NdeI and HindIII at the end. This was done so we would be able to purify the synthetic IDCase gene fragment simply from the restriction digested plasmid, without PCR. Molecular cloning was done to construct the pCAL-IDCase. The

insert IDCase from pUC57-SynIDCase was cut using restriction digestion enzymes NdeI and HindIII and ligated with the vector pCAL-n. The restriction digestion mix contained 2.9 μg of pUC57-synIDCase in 10 μL, 5 μL of NEB restriction buffer 2, 33 μL of sterile distilled water, 1 μL of HindIII and 2 μL of NdeI restriction enzymes. The vector pCAL-n was digested in a mix with 30 μL of pCAL-n (containing 1.2 μg), 5 μL of restriction buffer 2, 13 μL of sterile distilled water, 1 μL of HindIII and 1 μL of NdeI restriction enzymes. The 2 digestion mixes were then incubated at 37 °C for 3 h. A 0.8% agarose gel was run with the entire pUC57-synIDCase digestion mix. The 1.1 kbp fragment representing the IDCase gene was excised from the gel and purified using the Qiagen spin column purification kit with the additional reagents for a gel slice. Only spin column purification was done for the pCAL-n restriction digestion mix.

Purified DNA was used for preparing the ligation mix. The positive ligation mix contained 6 μL of the vector from spin column purification, 12 μL of the synthetic IDCase insert, 2 μL of the ligation buffer and 1 μL of T4 DNA ligase. The mixture was incubated at room temperature for 1 hr and the ligation mix was used to transform XL1 Blue *E. coli* cells as stated in the previous procedure. 100 μL of the transformation mix was spread on an LB/agar/ampicillin plate using a flame sterilized spreader.

Restriction digestion of plasmids to verify construction

Inoculation of the isolated colonies from the transformation plates was carried out for 18 samples, each in 5 mL LB+ampicillin overnight cultures. The plasmid mini-prep was done for the samples using the overnight cultures as stated in the previous chapter. A 0.8% agarose gel was run. Three 50-mL overnight cultures were grown for cells carrying suspected positives pCAL-synIDCase (numbered 1 and 4) and pCAL-IDCase *N. crassa*.

The 3 cultures were centrifuged at 3800 rpm and the cell pellet was subjected to QIAGEN HiSpeed Midi Kit purification. The presence of the plasmids was verified by running a 0.8% agarose gel. Restriction digestion was then done using the purified plasmids and with NdeI and HindIII restriction enzymes. A 1.2% agarose gel was run to see the size of the pCAL-n and the IDCase insert.

The plasmids were introduced into BL21 codon plus cells. Protein from the cells carrying synthetic IDCase, *N. crassa* IDCase and calmodulin binding protein (CBP)-fusion IDCase of *N. crassa* were isolated from the BL21 codon plus *E. coli* cells, and [*carboxy*-^{14}C] iso-orotate radioactivity assays [(4)] were done to determine the amount of activity from the different IDCase genes. The following factors were examined for their effects on the amount of decarboxylation activity: synthetic IDCase gene activity compared to *N. crassa* IDCase gene activity; the effect of different metal ions on the IDCase from *N. crassa* wild type and fusion plasmids, synthetic IDCase gene activity: at different growth temperatures for incubation, the effect of Zn^{+2} addition to the growth media, the effect of Zn^{+2} when added directly to the radioactive assay mix, the effect of freezing the cell pellet, and the effect when EDTA was added directly to the assay mix.

Standard Trial Growth Procedure: for Production/Isolation of IDCase protein in *E. coli* cells: IPTG Induction Time Course Experiments

Using sterile method, an isolated colony from the LB/agar/ampicillin plate was inoculated into an autoclaved 10 mL LB media, to which 10 μL of Ampicillin (50 mg/mL) was added after incubating for 1 h with shaking at 37 °C. The incubation was continued to obtain an overnight culture. The next day a 1 L secondary culture was started by adding the overnight culture (making a 1:100 dilution of culture) and 1000 μL

of ampicillin (50 mg /mL); incubation was continued at 37 °C with shaking for 3 h. The 1 L secondary culture flask was taken out from the incubator and 600 µL of 84 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Acros Organics) (final concentration = 50 µM) was added to induce the over-production of the IDCase protein. The culture was allowed to grow for 4-6 h at 37 °C with shaking.

Equal volumes of samples were collected after either every 1 h or every 2 h after the 50 µM IPTG induction, with the remainder of the culture continued at 37 °C with shaking. For other experiments other than the time point experiment, the secondary culture was allowed to grow for 4 h after 50 µM IPTG at 37 °C with shaking. The samples collected were centrifuged at 7000 x g rpm for 20 minutes and the supernatant was discarded. The large secondary cultures (1 L or 4 L) were centrifuged using Sorvall RC 5C PLUS centrifuge with Sorvall SLA-3000 SuperLite Rotor at 7000 x g rpm for 20 minutes at 4 °C.

The cell pellet was resuspended in enough Tris lysis buffer (2 mL for 2.5 g of wet cell mass). The cells are lysed following the "standard cell lysis procedure". For the experiment on the different growth temperatures the incubation temperatures for the secondary culture were 37 °C, 30 °C and 12 °C in the standard trial growth procedure. In another experiment 0.2 mM and 0.4 mM $ZnCl_2$ were added to the secondary culture at the beginning followed by the standard trial procedure.

Standard Cell Lysis Procedure

The secondary culture samples collected in the time point experiments were taken into 50-mL centrifuge tubes and centrifuged at 3800 rpm using bench top IEC Centra GP8 for 15 minutes. The wet cell pellet collected was resuspended in 2 mL of Tris lysis

buffer which contained 10 mM Tris adjusted to pH 7.0, 10% glycerol, 5 mM beta-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM pepstatin and 2 mM leupeptin. Cell lysis was done by taking the resuspended cell solution and adding 0.1 mm glass beads in 2 mL screw cap tubes. The cells were then lysed using three one-minute pulses at maximum speed of the Mini Bead Beater (BioSpec Products, Bartlesville, OK) interspersed with cooling the lysate on ice. After the pulses were finished, the lysate was allowed to sit on ice for 10 minutes to allow the glass beads to settle down in the tube. The lysate was then transferred into 1.5-mL Eppendorf tubes and centrifuged for 5 minutes at 13000 rpm in microcentrifuge at 4 °C. The clear lysate was pipetted into another Eppendorf tube from the cell debris pellet. The lysate was used for enzyme assay, Bradford assay and to run SDS-PAGE gel electrophoresis to check for the activity and quantify the amount of IDCase respectively.

For larger samples, the clear lysate was collected by transferring the lysate after bead beating into 20 mL screw cap centrifuge tubes and centrifugation using Sorvall RC 5C PLUS centrifuge with Sorvall SS-34 Rotor at 10000 rpm for 20 min at 4 °C.

Bradford Assay

To measure the amount of protein in the sample, a standard graph with known concentrations of Bovine Serum Albumin (BSA) protein is plotted with μg of protein versus absorbance. In duplicate five different amounts of 0.5 mg / mL BSA are added to 0.9 mL of Bradford Assay Reagent (Pierce), and diluted to 1 mL with 0.15 M NaCl. For the standards, amounts of 0, 5, 10, 15, and 20 μL of BSA and an amount of sample protein (normally 1 to 5 μL) were added to the 0.9 mL Bradford Assay reagent, diluting to 1 mL with 0.15 M NaCl. [8] The amount of sample protein is taken such that the

absorbance is between the standard absorbance values. The absorbance is measured using UV spectrophotometer at 595 nm. Using the standard curve, μg amounts of the protein sample can be determined.

SDS-PAGE Gels

SDS-PAGE gels were run by mixing 10 μg of protein and 10 μL SDS loading buffer and denaturing the protein at 100 °C for 5 minutes. The prepared samples were loaded on the gel and run at 80 V for first 30 min and at 110 V for 2 h. The gels are stained with a solution containing Coomassie Brilliant Blue and then destained for the protein bands to be visible.

Radioactivity Assays with [*carboxy*-^{14}C] Iso-orotate

IDCase activity was measured by adding different amounts of the protein present in the lysate to an assay mix containing 3000-5000 cpm of [*carboxy*-^{14}C] labeled IOA. If present in the protein lysate, IDCase would remove the carboxylate group from the IOA with the formation of uracil and release of $^{14}CO_2$, which is driven off from the reaction and absorbed onto a wick placed in a wick holding basket attached to the rubber septum. The wick is made of Whatman filter paper, which is moistened with 2 M KOH to absorb the CO_2. The following is the reaction assay mix in the 20 ml vial:

- 400 μL dH_2O (or more if less protein solution is used)
- 25 μL 1 M Tris pH 7.5
- 50 μL of the [*carboxy*-^{14}C] labeled IOA assay mix (500 mM IOA, 3000-5000 cpm)
- 25 μL protein solution (this amount was varied depending on the amount of enzyme present in the lysate)

Tris solution, dH_2O, and [*carboxy*-^{14}C] labeled IOA assay mix were added first to the scintillation vial and capped with the rubber septum holding the moistened wick in the basket. The required amount of protein solution was now added to initiate the reaction. The contents are mixed by gentle swirling and placed in shaking water bath for 1 to 5 minutes depending on the experiment. After the set time, 0.1 mL of 2 M HCl was used to quench the reaction. The vials were left overnight. The wicks were removed and dried in vacuum oven set at 72 °C for about 20 minutes. (drying should not continue so far that the wicks turn to yellow color). The dried wicks were transferred separately into a pre-counted vial containing scintillation fluid (Scinti Safe Econo 1) and the ^{14}C was measured with a scintillation counter.

IDCase activity from pCAL-IDCase *N. crassa*

200 mL of LB+ampicillin secondary culture was started and a time point induction up to 6 h was carried out as stated in the standard trial growth procedure. 50-mL samples were collected after every 2 h. The lysates obtained at 0 h, 2 h, 4 h, and 6 h were prepared following the standard cell lysis procedure. The lysate samples were assayed for activity of the IDCase. The Bradford assay was done to determine the amount of protein in the lysate at the different time points.

IDCase activity from pCAL-IDCase *N. crassa* and pCAL-fusion IDCase *N. crassa* with Varying Metal Ions Added to Cultures

^{14}C assays were carried out on protein samples from cells carrying pCAL-IDCase *N. crassa* and pCAL-fusion IDCase grown in the presence of different metal ions. The metals were added as follows: 10 μL of 1 M solution of $CoCl_2$, $MgCl_2$ and $ZnCl_2$ (final concentration = 200 μM) separately to 50 mL secondary culture flasks. The secondary

culture was allowed to grow up to 6 h after 50 μM IPTG addition. The lysate samples were assayed for any increase in the IDCase activity. SDS-PAGE gels were run for all the lysate samples.

Radioactivity Assays on Synthetic IDCase gene (pCAL-synIDCase):

Cells carrying the suspected plasmids pCAL-synIDCase # 1, # 4 and pCAL IDCase *N. crassa* were used for a time point induction experiment with three separate 300 mL secondary cultures. 50 μM ITPG induction was used as stated in the previous procedure and the cell pellet was collected after 6 h of growth. The lysate was assayed for activity of the IDCase.

Cells carrying pCAL-synIDCase # 1 were used to examine the effect of different growth temperatures. Three 200-mL secondary cultures were kept at 3 different temperatures of growth : 37 °C, 30 °C and 12 °C - with 50 μM IPTG time point induction. The lysate was obtained as stated in the previous procedure for 0 h, 2 h and 4 h samples collected. The assay was done for activity of the IDCase and SDS-PAGE gels were run for all the samples to check for the amount of IDCase expressed at the different temperatures.

Cells carrying pCAL-synIDCase # 1 were used to examine the effect of different amounts of Zn^{+2} added to LB culture media. Three 50-mL secondary cultures were induced with 50 μM IPTG. To one flask no Zn^{+2} was added, to the second flask 10 μL of 1 M $ZnCl_2$ was added (final concentration = 200 μM) and to the third flask 20 μL of 1 M $ZnCl_2$ was added (final concentration = 400 μM) at the start of the secondary culture. The cultures were allowed to grow for 4 h after IPTG induction. The lysate was collected

separately for the 3 flasks and assayed for the IDCase activity. SDS-PAGE gels were run for the 3 samples.

Cells carrying pCAL-synIDCase # 1 were used to examine the effect of different amounts of Zn^{+2} added to the assay mixes. Final concentrations of 4 mM, 10 mM and 20 mM $ZnCl_2$ were added directly to the assay mix instead of adding to the secondary culture LB+ampicillin media. The volume of dH_2O added in the reaction mix was adjusted so that the final volume of the reaction mix remained 500 µL. The lysate used was collected from 50 mL secondary culture induced with 50 µM IPTG and 4 h of growth after induction.

Cells carrying pCAL-synIDCase # 1 were used to examine the effect of EDTA added to Assay mix. Concentrations of 0, 0.1, 1 and 5 mM EDTA were added directly to the assay mix in separate vials for each. The volume of dH2O was adjusted, after the addition of EDTA to the assay mix so that the final volume of the mix remained 500 µL. The lysate as protein source in the assay mix was obtained from a 50 mL secondary culture induced with 50 µM IPTG and 4 h growth time after induction.

Cells carrying pCAL-synIDCase # 1 were used to examine the effect of freezing the cell pellet on the amount of IDCase activity. A 50-mL secondary culture was grown as stated in the previous procedure with 50 µM IPTG induction and 4 h growth after induction. The cell pellet was collected after centrifugation and stored at 4 °C for 18 h. The cell lysates were collected as stated in the previous procedure: one that was frozen and one that was not frozen. The two lysates were assayed to see if there is a decrease in activity of the IDCase upon freezing.

Results and Discussion

The positive ligation plate showed 9 isolated colonies per plate. The 0.8% agarose gel from the plasmid mini-prep for pCAL-synIDCase *M. grisea* showed positive bands for 1, 2, 4, 6-13 plasmid mini-preps. The bands 2 and 6-13 were of the same size. Bands 1 and 4 looked like the same size.

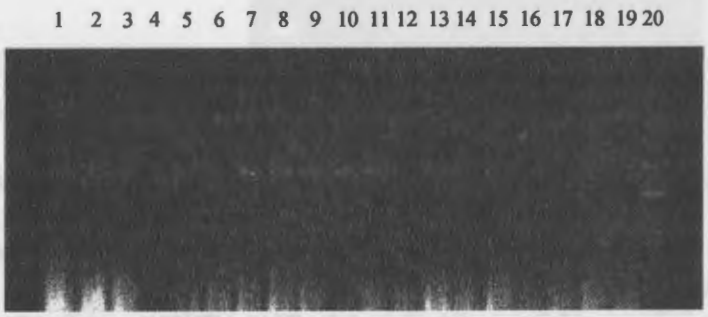


Figure 4- 3: Agarose gel for positive plasmid mini-preps.
Lane 1: pCAL- IDCase *N. crassa*
Lane 2-19: Potential pCAL-synIDCase plasmids which are renumbered as 1-18.
Lane 20: 1 kb DNA ladder.

Purification of the plasmids 1, 2, 4 and *N. crassa* was done using Qiagen HiSpeed Midi kit and the 0.8% agarose gel showed bands of the same size for 1 and 4. The band size of 2 was smaller and may not have the correct size insert.

The restriction digested plasmids pCAL-synIDCase 1 and 4 when digested with the restriction enzymes NdeI and HindiIII showed the correct size of IDCase insert. Molecular cloning was successful. This was confirmed with restriction digestion of the pUC57-synIDCase, pCAL-synIDCase 1 and 4. The 1.2% agarose gel run with the restriction digested plasmids showed the correct size insert in both the plasmids pCAL-synIDCase 1 and 4.

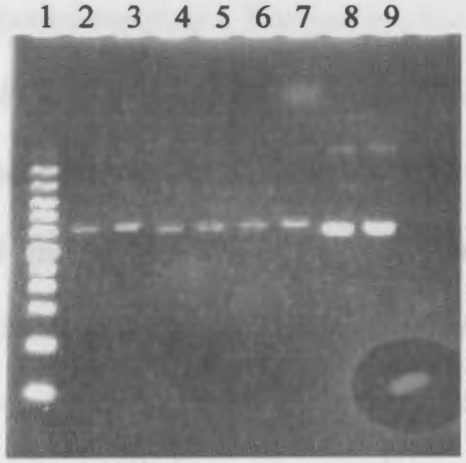


Figure 4-4: 0.8% Agarose gel showing the purified plasmids pCAL-synIDCase #1, #2, #4 and pCAL-IDCase *N. crassa*.
Lane 1: 1 kb DNA marker.
Lane 2: 1 μL pCAL-synIDCase#1. Lane 3: 10 μL pCAL-synIDCase#1.
Lane 4: 1 μL pCAL-synIDCase#2. Lane 5: 10 μL pCAL-synIDCase#2.
Lane 6: 1 μL pCAL-synIDCase#4. Lane 7: 10 μL pCAL-synIDCase#4.
Lane 8: 1 μL pCAL-IDCase *N. crassa*. Lane 9: 10 μL pCAL-IDCase *N. crassa*.

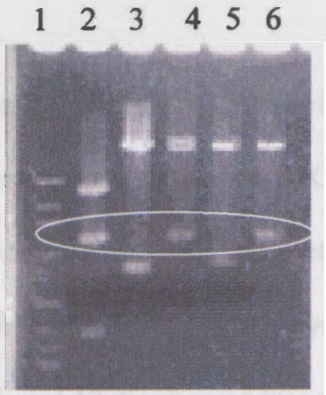


Figure 4-5: 1.2% agarose gel of NdeI and HindIII-digest of plasmids, which showed the correct size of the synthetic IDCase DNA fragment in pCAL-synIDCase#1 and #4. The fragments of the synthetic IDCase gene are circled.
Lane 1: 100 bp ladder DNA marker
Lane 2: Digested pUC57-IDCase. Lane 3: Digested pCAL-IDCase *N. crassa*.
Lane 4: Digested pCAL-synIDCase #1.
Lane 5: Digested pCAL-synIDCase #2.
Lane 6: Digested pCAL-synIDCase #4.

The radioactivity assay showed a substantial increase in IDCase activity for the cells carrying the synthetic IDCase gene. Time point induction of pCAL-IDCase *N.*

crassa (wild type) for 2 h induction sample in a 2-minute reaction shows specific activity of 0.009 nmol/μg/min. The IDCase activity is greatly increased in the 2 hr induction sample of cells with the synthetic gene of *M. grisea*, which is 0.8 nmol/μg/min with pCAL-synIDCase # 1 and 0.4 nmol/μg/min with pCAL-synIDCase #4. We compared the synthetic IDCase from *M. grisea* with *N. crassa* IDCase and not with the naturally-occurring IDCase from *M. grisea*, since there was a restriction site in the IDCase *M. grisea* wild type gene and the IDCase full fragment cannot be easily obtained for cloning with pCAL-n. By comparing the Synthetic IDCase *M. grisea* with IDCase *N. crassa* we saw the effect of rare codons in over-expression of the IDCase in *E. coli* system. The very low specific activity of IDCase *N. crassa* (Table A-1, Appendix) is indicative of the effect of rare codons in *E. coli* cells for over-expression of protein with heterologous gene.

The radioactivity assay with the addition of metal ions showed increase in activity for pCAL-IDCase *N. crassa* and pCAL-fusion IDCase *N. crassa* grown in the presence of added $ZnCl_2$ compared to the addition of other metals (Table A-4, A-5, Appendix).

The ^{14}C assay with cells carrying pCAL-synIDCase # 1 grown at different temperatures 37 °C, 30 °C and 12 °C showed less active protein at 12 °C and 30 °C. (Table A-6, A-7 Appendix). The SDS-PAGE gel also showed less protein being produced at 30 °C and 12 °C (data not shown). The activity of the IDCase produced at 37 °C showed high activity and was maximum at 4 h growth after IPTG induction (Table A-8, Appendix). The SDS-PAGE gel also showed the over-expressed protein band with the samples collected at 37 °C (data not shown). The results showed that the ideal temperature for IDCase over-expression was 37 °C.

The ^{14}C assay with cells carrying pCAL-synIDCase # 1 grown with different amounts of Zn^{+2} added to LB media showed high activity of IDCase in all samples (Table A-9, Appendix). SDS-PAGE gel showed over-expressed IDCase bands at 42.9kDa. Protein with the expected size of IDCase was present in nearly equal amount in both whole cell samples and lysates.

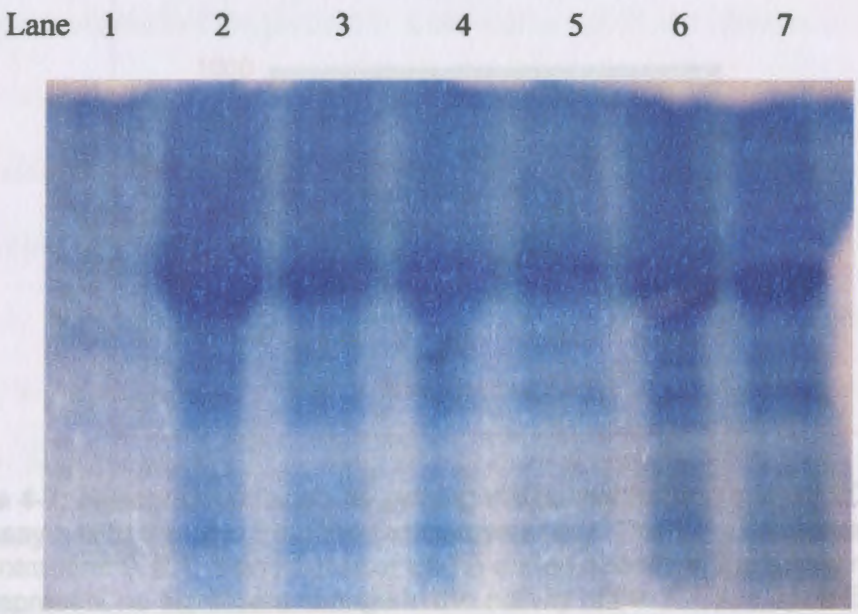


Figure 4-6: Effect of added Zn^{+2} on production of IDCase protein. Large bands at 42.9 kDa seen for all the samples are IDCase bands. BL21 codon plus *E. coli* cells with pCAL-synIDCase #1 plasmid were used to produce the protein.
Lane 1: protein marker (not clearly visible)
Lane 2: whole cell sample, no Zn^{+2} added
Lane 3: lysate sample, no Zn^{+2} added
Lane 4: whole cell sample, 200 μM Zn^{+2} added to the growth media
Lane 5: lysate sample, 200 μM Zn^{+2} added to the growth media
Lane 6: whole cell sample, 400 μM Zn^{+2} added to the growth media
Lane 7: lysate sample, 400 μM Zn^{+2} added to the growth media

Surprisingly, the ^{14}C assay with millimolar amounts of Zn^{+2} added to the assay mix showed highly diminished IDCase activity (Table A - 10, Appendix). This is likely due to the ability of iso-orotate to chelate divalent metal ions. [21] Apparently this chelation prevents the substrate from binding to the active site.

The ^{14}C IDCase assays with EDTA added directly to the assay mix showed negligible variation in activity with concentrations of 0, 0.1, 1 and 5 mM. The activity was high and did not decrease even on increased concentration of EDTA.

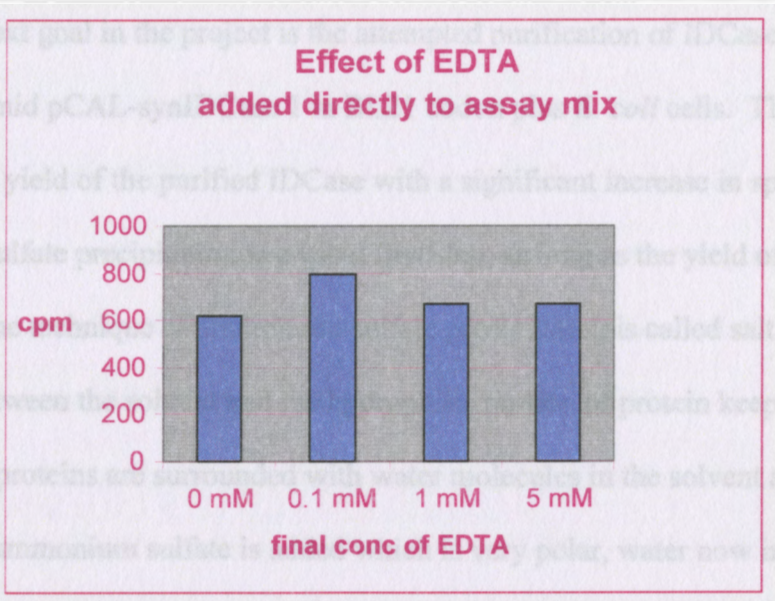


Figure 4-7: Histogram obtained by plotting the concentration (mM) of EDTA added to the assay mix to the cpm from the radioactive assay. The x – axis represents concentrations 0, 0.1, 1 and 5 mM of EDTA added directly to the assay mix. All the 4 bars represent no significant change in the activity of the IDCase upon EDTA addition.

Although the presence of a metal ion in IDCase is implied by the increase in activities with addition of Zn^{+2} to the growth media, this metal ion, if indeed present, is not removed from the protein by the addition of EDTA. This result is not uncommon for metal ion-containing proteins.

Chapter 5

Purification of IDCase using DEAE Sephacel Column Chromatography

Introduction

The next goal in the project is the attempted purification of IDCase produced from the plasmid pCAL-synIDCase 1 in BL21 codon plus *E. coli* cells. The intent is to get maximum yield of the purified IDCase with a significant increase in specific activity. Ammonium sulfate precipitation is a usual first step, as long as the yield of activity is reasonable. The technique of ammonium sulfate precipitation is called salting out. The interaction between the solvent and the hydrophilic portion of protein keeps the protein in solution. The proteins are surrounded with water molecules in the solvent and thus are stable. When ammonium sulfate is added which is very polar, water now interacts with $(NH_4)_2 SO_4$ molecules leaving the proteins. With less water available to stabilize the protein, the proteins begin to interact with each other to form aggregates and thus precipitate. Some proteins precipitate at low concentration of $(NH_4)_2 SO_4$ and some at high concentration. [20] In an attempt to find where the IDCase precipitates out and the high activity of the IDCase protein was retained. Ammonium sulfate addition was done separately at different percentages of saturation from 35% to 80%. Then the ammonium sulfate fractionation was done from 40% to 65% to determine the specific activity, fold purification, total activity and yield of the IDCase protein.

Diethylaminoethyl (DEAE) Sephacel Column is a weak anion exchange column. The negatively charged protein binds to the column; when eluted with a salt gradient, different proteins come off the column with different retention times. [20] IDCase has an

overall negative charge, according to the amino acid sequence, and binds to the DEAE Sephacel column and elutes when the critical salt concentration is reached.

Materials and Methods

Ammonium Sulfate [$AmSO_4$] Precipitation

Two 1 L secondary cultures of *E. coli* were grown as stated in the previous procedure with 50 μM IPTG induction and with growth time of 4 hrs to obtain 50 ml lysate. The lysate was divided into ten 5-ml portions. Keeping the samples cold, the following amounts of $AmSO_4$ were added slowly for over 10 min, with thorough stirring and continued stirring for 30 minutes after addition of the $AmSO_4$.

1) 35% - 0.97 g; 2) 40% - 1.13 g; 3) 45% - 1.29 g; 4) 50% - 1.46 g; 5) 55% - 1.63 g; 6) 60% - 1.81 g; 7) 65% - 1.99 g; 8) 70% - 2.18 g; 9) 75% - 2.38 g; 10) 80%- 2.58 g

The samples are then centrifuged as stated previously to obtain a pellet and clear supernatant. The pellet was redissolved in Tris lysis buffer and the activity of IDCase was checked for in all the 10 redissolved pellet samples and the 10 supernatant samples.

$AmSO_4$ Fractionation

A 1-L secondary culture was grown with 50 μM IPTG induction and conditions as stated previously. The volume of the lysate was measured as 13 ml after bead beating. The lysate was saturated to 45% $AmSO_4$ by adding 3.354 g of $AmSO_4$ and the addition of $AmSO_4$ was done as stated previously. The sample was centrifuged and the supernatant was saturated to 65% $AmSO_4$ by adding 1.599 g of $AmSO_4$ for 13 ml of the supernatant. The addition was done as previously stated. The sample was centrifuged and the pellet was redissolved in 2 ml Tris lysis buffer. The redissolved pellet sample and lysate sample

were assayed for IDCase activity to find the specific activity, total activity, yield and fold purification.

Anion Exchange Chromatography

A column (25 cm × 2.5 cm diameter) of DEAE Sephacel was washed with 200 mL 1 M NaCl to clean the column and then washed again with 50 mM MOPS buffer, pH 6.5. All MOPS buffers used in these procedures were the same composition with the exception of added NaCl where noted. The protein solutions were applied to the column using a flow adapter with pump operating at 2 mL/min. In the first trial the column was then equilibrated with MOPS buffer plus 100 mM NaCl. 30 ml of the lysate obtained from a 4-L culture as previously stated procedure, was loaded to the column. The column was washed with MOPS buffer plus 100 mM NaCl for up to 25 fractions and then with MOPS buffer plus 200 mM NaCl, until the no more protein came off the column (as detected by Bradford assays of fractions). Elutions were collected in 13 x 100 mm test tubes using fraction collector. About 6-8 mL was collected in each test tube. The Bradford assay was done for all the fractions collected and SDS-PAGE gels were run. Trial 1 was done to determine the gradient to be used. The column was then washed with 1 M NaCl first and then with 50 mM MOPS buffer to clean the column for trial 2. In trial 2 the column was equilibrated with MOPS buffer plus 20 mM NaCl. About 35 mL of concentrated protein was loaded to the column and then washed with a salt gradient of MOPS buffer plus 20 mM NaCl to MOPS buffer plus 200 mM NaCl. The elutions were collected in 13 x 100 mm test tubes using fraction collector. About 6-8 mL of elute was collected in each test tube. The Bradford assay was done for every 3rd fraction. An SDS-PAGE gel was run with the samples showing high amounts of protein from the Bradford

assay. The lysate and the eluted fractions were checked for specific activity by the UV spectrophotometric assay. [4] The fractions that showed the presence of IDCase from the SDS-PAGE gel were pooled.

Results and Discussion

There was a steady increase in IDCase activity in $AmSO_4$ pellets as the percentage of $AmSO_4$ was increased. Assays with 1 minute reaction time and 2 μL of the protein added showed a steady increase in the activity from 35% to 50% $AmSO_4$ ppt. and increased significantly from 55% to 80% $AmSO_4$. The histogram plotted with the percentage saturation verses the cpm from the radioactive assay, and the SDS-PAGE gel run with the different resuspended samples, showed that the IDCase is precipitating at 55% to 70% $AmSO_4$.

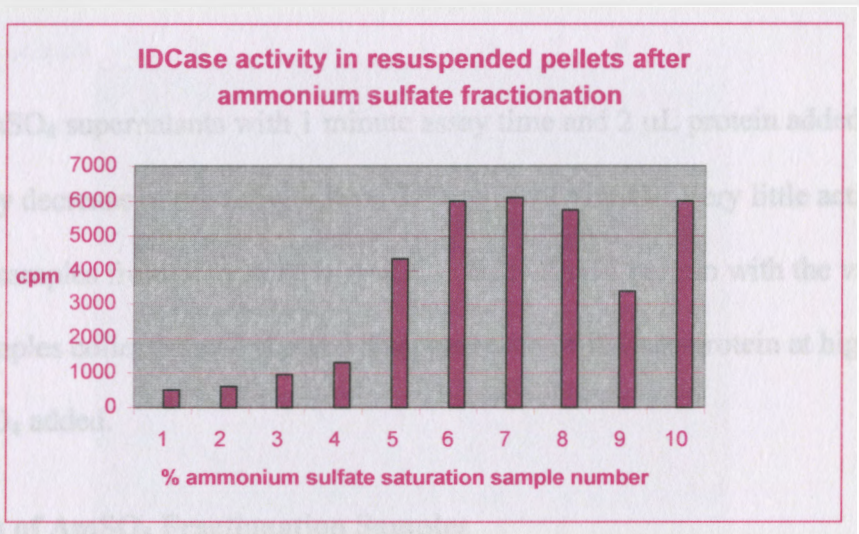


Figure 5-1: Histogram of resuspended ammonium sulfate fractionation. Numbered samples represent 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% and 80% $AmSO_4$ saturation. The increase in activity can be seen from 55% to 80%. BL21 codon plus *E. coli* cells with pCAL-synIDCase #1 plasmid were used to produce the samples.

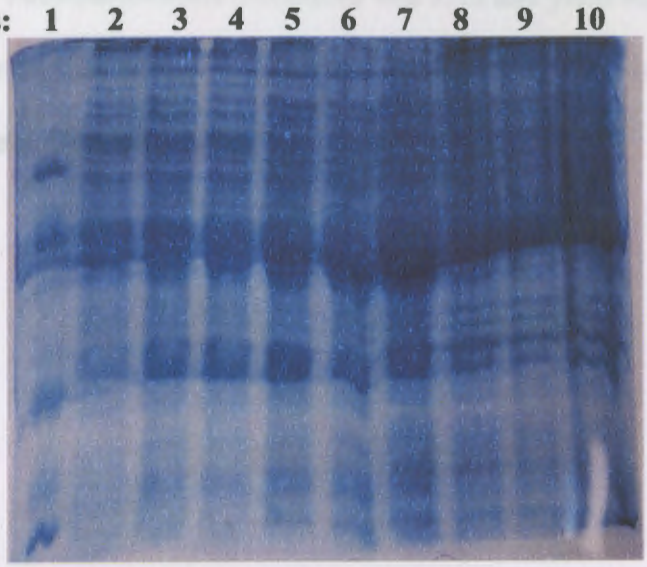


Figure 5-2: Increased appearance of IDCase in progressively higher $AmSO_4$ pellets.
Lane 1: protein marker
Lanes 2 – 10: resuspended pellet of 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% $AmSO_4$ saturation. The SDS-PAGE gel showed the presence of IDCase bands at 42.9 kDa in lane 5, 6 and 7.

The $AmSO_4$ supernatants with 1 minute assay time and 2 µL protein added showed a steady decrease in the activity from 35% to 50% $AmSO_4$. Very little activity was present in samples from 55% to 80% $AmSO_4$. SDS-PAGE gel run with the various supernatant samples collected also showed disappearance of IDCase protein at high levels of $AmSO_4$ added.

IDCase Assays of $AmSO_4$ Fractionation Samples

Protein produced from cells carrying pCAL-synIDCase # 1 was used in a 45% to 60% $AmSO_4$ fractionation, as maximum amount of IDCase protein based on the previous data. The specific activity of lysate was calculated as 1.09 nmol/µg/min, and the specific activity of the resuspended pellet after 45% to 60% $AmSO_4$ fractionation was calculated

as 1.2 nmol/μg/min. Fold-purification calculated was 1.13 and yield was 37% which was not promising. So we decided to carry on the protein purification with DEAE Sephacel anion exchange column chromatograph.

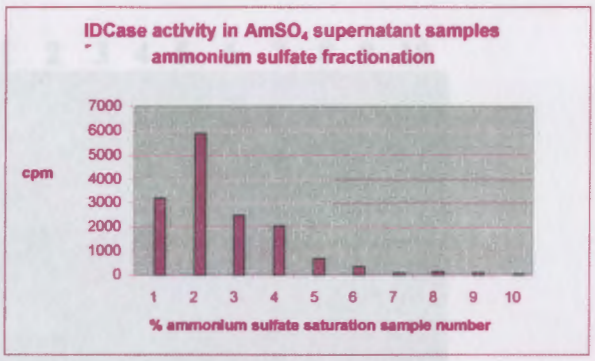


Figure 5-3: IDCase activity in supernatants following $AmSO_4$ fractionation. Numbered samples represent 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% and 80% $AmSO_4$ saturation fractions. The decrease in activity can be seen from 55% to 80%. BL21 codon plus *E. coli* cells with pCAL-synIDCase #1 plasmid were used to get the samples.

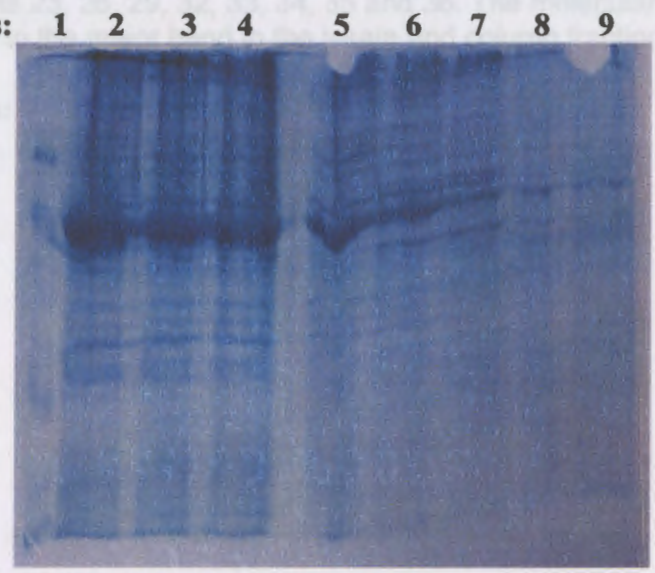


Figure 5-4: Disappearance of IDCase in supernatants from progressively higher $AmSO_4$ addition.
Lane 1: protein marker
Lanes 2 – 10: supernatants of 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% fractions. The SDS-PAGE gel showed the presence of IDCase bands at 42.9 kDa in lane 2, 3, 4 and 6.

Anion Exchange Chromatography

In trial 1 of DEAE Sephacel column chromatography, the 29th to 43rd fractions showed the presence of purified IDCase bands at 42.9 kDa in SDS-PAGE gels.

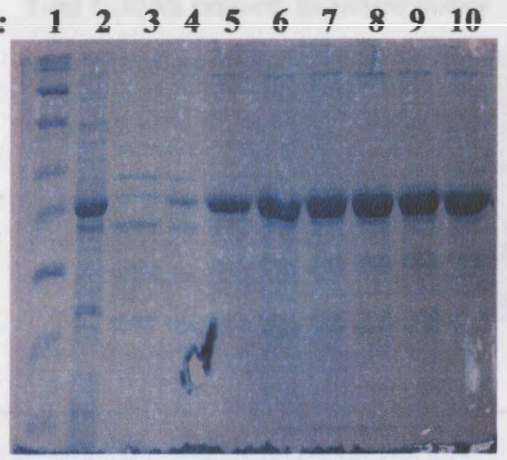


Figure 5-5: DEAE Sephacel column purification of IDCase trial 1 (fractions 23-36). Lane 1: protein marker. Lane 2: original lysate before column. Lane 3 – 10: Fractions 23, 26, 29, 32, 33, 34, 35 and 36. The molecular weight for IDCase corresponds to the major band in the lysate and column fractions.

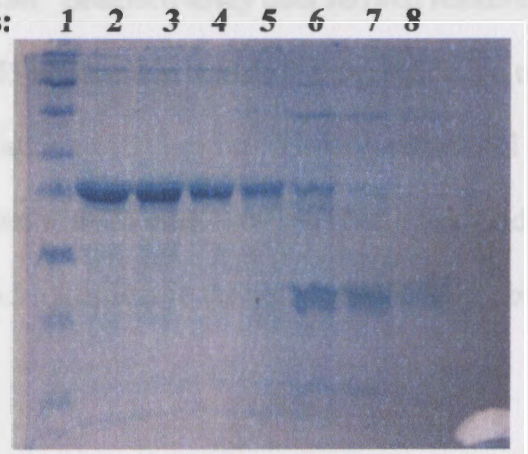


Figure 5-6: DEAE Sephacel column purification of IDCase trial 1 (fractions 37-48). Lane 1: protein marker. Lanes 2 – 8: Fractions 37, 38, 41, 43, 45, 46 and 48. The molecular weight for IDCase corresponds to the major band in column fractions 37 – 43.

Bradford assay of the purified protein fractions showed high amount of the protein concentration from fractions 29th to 43rd which also show the presence of IDCase

in those fractions from the SDS-PAGE gels. This indicates that the samples from Bradford assay which show high amount of concentration of the protein had the purified IDCase in those fractions.

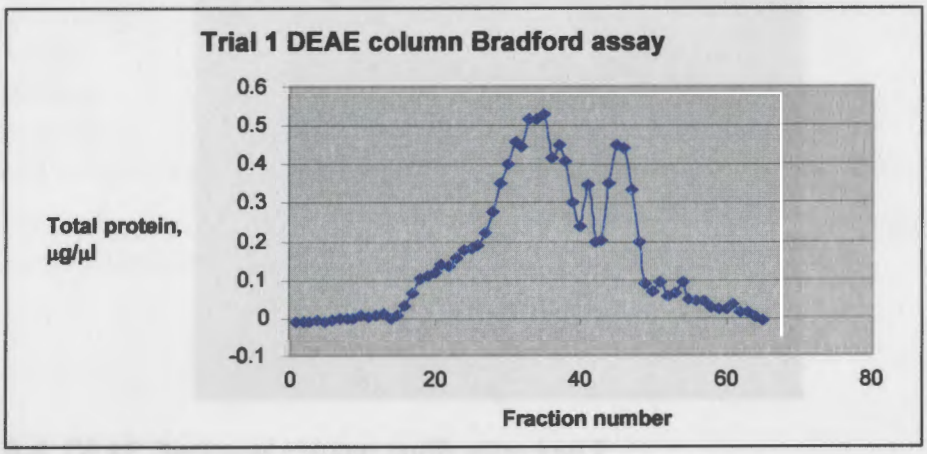


Figure 5-7: Protein in fractions from DEAE Sephacel chromatography.

In trial 2 the fractions from 43 to 55 showed purified IDCase bands when the SDS-PAGE gel was run. Bradford assay data showed maximum amount of the protein from fractions 43 to 55 collected in trial 2. Fold-purification calculated was 1.2 and yield was 15%. The purification of IDCase was partially successful using DEAE Sephacel anion exchange column with 20 mM - 200 mM NaCl salt gradient with the 50 mM MOPS buffer at pH 6.5 according to the gel electrophoresis, but the total activity and the yield were not good.

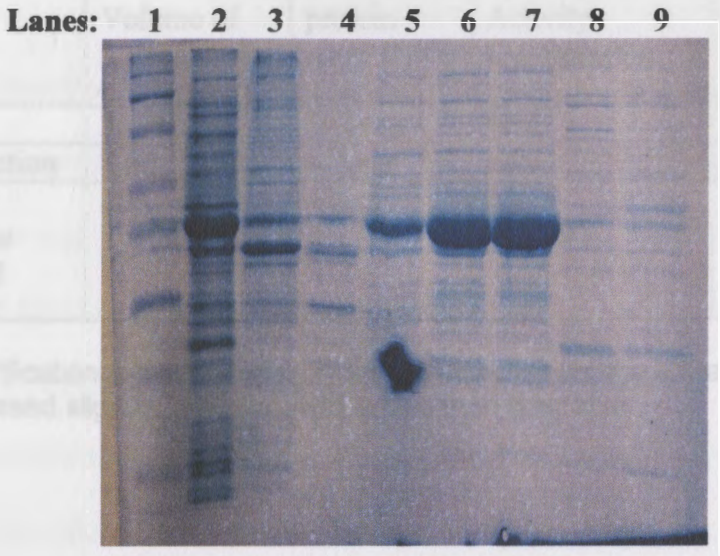


Figure 5-8: DEAE Sephacel column purification trial 2.
Lane 1: protein marker. Lane 2: original lysate before column.
Lanes 3 – 9: Fractions 10, 25, 43, 46, 49, 67 and 73. The molecular weight for IDCase corresponds to the major band in the lysate and column fractions.

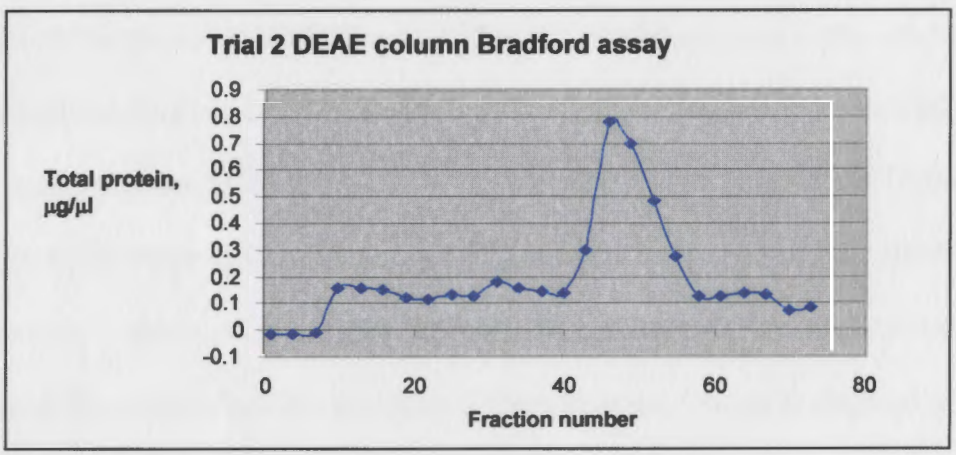


Figure 5-9: Protein in fractions from DEAE Sephacel chromatography.

Sample	Total Volume of lysate	Total protein	Specific Activity nmol/min/μg	Total activity nmol/min
Lysate	35 mL	397 mg	0.88	349
46^{th} fraction	8 mL	6.2 mg	0.96	6.0
Pooled fractions 40 to 55	100 mL	47 mg	1.1	52

Table 5-1: Purification table for trial 2. The specific activity of the IDCase in the pooled fractions increased slightly, but total yield is less than desirable.

Chapter 6

Conclusions

In this laboratory, the IDCase gene from *N. crassa* was isolated but it did not produce large amounts of the protein in *E. coli* cells. This research work started with the attempt to isolate a new IDCase gene in cDNA library from *R. glutinis*. The sequencing data for the two plasmids 7-1 and 1P1 obtained did not show gene sequence that could be that for IDCase. Production of an IDCase-THase fragment from the PCR of genomic DNA of *R. glutinis* was attempted. This attempt was also not successful for the isolation of the new IDCase gene from *R. glutinis*.

Efforts were then shifted to see if the IDCase gene in *M. grisea* would give high activity of the protein on radioactive assay. Because of the rare codon effect in *E. coli* cells we ordered the synthetic IDCase gene of *M. grisea*. Molecular cloning of the synthetic IDCase gene to produce the plasmid pCAL-synIDCase was done, which was used to transform BL21 codon plus *E. coli* cells. The synthetic IDCase gene of *M. grisea* showed overproduction of IDCase in BL21 *E. coli* cells. The addition of Zn^{+2} increased the activity of *N. crassa* IDCase and retained the activity produced from the synthetic gene, when Zn^{+2} was added to the growth media. The radioactivity assays done to get the properties of the protein from the synthetic IDCase gene were partially successful. The purification by ammonium sulfate fractionation showed only 37% yield, DEAE Sephacel column purification was partially successful which can be improved in further experiments. Our lab is the first to get the overproduced IDCase and further experiments will be conducted to get the IDCase crystal structure and the catalytic mechanism of the IDCase enzyme.

References

1. http://www.visionlearning.com/library/module_viewer.php?mid=63
2. *http://www.indstate.edu/thcme/mwking/nucleic-acids.html*
3. Voet, D., and Voet, J. G. *Biochemistry*. **1995**, *2nd Ed.*
4. Smiley, J. A., Angelot, J. M., Cannon, R. C., Marshall, E. M., and Asch, D. K. *Anal. Biochem*. **1999**, *266*, 85-92.
5. Jones, M. E. *Ann. Rev. Biochem*. **1980,** *49*, 253-279.
6. Palmatier, R. D., McCroskey, R. P., and Abbott, M. T. *J. Biol. Chem*. **1970,** *245*, 6706-6710.
7. Wondrack, L. M, Chin, A. H., and Abbott, M. T. *J. Biol. Chem*. **1978**, *253*, 6511-6515.
8. Bradford, M. M. *Anal. Biochem*. **1976**, *72*, 248-254.
9. Clotech – TriplEx & TriplEx2 Libraries User Manual. (2000) PT3003-1(PR09529).
10. www.beckmancoulter.com
11. http://www.broad.mit.edu
12. Smiley, J. A., Kundracik, M., Landfried, D. A., Barnes Sr., V. R., and Axhemi, A. A. *Biochim. Biophys. Acta*. **2005**, *1723*, 256-64.
13. Armend A. Axhemi, Master's Thesis. **2005**, Youngstown State University, Department of Chemistry.
14. Daniel A. Lanfried, Master's Thesis. **2003**, Youngstown State University, Department of Chemistry.
15. James F. Kane. *Current Opinion in Biotechnology*. **1995**, *6*, 694-500.

16. Bagnoli F, Lio P. *J Theor Biol.* **1995**, *173*, 271-281.

17. Spanjaard R.A, Van Duin J. *Proc Natl Acad Sci USA.* **1988**, *85*, 7967-7971.

18. Spanjaard R.A, Chen K., Walker JR, Van Duin J. *Nucleic Acids Res.* **1990**, *18*, 5031-5036.

19. Clara M. Seibert, Frank M. Raushel. *J. Biochem.* **2005**, *44*, 6383-6391.

20. Shawn O. Farrell, Lynn E. Taylor. *Experiments in Biochemistry A Hands-On Approach. 2nd Ed.*

21. Hueso-Urena F., Moreno-Carretero M. N., Romero-Molina M. A., Salas-Peregrin J. M., Sanchez M. P., Alvarez de Cienfuegos-Lopez, and Faure R. *J. Inorg. Biochem.* **1993**, *51*, 613-632.

APPENDIX

Codon Usage in Wild type *M. grisea* IDCase versus Synthetic IDCase Gene

	Cysteine codons	
	TGT	TGC
IDCase *M. grisea*	0	4
Synthetic IDCase *M. grisea*	2	2

	Threonine codons			
	ACT	ACC	ACA	ACG
IDCase *M. grisea*	3	5	3	5
Synthetic IDCase *M. grisea*	0	0	10	6

	Aspartic acid codons	
	GAT	GAC
IDCase *M. grisea*	8	17
Synthetic IDCase *M. grisea*	23	2

	Glutamic acid codons	
	GAA	GAG
IDCase *M. grisea*	6	11
Synthetic IDCase *M. grisea*	17	0

	Tyrosine codons	
	TAT	TAC
IDCase *M. grisea*	2	8
Synthetic IDCase *M. grisea*	6	4

	Histidine codons	
	CAT	CAC
IDCase *M. grisea*	2	12
Synthetic IDCase *M. grisea*	9	5

	Glutamine codons	
	CAA	CAG
IDCase *M. grisea*	2	3
Synthetic IDCase *M. grisea*	0	5

	Asparagine codons	
	AAT	AAC
IDCase *M. grisea*	5	8
Synthetic IDCase *M. grisea*	5	8

	Lysine codons	
	AAG	AAA
IDCase *M. grisea*	10	2
Synthetic IDCase *M. grisea*	0	12

	Serine codons					
	TCT	TCC	TCA	TCG	AGT	AGC
IDCase *M. grisea*	3	5	6	5	6	4
Synthetic IDCase *M. grisea*	3	4	5	2	5	10

	Phenyl alanine codons	
	TTT	TTC
IDCase *M. grisea*	7	8
Synthetic IDCase *M. grisea*	9	6

	Glycine codons			
	GGT	GGC	GGA	GGG
IDCase *M. grisea*	7	15	7	5
Synthetic IDCase *M. grisea*	19	15	0	0

	Valine codons			
	GTT	GTC	GTA	GTG
IDCase *M. grisea*	5	9	2	6
Synthetic IDCase *M. grisea*	11	4	0	7

	Alanine codons			
	GCT	GCC	GCA	GCG
IDCase *M. grisea*	7	10	11	10
Synthetic IDCase *M. grisea*	3	13	9	13

Incubation time with IPTG	Volume of Protein	Time of Reaction	cpm	Specific Activity nmol/min/μg
1 h	10 μL	2 minutes	78	0.014
1 h	10 μL	5 minutes	74	0.005
1 h	20 μL	5 minutes	89	0.003
2 h	10 μL	2 minutes	76	0.009
2 h	10 μL	5 minutes	86	0.004
2 h	20 μL	5 minutes	138	0.003
4 h	10 μL	2 minutes	427	0.185
4 h	10 μL	5 minutes	746	0.128
4 h	20 μL	5 minutes	647	0.054
6 h	10 μL	2 minutes	499	0.332
6 h	10 μL	5 minutes	1199	0.319
6 h	20 μL	5 minutes	2258	0.3

Table A - 1: IPTG induction time points for cells carrying pCAL-IDCase *N. crassa*.

Incubation time with IPTG	Volume of Protein	Time of Reaction	cpm	Specific Activity nmol/min/μg
1 h	10 μL	5 minutes	3458	0.209
1 h	20 μL	5 minutes	3680	0.108
2 h	10 μL	2 minutes	2629	0.875
2 h	10 μL	5 minutes	3316	0.442
2 h	20 μL	5 minutes	3492	0.233
4 h	10 μL	2 minutes	3774	0.553
4 h	10 μL	5 minutes	3589	0.211
4 h	20 μL	5 minutes	3384	0.1
6 h	10 μL	2 minutes	3353	0.594
6 h	10 μL	5 minutes	3544	0.250
6 h	20 μL	5 minutes	4013	0.141

Table A - 2: IPTG induction time points for cells carrying pCAL-synIDCase#1.

Incubation time with IPTG	Volume of Protein	Time of Reaction	cpm	Specific Activity nmol/min/μg
2 hr	10 μL	2 minutes.	1906	0.361
2 h	10 μL	5 minutes	2871	0.221
2 h	20 μL	5 minutes	3553	0.128
4 h	10 μL	2 minutes	3260	1.13
4 h	10 μL	5 minutes	3114	0.428
6 h	10 μL	2 minutes	2600	0.302
6 h	10 μL	5 minutes	3094	0.144
6 h	20 μL	5 minutes	2978	0.069

Table A - 3: IPTG induction time points for cells carrying pCAL-synIDCase #4.

Metal added (200 μM each)	Volume of Protein	Time of Reaction	cpm	Specific Activity nmol/min/μg
$CoCl_2$	5 μL	1 minute	145	0.054
$CoCl_2$	10 μL	2 minutes	406	0.038
$CoCl_2$	10 μL	5 minutes	938	0.035
$CoCl_2$	20 μL	5 minutes	1494	0.027
$MgCl_2$	5 μL	1 minute	155	0.077
$MgCl_2$	10 μL	2 minutes	448	0.056
$MgCl_2$	10 μL	5 minutes	817	0.040
$MgCl_2$	20 μL	5 minutes	923	0.023
$ZnCl_2$	5 μL	1 minute	564	0.112
$ZnCl_2$	10 μL	2 minutes	1718	0.085
$ZnCl_2$	10 μL	5 minutes	2795	0.055
$ZnCl_2$	20 μL	5 minutes	3383	0.034

Table A - 4: Effect of metal addition on the activity of IDCase, when metal was added to the growth media for cells carrying pCAL-IDCase *N. crassa*.

Metal added (200 μM each)	Volume of Protein	Time of Reaction	cpm	Specific Activity nmol/min/μg
$CoCl_2$	5 μL	1 minute	1132	0.290
$CoCl_2$	10 μL	2 minutes	2691	0.172
$CoCl_2$	20 μL	5 minutes	3661	0.043
$MgCl_2$	5 μL	1 minute	1668	0.342
$MgCl_2$	10 μL	2 minutes	2856	0.146
$MgCl_2$	10 μL	5 minutes	3275	0.067
$MgCl_2$	20 μL	5 minutes	3232	0.033
$ZnCl_2$	5 μL	1 minute	442	0.141
$ZnCl_2$	10 μL	2 minutes	2690	0.215
$ZnCl_2$	10 μL	5 minutes	2694	0.086
$ZnCl_2$	20 μL	5 minutes	2953	0.047

Table A - 5: Effect of metal addition on the activity of IDCase, when metal was added to the growth media for cells carrying pCAL-fusion IDCase *N. crassa*.

Incubation time with IPTG	Volume of Protein	Time of Reaction	cpm
0 h	10 µL	2 minutes	0
0 h	10 µL	5 minutes	82
0 h	20 µL	5 minutes	106
2 h	10 µL	2 minutes	33
2 h	10 µL	5 minutes	106
2 h	20 µL	5 minutes	192
4 h	10 µL	2 minutes	64
4 h	10 µL	5 minutes	131
4 h	20 µL	5 minutes	298

Table A-6: IPTG induction time points for cells carrying pCAL-synIDCase *M. grisea* with 12 °C growth temperature. BL21 codon plus *E. coli* cells with pCAL-synIDCase #1 plasmid were used to get the samples.

Incubation time with IPTG	Volume of Protein	Time of Reaction	cpm
0 h	10 µL	2 minutes	21
0 h	10 µL	5 minutes	297
0 h	20 µL	5 minutes	245
2 h	10 µL	2 minutes	84
2 h	10 µL	5 minutes	225
2 h	20 µL	5 minutes	512
4 h	10 µL	2 minutes	-18
4 h	10 µL	5 minutes	423
4 h	20 µL	5 minutes	868

Table A-7: IPTG induction time points for cells carrying pCAL-synIDCase *M. grisea* with 30 °C growth temperature. BL21 codon plus *E. coli* cells with pCAL-synIDCase #1 plasmid were used to get the samples.

Incubation time with IPTG	Volume of Protein	Time of Reaction	cpm
0 h	10 µL	2 minutes	36
0 h	10 µL	5 minutes	116
0 h	20 µL	5 minutes	171
2 h	10 µL	2 minutes	917
2 h	10 µL	5 minutes	1594
2 h	20 µL	5 minutes	1782
4 h	10 µL	2 minutes	1954
4 h	10 µL	5 minutes	1965

Table A-8: IPTG induction time points for cells carrying pCAL-synIDCase *M. grisea* with 37 °C growth temperature. BL21 codon plus *E. coli* cells with pCAL-synIDCase #1 plasmid were used to get the samples.

Concentration of added $ZnCl_2$	Volume of Protein	Time of Reaction	cpm
0	5 µL	1 minute	1395
0	10 µL	2 minutes	4298
0	10 µL	5 minutes	4663
0	20 µL	5 minutes	4949
0.2 mM	5 µL	1 minutes	790
0.2 mM	10 µL	2 minutes	3043
0.2 mM	10 µL	5 minutes	4767
0.2 mM	20 µL	5 minutes	4666
0.4 mM	5 µL	1 minutes	1377
0.4 mM	10 µL	2 minutes	3904
0.4 mM	10 µL	5 minutes.	4903
0.4 mM	20 µL	5 minutes	4816

Table A -9: Effect of $ZnCl_2$ addition to the growth media on IDCase activity, for BL21 codon plus *E. coli* cells with pCAL-synIDCase #1 plasmid.

Concentration of $ZnCl_2$ in reaction mix	Volume of Protein	Time of Reaction	cpm
4 mM	5 µL	1 minute	67
4 mM	10 µL	2 minutes	58
4 mM	10 µL	5 minutes	53
4 mM	20 µL	5 minutes	141
10 mM	5 µL	1 minute	22
10 mM	10 µL	2 minutes	43
10 mM	10 µL	5 minutes	29
10 mM	20 µL	5 minutes	89
20 mM	5 µL	1 minute	14
20 mM	10 µL	2 minutes	-1

Table A - 10: Effect of $ZnCl_2$ added to the reaction assay mix. The cpm shows diminished activity of IDCase when $ZnCl_2$ was added directly to the assay mix.

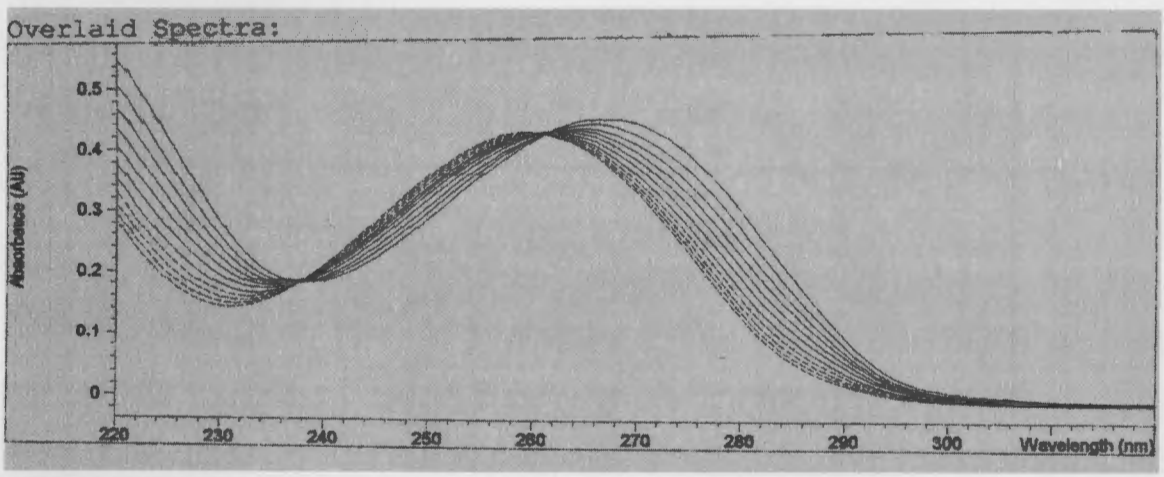


Figure A-1: The U.V spectrophotometer assay of Synthetic IDCase *M. grisea*. Conversion of Iso-orotate 270 nm to Uracil 260 nm by IDCase can be clearly seen.