STRUCTURAL STUDIES OF ESCHERICHIA COLI

α-GALACTOSIDASE

By

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DEDICATION

For my Mom and Dad who offered me unconditional love and support throughout all those years

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ABBREVIATIONS

DNA	Deoxyribose nucleic acid
GH4	Glycosyl hydrolase 4 family
NAD	Nicotinamide adenine dinucleotide
Mn^{2+}	Manganese (II) ion
LB media	Luria-Bertani medium
EK	Enterokinase
LIC	Ligation independent cloning
PCR	Polymerase chain reaction
AMP+	Ampicillin
MW	Molecular weight
IPTG	Isopropyl β-D-1-thiogalactopyranoside
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
DTT	Dithiothreitol
His	Histidine

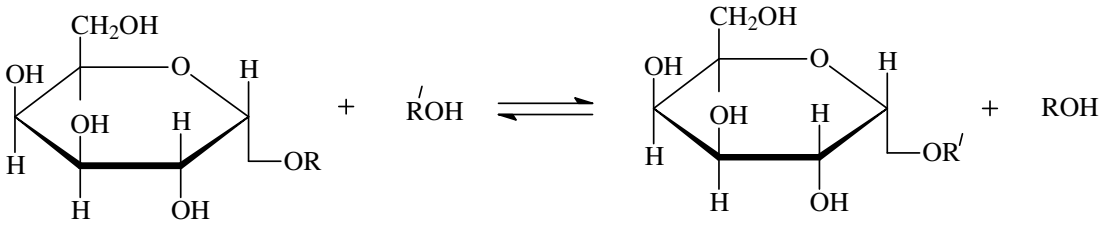
CHAPTER I

BACKGROUND AND REVIEW OF LITERATURE

1.1 Introduction

An enzyme preparation (melibiase) that hydrolyzes the disaccharide melibiose was first isolated in 1895 by Bau[1] and Fischer and Lindner[2] from bottom yeast. Bottom yeast are species of *Saccharomyces* used in the brewing industry in order to prevent foam formation at the top levels of the fermenter.[3] In later years Weidenhagen, who studied the melibiase enzyme specificity using a variety of sugars possessing non-reducing terminal α-D-galactosyl residues changed the name melibiase to α-galactosidase.[4,5]

α-Galactosidases [α-D-galactoside galactohydrolase (EC 3.2.1.22)] catalyze the following reaction.[6]



R and R' can be aliphatic or aromatic groups. Usually the hydroxylic acceptor molecule, R'OH, is water. Based on the R and R' groups the enzyme can hydrolyze a variety of substrates from simple α-galactosides to complex oligo and polysaccharides.[6]

α-Galactosidases have been reported to occur widely in nature, such as in bacteria, fungi, plant seeds and tissues and animal tissues. The distribution of the enzyme and cellular localization has been found to vary with the organism. The *Escherichia coli* enzyme is cytoplasmic, whereas the human enzyme is lysosomal and the yeast enzyme is extracellular.[7] In most organisms, α-galactosidase is found in the soluble fraction of the cellular extracts.

Physiological significances of α-galactosidases have been widely studied. For example, α-galactosidases are involved in the plant kingdom for hydrolyzing the oligosaccharides that serve as a soluble and readily metabolizable energy reserve[8] during seed germination. Some studies have revealed that α-galactosidases can be involved in plant galacto-lipid metabolism.[9]

In the past, studies with α-galactosidases have mostly been carried out using relatively crude enzyme extracts due to the inability of obtaining the pure enzyme. α-Galactosidases from various sources have been isolated using conventional methods of extraction.[6,10] In the cell, α-galactosidases are found to be associated with other glycosidases, thus making it difficult to isolate.[6] The techniques that have been used for the isolation of α-galactosidases include ammonium sulphate[11-14] and organic solvent

fractionations,[13-15] heat treatment,[11,12] acidification,[14,15] ion exchange,[12,15] gel chromatography[11,14,15] and iso-electro focusing.[16] Highly purified, homogeneous α-galactosidases have only been isolated on a few occasions.[14] The first crystalline form of α-galactosidase was reported in 1970 from the mycelia of *Mortierella vinacea.*[11]

The α-galactosidase enzyme has shown various potential technological and medicinal applications. Presently the most important industrial applications of α-galactosidase include the beet sugar industry, soy food processing, the pulp and paper industry, dietary industry and animal feed processing. As for medicinal applications, α-galactosidases are currently used in blood group transformation, treatment of Fabry's disease and in xenotransplantation.

1.2 Industrial Applications of α-galactosidase

1.2.1 Soy based food industry

Soy based foods have become a promising nutrient supplement to overcome the current protein-calorie malnutrition problem.[17] Soy milk is a low-cost substitute for dairy milk in developing countries and a nutritive supplement for the lactose intolerant population.[18] Soy usage has been limited due to the anti-nutritional compounds that diminish the nutritive value and lower the acceptance of soy based products. One such anti-nutritional factor is flatus causing oligosaccharides.[19] Soy beans contain 9-12% total sugars including 4-5% sucrose, 1-2% raffinose, 3.5-4.5% stachyose and smaller quantities of melibiose and verbascose.[17] Monogastric animals including humans are

incapable of hydrolyzing α-galactosides[20] (raffinose and stachyose) in soy beans that cause flatulence and gastrointestinal disturbance, which reduce the feed efficiency and consumer acceptance of those products.[21] Due to those reasons α-galactoside-free soy bean products have become increasingly popular.

Industrially, the α-galactoside removal is achieved by treatment of soy milk with a crude extract of α-galactosidase obtained from the fungus *Aspergillus oryzea*.[20,22] In early methods, the enzyme was immobilized on polyacrylamide[23, 24] but in novel methods the enzyme is immobilized on calcium alginate in the presence of glutaraldehyde which is used as a hardner.[20] The use of calcium alginate has been found to be comparatively safe, simple, and cheap with durable enzyme activity.[20]

Another related application of α-galactosidase is in the form of a dietary supplement to overcome flatulence, bloating and abdominal discomforts.[25] The enzyme works in the digestive tract to break down legumes and cruciferous vegetables to simpler sugars reducing flatulence. One such dietary supplement is Beano® in which the source of α-galactosidase is the fungus *Aspergillus niger*.[26]

1.2.2 Sugar beet industry

From the global sugar production, 30% of the sugar is produced using sugar beet, a root crop which is grown in the temperate zone.[27] In colder temperatures, raffinose biosynthesis in sugar beet occurs by reaction of an inositol galactoside called galactinol

with sucrose.[28, 29] During the manufacture of beet sugar, which is a crystallization process, the raffinose content increases up to > 6% by weight.[30] This high concentration of raffinose inhibits the rate of sucrose crystallization as well as modifies the sucrose crystals to a needle like shape that lessens the consumer acceptance.[31] Therefore the removal of raffinose from the manufacturing process was economical. Over the past decade, mycelia containing α-galactosidase have been used commercially to hydrolyze raffinose into sucrose and galactose. *Mortierella vinacea*[30] and *Absidia greisola*[32] are examples of α-galactosidase containing mycelia producing fungal sources in this industry.

1.2.3 Enzymatic conversion of blood group B to O in human erythrocytes

Accidental transfusion of ABO-incompatible erythrocytes causes fatal reactions. To overcome this and to create a universal blood supply using enzymatic methods has been initiated by Goldstein and colleagues in the 1980s.[33,34] The enzymatic conversion of group B red blood cells to group O red blood cells *in vitro* is attained by the use of α-galactosidase from coffee beans.[35, 36] Group B antigens differ structurally from group O antigen only by an addition of one terminal alpha-linked galactose residue.[37,38] α-Galactosidase isolated from green coffee beans has shown a high activity towards removing the alpha-linked galactose residue from the group B red blood cell surface.[39] The treated group B red blood cells have been successfully transfused to both group A and O recipients.[40]

1.2.4 Enzyme therapy for Fabry disease

Fabry disease is an X-linked recessive lysosomal storage disease resulting from the deficiency of α-galactosidase A in human.[41, 42] Patients with this inborn error accumulate globotriaosylceramide and related glycosphingolipids[43] in lysosomal plasma and tissues which would eventually leads to painful neuropathy with progressive renal, cardiovascular, and cerebrovascular dysfunction and early death.[44]

Recent studies have suggested that enzyme replacement therapy for Fabry disease was well tolerated and more effective over the already existing treatment methods. Two differently produced enzyme preparations have been examined in clinical investigations. One enzyme, which is called Fabrazyme, is produced by Chinese hamster ovary cells with classic recombinant technology, and the other enzyme, known as Replagal, is produced by cultured human skin fibroblasts with an activated promoter of the α-galactosidase A gene.[44] In both studies, promising lipid substrate reductions in tissue biopsies have been observed. The availability of the recombinant enzyme offers the potential of a safe and effective targeted treatment approach.

1.2 *Escherichia coli* α-galactosidase

Mel Operon

In *E. coli* the melibiose utilization is dependant on the melibiose operon that is located at 93 min on the genetic map.[45] The mel operon consists of at least two structural genes, *melA* and *melB*.[45,46] Both proteins are necessary for the metabolism of melibiose.[47] *melA,* which is the promoter proximal gene, codes for the α-galactosidase enzyme that hydrolyses melibiose into glucose and galactose[7] while the *melB* gene codes for the melibiose carrier protein located on the membrane, which is involved in melibiose transportation into the cell.[48] The mel operon is found to be an inducible operon suggesting that it could be controlled in a similar way as the well studied Lactose operon.[47] Recent studies show the *E. coli* MelR protein is a transcription activator, which is essential for melibiose dependant expression of *melAB* genes.[49-51] These studies also show that the cyclic AMP receptor protein (CRP) interacts with the *melAB* promoter and increases MelR-dependent transcription activation.[49] However the mel operon and its gene products are still poorly understood and the presence of a repressor gene is not yet proven.[47]

In 1971, Burstein and Kepes reported the partial purification of α-galactosidase from *E. coli*[52] and the cofactor requirement was studied using the crude enzyme. In 1988, the α-galactosidase was purified but the enzyme was found to be unstable.[53] Even though the

eukaryotic α-galactosidase structures and mechanisms are extensively studied, the prokaryotic α-galactosidase structure and mechanism are not yet available.

CHAPTER II

METHODOLOGY

2.1 Material and Methods

2.1.1 Introduction

The *melA* gene (Appendix 1) product is the α-galactosidase protein of 451 amino acids in length (Appendix 2) and has a molecular weight of 50,657 Da.[11, 14, 15] The protein has a theoretical pI of 5.52[15] and has been shown to be composed of alternating hydrophilic and hydrophobic regions with an average hydropathy of -0.18 which is consistent with α-galactosidase being a soluble protein.[7]

α-Galactosidase belongs to the glycosyl hydrolase 4 family of enzymes (GH4).[11,15] GH4 family enzymes are unique in their requirement for NAD(H) and a divalent metal ion for activity.[54] α-Galactosidase of *E. coli* was reported to require NAD^+ and Mn^{2+} for activity.[10]

The basic experimental outline consists of genomic DNA isolation from *Escherichia coli*, recombinant plasmid construction, *melA* gene expression, α-galactosidase protein purification, enzyme activity studies and crystallization studies.

2.1.2 Genomic DNA isolation from *E. coli*

The genomic DNA was obtained from One Shot TOP10 *E. coli* strain (Invitrogen). To begin the genomic DNA isolation procedure, 5 ml of fresh Luria-Bertani (LB) liquid media containing 50 µg/ml ampicillin was inoculated with the above *E. coli* strain and cultured for 12 hours at 37°C with shaking (200 rpm, MaxQ 500 shaking incubator, Barnstead Lab-Line). A 1.5 ml sample of this culture was spun down (Marathon microcentrifuge, Fisher Scientific) until a compact pellet formed and the supernatant was discarded. The pellet was resuspended in 567 µl TE buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA) and 30 µl of 10% SDS, followed by the addition of 3 µl of a 20mg/ml proteinase K solution. The mixture was incubated 1 hour at 37°C. After incubation 100 µl of 5 M NaCl was added and mixed thoroughly, followed by adding 80 µl of 10%CTAB (hexadecyltrimethyl ammonium bromide) in 0.7 M NaCl and incubated for 10 min at 65°C. An approximate equal volume (0.7-0.8 ml) of 24:1 choloroform/isoamyl alcohol was added, mixed and spun for 4-5 minutes in a microcentrifuge. Afterwards, the aqueous, viscous supernatant was removed to a fresh microcentrifuge tube and an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol was added and extracted thoroughly and microcentrifuged for 5 min. The supernatant was transferred to a fresh tube and the genomic DNA was precipitated by adding 0.6 vol of isopropanol. The DNA was spooled out and washed with 70% ethanol and microcentrifuged for 5 minutes, and the resulting supernatant was decanted and the pellet was dried. The dried DNA pellet was re-dissolved in 100 µl TE buffer and stored in -20°C.

2.1.3 Recombinant plasmid construction

Initially, in order to construct the recombinant plasmid with the *melA* insert, Champion pET Directional TOPO Expression vectors (Invitrogen) were used. After many trials and failing to isolate the desired product using the above kit for one year, the pET-46 EK/LIC vector kit (Novagen) was used. Those two vector kits differ from each other mainly at the insertion step of the PCR product into the vector. In the pET Directional TOPO expression system, the recombinant molecule is produced by the insertion of the double stranded blunt ended insert to the already produced overhangs with Topoisomerase enzyme on the linearized vector by displacing four bases in the 3' strand.

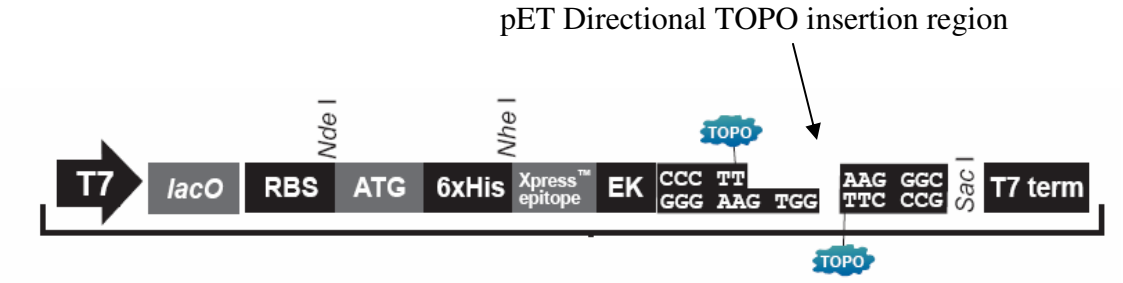


Figure2-1 Cloning region for the pET Directional TOPO indicationg the poly His tag.[55]

pET-46 EK/LIC vectors are ligation independent and were developed for the directional cloning of the polymerase chain reaction (PCR) product without the need for restriction enzyme digestion or ligation reactions.[56,57] The EK/LIC vectors express the target protein immediately downstream of an enterokinase cleavage site so that the vector encoded fusion tag can be removed from the purified protein.[58]

The ligation independent cloning (LIC) method takes advantage of the 3′→5′ exonuclease activity of T4 DNA polymerase to create very specific 13-14 base single stranded overhangs in the EK/LIC vector.[58] PCR products with complementary overhangs were created by building appropriate 5' extensions into the primers.

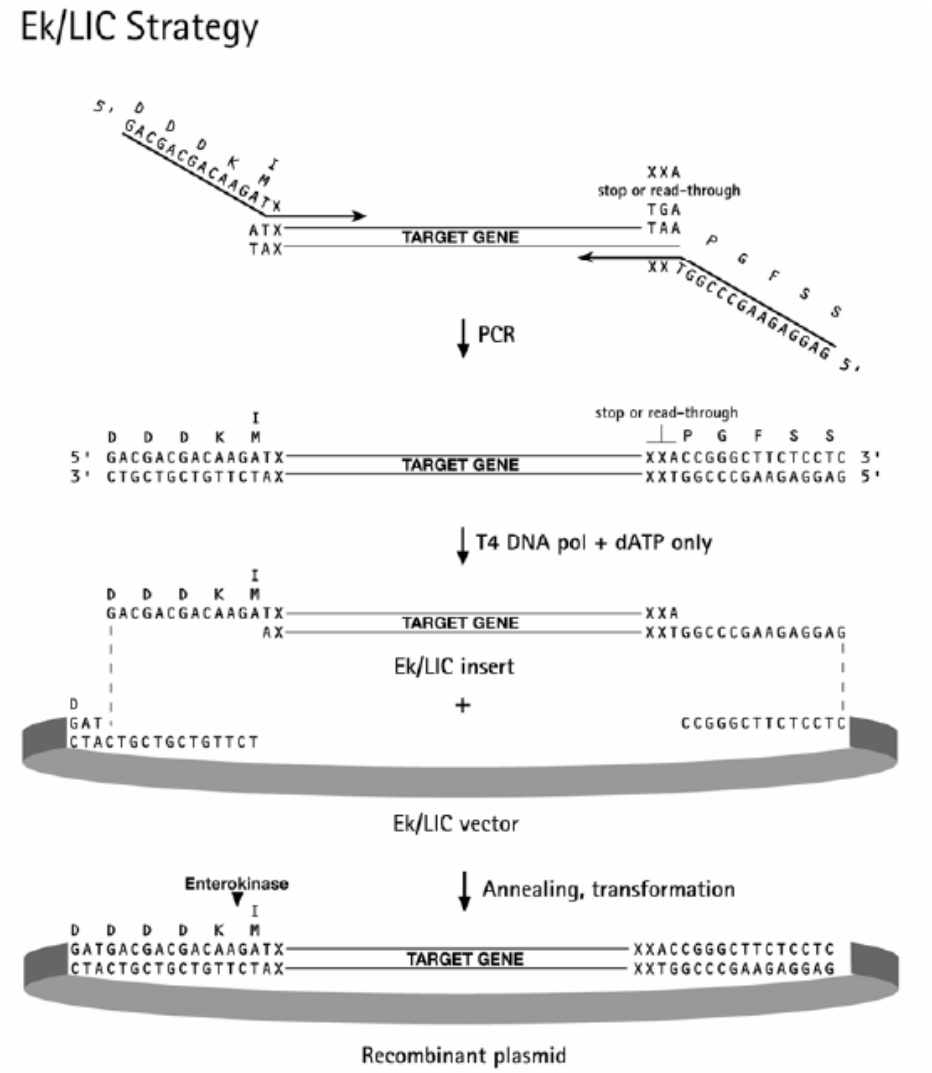


Figure 2-2. EK/LIC strategy. After amplification with primers that include the indicated 5' LIC extensions, the PCR insert is treated with LIC-qualified T4 DNA polymerase, annealed to the EK/LIC vector and transformed (Novagen).[59]

α-Galactosidase forward and reverse primers were designed using the nucleotide sequence provided for *E. coli* K-12 substr. MG1655 in NCBI database with the gene ID NC 000913. In order to work with the LIC system, for the forward primer, GACGACGACAAGAT sequence and for the reverse primer GAGGAGAAGCCCGGT sequence were added at the 5' ends. The final primer sequences with the *melA* gene were, forward primer 5'-GACGACGACAAGATGATGTCTGCACCCAAAATT-3', reverse primer 5'-GAGGAGAAGCCCGGTTTAACGGTGCAACCAACG-3') which were custom made by Invitrogen.

2.1.4 PCR reaction and gel extraction of the PCR product

The *melA* gene was amplified from isolated *E. coli* genomic DNA by PCR using the PTC-150 Minicycler (MJ Research). Each of the primers was diluted to the concentration of 20 µM before PCR setup. Each 50 µl PCR mixture consisted of 3 µl of 20 µM forward primer, 3 µl of 20 µM reverse primer, components of the Invitrogen AccuPrime Taq DNA polymerase system (5 µl of 10X AccuPrime PCR buffer II and 1.5 µl of Taq DNA polymerase solution), 1.5 µl of isolated genomic DNA, and 36 µl of sterilized water. The genomic DNA was initially denatured for 6 minutes at 95°C, annealed for 45 seconds at 60°C and extended for 1 min 30 sec at 72°C. Thereafter, from the second to the 35th PCR cycle, the following steps were included: 45 seconds denaturation at 95°C, 30 seconds annealing at 60°C, 1 minute 30 second extension at 72°C. The final extension was done at 72°C for 7 minutes.

The PCR product (~1389 bp) was loaded onto a 1% agarose gel (total of 8 lanes) containing 0.5 µg/ml ethidium bromide and verified through agarose gel electrophoresis (100 V, 2 hours, Sub-Cell GT agarose gel electrophoresis, BioRad). The bands coresponding to the correct size of the PCR product were visualized with UV light.

To purify the PCR product, the Invitrogen PureLink Quick Gel Extraction Kit was utilized. This purification procedure removes primers, nucleotides, salts, agarose, ethidium bromide and other impurities from the amplified DNA sample. The bands containing the PCR product were cut from the gel and divided into two 1.5 ml microcentrifuge tubes to dissolve the gel material. The Gel Solubilization Buffer (598.9 µl) was added into the tube which contained 199.6 mg of the excised gel slice. The other tube contained 262.1 mg of gel slice so that 786.3 µl of Gel Solubilization Buffer was added. The tubes were incubated at 50°C for 15 min with mixing every 3 minutes. An additional 5 min incubation at 50°C was done to ensure that the gel completely dissolved.

The solutions were transferred by pipette, respectively, into two Quick Gel Extraction Columns, which were placed onto the Wash Tubes, and centrifuged in the microcentrifuge for 1 min at maximum speed. The flow-through was discarded. Wash Buffer (700 µl) containing ethanol was added into each of the Spin Columns. After 5 min of incubation at room temperature, the solutions were centrifuged for 1 min at maximum speed, and the flow through was discarded. After one more round of centrifugation to assure removal of all liquid, the columns were transferred onto new 1.5 ml Recovery

Tubes. The Nuclease-Free water (50 µl) was then added into each of the columns, and the PCR products bound to the resin of the columns were eluted into the Recovery Tubes.

A comparison between the PCR product and the DNA mass ladder (Invitrogen), it was deduced from the band intensity that the concentration of the gel purified PCR product was 4 ng/µl. The purified PCR product will be used with the pET-46 EK/LIC vector from Novagen to create the α-galactosidase recombinant protein expression vector.

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2.1.5 T4 DNA Polymerase Treatment of the Insert

T4 DNA polymerase treatment generates compatible overhangs on the PCR product. In order to ligate with the sticky-ends of the pET-46 EK/LIC vector, the insert has to contain complementary sticky ends. T4 DNA polymerase reagents available with the pET-46 EK/LIC vector kit (Novagen) were assembled (2 µl of 10X T4 DNA polymerase buffer, 2 µl of 25 mM dATP, 1 µl of 100 mM DTT, and 0.4 µl of T4 DNA polymerase enzyme) in a 1.5 ml micro-centrifuge tube and enough purified PCR product was added (14.6 µl) to obtain a total volume of 20 µl. The reaction was mixed with pipetting and incubated at 22°C for 30 minutes. The enzyme was heat inactivated by incubating at 75°C for 20 minutes. The prepared insert was stored at -20°C.

During the annealing procedure, 1 µl of pET-46 EK/LIC vector and 2 µl of T4 DNA polymerase treated insert were mixed in a 1.5 ml micro-centrifuge tube and incubated at

22°C for 5 mins. Then, 1 µl of 25 mM EDTA was added and mixed by stirring with the pipette tip and incubated for a further 5 min at 22°C.

2.1.6 Transformation

For the transformation, NovaBlue GigaSingles Competent Cells that were provided with the pET46 EK/LIC vector kit were removed from the -20°C freezer and placed on ice for ~5 min. A 1 µl sample of the annealing reaction was added directly to the competent cells and gently stirred and returned to the ice. The reaction tubes were incubated on ice for 5 min. Tubes were transferred to a water bath at 42°C and kept exactly for 30 seconds without shaking to heat shock the cells to make them more receptive to the vector plasmid. The tubes were transferred immediately onto ice. To each transformation mixture, 250 µl of room temperature SOC medium (EK/LIC vector kit, Novagen) was added followed by incubation at 37°C while shaking at 250 rpm for 60 min prior to plating on selective medium.

Selection for transformants was accomplished by plating 150 µl of the above reaction mixture onto a pre-warmed (37°C) LB agar plate containing 50 µg/ml ampicillin with the use of a bent glass rod to spread the culture onto the plate. The plasmid contained a gene to encode drug resistant against ampicillin. The plates were incubated overnight at 37°C.

2.1.7 Verification of the correct recombinant plasmid

From the LB/Amp agar plates, single, well-isolated colonies were picked (using sterile toothpicks), and each colony was used to inoculate 5 ml LB/Amp media in 15 ml conical bottom vials. The cultures were grown for 16 hrs at 37°C and 200 rpm. An aliquot (1 ml) of the overnight cell culture was mixed with 0.5 ml of a sterile 60% glycerol solution in a 2 ml cryo-vial and stored at -80°C for future propagation and maintenance of gene construct.

The remaining culture was used to harvest cells with centrifugation at 4500 rpm for 10 min at 4°C (Allegra X-15R Benchtop Centrifuge, Beckman Coulter) for the plasmid isolation process using the Wizard Plus SV Miniprep DNA Purification System (Promega). The supernatant was discarded and the pellet was resuspended completely in 250 µl of Cell Resuspension Solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, and 100 µg/ml RNase A) by vortexing. Afterwards, the resuspended pellet was transferred into a 1.5 ml microcentrifuge tube and mixed with 250 µl Cell Lysis Solution (0.2 M NaOH and 1% SDS) by inverting the tube 4 times. Partial clearing of the lysate was observed after 5 min incubation. To quench the endonucleases and other proteins released during the lysis, Alkaline Protease Solution (10 µl) was added into the lysate, mixed by inverting the tube 4 times and incubated for 5 min. After incubation, the cell solution was mixed with 350 µl Neutralization Solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, and 2.12 M glacial acetic acid) by inverting the vial 4 times for solution neutralization. The mixture was centrifuged (Marathon Microcentrifuge, Fisher Scientific) at maximum

speed for 10-30 min until the cell debris was pelleted. The cleared lysate was then decanted into a Spin Column, which was inserted into a 2 ml Collection Tube for the plasmid DNA purification. The contents in the Spin Column were centrifuged at maximum speed for 1 min, and the flow-through from the Collection Tube was discarded. The Column Wash Solution (750 µl, 60% ethanol, 60 mM potassium acetate, 8.3 mM Tris-HCl, 0.04 mM EDTA) was added into the Spin Column and centrifuged at maximum speed for 1 min, and the flow through was discarded. Ethanol will precipitate the plasmid DNA, which was collected by the resin of the Spin Column. The wash was repeated using 250 µl Column Wash Solution and centrifuged for 2 min at maximum speed. The Spin Column was transferred to a new 1.5 ml microcentrifuge tube. By adding 100 µl Nuclease-Free water into the column with 1 min centrifugation at maximum speed, the plasmid DNA was eluted into the 1.5 ml microcentrifuge tube. The Spin column was discarded, and the plasmid DNA samples were stored at -20°C.

To verify the presence and the concentration of the plasmid, a 1% agarose gel electrophoresis was carried out with 8 lanes. The expected linear plasmid size was ~ 6589 bp. With comparison to the Mass marker, the concentration of isolated plasmids was determined to be 15-20 ng/µl. From the isolated plasmid samples, five samples were used in restriction enzyme analysis.

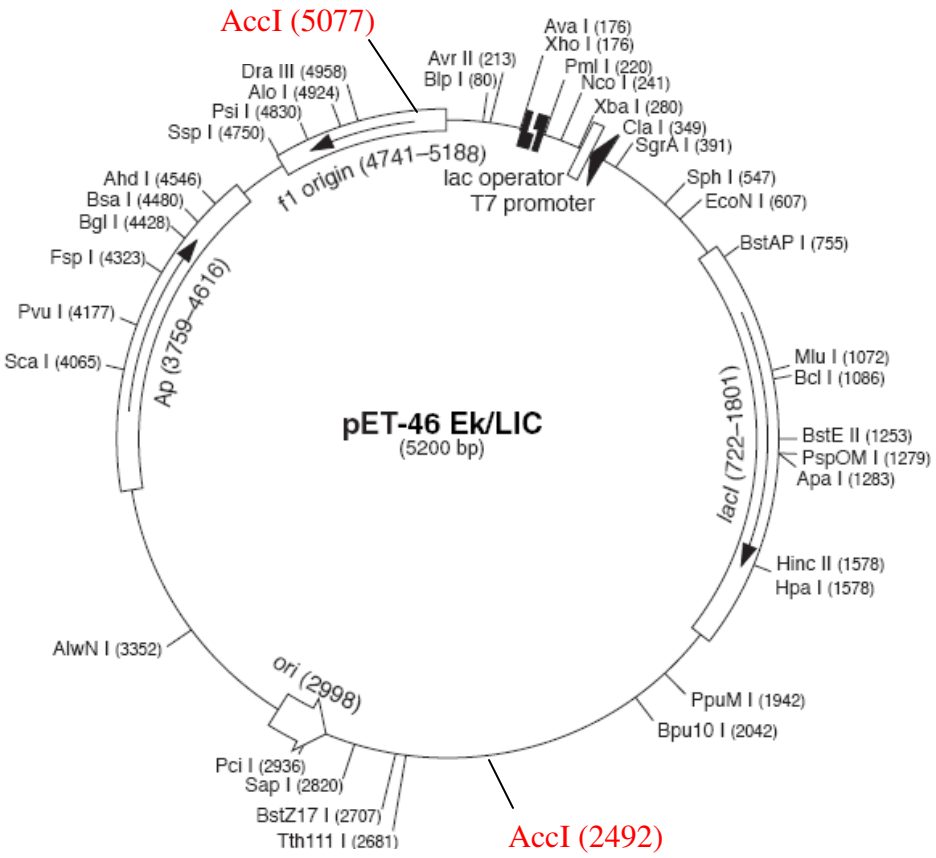

Figure 2-3 The restriction sites of the pET-46 EK/LIC vector. AccI restriction sites are located at 2492 and 5077.[59]

In the restriction enzyme analysis, AccI (Promega) was used to digest the recombinant plasmid twice in two different locations (on the map at positions 2492 and 5077) for the verification of the correct insertion. In a 1.5 ml micro-centrifuge tube, 7 µl of sterile water, 2 µl Restriction Enzyme 10X buffer, 0.5 µl of BSA, and 0.5 µl of Acc I restriction enzyme (all provided by Promega) were mixed with 10 µl of 4 ng/µl DNA and digested for 2 hrs at 37-42°C in a water bath. After digestion, 10 µl out of the total 20 µl of the products were loaded onto a 0.9% agarose gel and electrophoresed at 100 V for 2 hrs.

The integrity of the plasmid was further checked with DNA fluorescent sequencing by the Recombinant DNA/Protein Resource Facility of the Biochemistry and Molecular Biology Department. The T7 promoter and terminator primers, which lie on either side of the inserted gene and control the expression of the recombinant protein, was performed to confirm the *melA* gene was cloned into the pET-46 EK/LIC vector with the correct size and orientation.

2.1.8 Over expression of α-galactosidase and purification

The verified recombinant plasmids were transformed into *E. coli* BL21 Star DE3 competent cells (Invitrogen). The BL21 Star DE3 *E. coli* cells are genetically engineered to improve T7 RNA polymerase producing mRNA stability which enhances the targeted protein production significantly since the targeted gene transcription promoter is T7 based.

The transformants were grown in 10 ml of LB media containing 50 µg/ml ampicillin (Amp) at 37°C and shaking at 200 rpm overnight. The small cultures were used to inoculate 250 ml LB/Amp media and grown at 37°C at 200 rpm until an optical absorbance of the culture at 600 nm (A_{600}) was between 0.6 and 0.8. Then the expression of the recombinant protein was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma), and the temperature was decreased to 25°C. The bacterial culture was incubated at 25°C for 20 hrs with shaking at 200 rpm. Cells were harvested by centrifugation (Allegra X-15R benchtop centrifuge, Beckman Coulter) at

4500 rpm for 10 min at 4°C. The supernatant was discarded and the pellets were frozen at -20°C until needed.

The cell pellet from 250 ml of culture was resuspended in 25 ml of ice-cold lysis buffer (40 mM Tris-HCl pH 8, 500 mM NaCl, and 0.2 mg/ml lysozyme) with votexing. The lysate was sonicated (Sonifier 150 Liquid processor, Branson) for 30 seconds at analog speed setting of "2" followed by cooling on ice for 30 seconds. Both the sonication and cooling steps were repeated three more times. The sonicated lysate was centrifuged at 3500 rpm for 50 min at 4°C until a clear supernatant was visible. α-Galactosidase was a soluble protein, thus it will be present in the supernatant of the sonicated cell suspension. The centrifuged clear solution was transferred to a fresh conical tip centrifuged tube.

For the purification of α-galactosidase, the Biologic Duo-Flow Chromatography System (Bio-Rad) was used. Immobilized metal affinity chromatography was used in the purification process. Prepackaged and precharged Ni Sepharose™ containing HisTrap™ HP Columns were purchased from GE Healthcare. The recombinant α-galactosidase contains an N-terminal poly-histidine tag, which would enable the protein to bind with the Ni^{2+} in the column. Since the proteins without the His tag would bind weakly or not at all to the Ni^{2+} column, they would be eluted first at a low imidazole concentration leaving the His tag containing proteins to elute at a higher imidazole concentration.

A considerable amount of β-lactamase protein, coded by the ampicillin resistant gene, was produced. A step-wise purification, where the imidazole concentration is varied in a step-wise manner, was done in order to minimize the impurities including β-lactamase and to get a much purer sample of the α-galactosidase protein. A nickel(II) chelating column with a 5 ml bed volume was preloaded with the 25 ml of crude protein extract using a Model EP-1 Econo Pump (Bio-Rad) at a 1 ml/min flow rate. The preloaded nickel(II) column was mounted onto the Biologic Duo-Flow Chromatographic system for the purification and elution of the α-galactosidase.

The buffers used in the purification of alpha galactosidase using the Biologic Duo-Flow system are 40 mM Tris-HCl with 500 mM NaCl, 40 mM Tris Base with 500 mM NaCl, 500 mM imidazole (pH 8), and degassed water. Thorough out the purification, the buffer blending mode was used, which mixes the Tris solutions and the water/imidazole solutions separately and then mixes them together. Furthermore, the Tris solution is titrated to a pH of 8. Therefore, the final solution will contain 20 mM Tris (pH 8) and varying amounts of imidazole from 0 to 250 mM, depending on the percentage specified in the program. Initial non-bound impurities were eluted using 16 ml of 5% 12.5 mM imidazole at the rate of 2 ml/min. Most of the unbound impurities were eluted at this imidazole concentration. The column was further washed using 26 ml of 25% 62.5 mM imidazole at the rate of 2 ml/min. Tightly bound α-galactosidase was eluted next with 40 ml of 250 mM imidazole at the rate of 2 ml/min. After the elution of the proteins, the column was washed further with 10 ml of 25% 62.5 mM Imidazole at the rate of 2 ml/min.

The collected fractions were verified for the presence of α-galactosidase protein using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis using Mini-Protein 3 Cell electrophoresis apparatus (Bio Rad) with a Precision Plus Protein Standards marker (Bio Rad).

The fractions containing the α-galactosidase were collected and further purified using a desalting column (HiPrep 26/10 Desalting GE Healthcare). In the desalting process 60 ml of 20 mM Tris HCl at pH 7 was used at the rate of 3 ml/min. Eluted fractions were analyzed using 15% SDS PAGE gels. The bands of the resulting gel were further analyzed using Matrix-ssisted laser desorption ionization mass spectrometry time of flight (MALDI-TOF) mass spectroscopy at the recombinant DNA/ Protein Resource Facility of the Biochemistry and Molecular Biology Department.

The purified protein fractions were concentrated to 1.5 ml using a 20 ml centricon with 10,000 MW cut off (Vivaspin, Vivascience) with centrifugation at 3000 rpm and 4°C. The final concentration was determined by the Bradford assay using bovine serum albumin as the standard. In the Bradford assay, Coomassie brilliant Blue G-250 binds to the protein and produce absorbance at 600 nm. The protein concentration is proportional to the absorbance and can be calculated using a calibration plot of absorbance vs. concentration.

It was found that the stability and the activity of α-galactosidase require Mn^{2+} and NAD^{+}.[10] Furthermore activity of the enzyme appeared to be maximum in reducing

conditions and therefore, 2-mercaptoethanol[10] or dithiothreitol[60] (DTT) were added and the activity of the enzyme was checked as suggested in the literature.

To the purified α-galactosidase (50 µg/ml), 0.5 mM Mn2+, 0.9 mM NAD+ and 10 mM DTT were added and the volume was adjusted to 450µl using 20 mM Tris HCl pH 7, mixed well and incubating for 5 min at room temperature. A 50 µl sample of 10 mM p-nitrophenyl-α-D-galactopyranoside (Sigma) was added and incubated at room temperature for 5 min. A dark yellow color development indicated the presence of active enzyme.[61]

PNP α-D-galactoside + H_2O → p-Nitrophenol + D-Galactose

2.1.9 Crystallization

X-ray crystallography is widely used in the study of proteins at atomic resolution. In order to solve the structure of the protein, X-ray diffraction data of a well grown, good quality single crystal must be obtained.[62,63] The most common setup to grow protein crystals is by the hanging drop technique. The hanging drop method is commonly used in screening a large number of potential crystallization conditions.

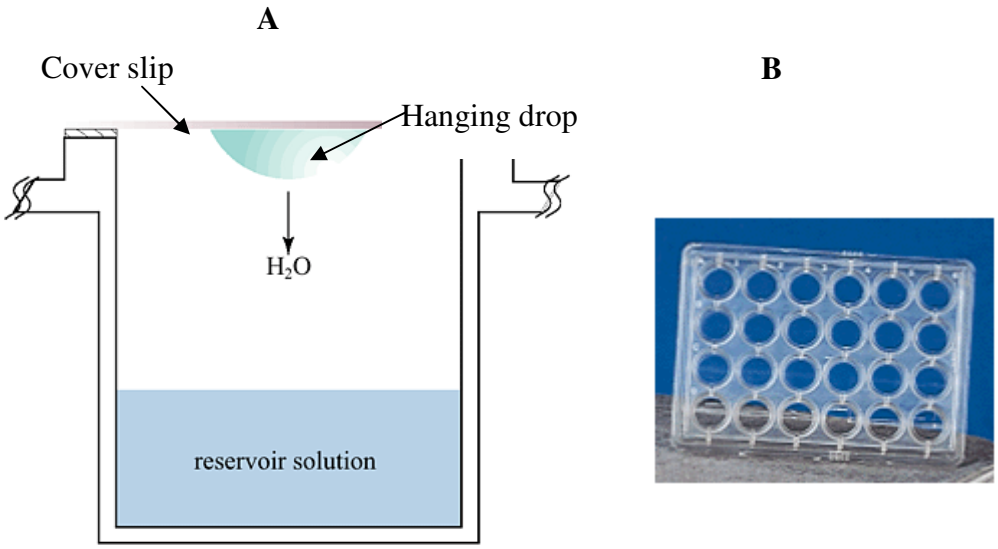

Figure 2-4. A diagram of the hanging drop vapor diffusion method A) Vacuum grease sealed experimental set up with a droplet on an inverted coverslip. B) A picture of a Linbro plate used in crystal screening process.

In the hanging drop method, bringing a protein solution to a saturation point is controlled by the vapor diffusion with the use of precipitating agents. A small droplet (~5µl) of protein containing buffer and precipitating agent is suspended on a glass or microscope coverslip over a well containing a much larger volume (~300 – 1000 µl) of the same buffer and precipitation agent solution with a large osmolarity. Over time, a net evaporation of the water from the protein sample droplet and a net condensation in the reservoir solution equalize the osmolarities of the two solutions. The evaporation of water from the protein sample droplet results in lowering the solubility of protein and the precipitating agent concentration that would induce the formation of protein crystals. Even though the process of the hanging drop method is not technically difficult, obtaining

a single protein crystal with good diffraction quality involves the optimization of many chemical and physical parameters.

Crystallization trials were done using the crystal screen kits provided by Hampton Research and ammonium sulfate percent saturation series as the precipitating agent.[62] The hanging drop vapor diffusion method was used with purified α-galactosidase at 1.2 mg/ml and 3 mg/ml concentration in 20 mM Tris-HCl (pH 7), 0.5 mM Mn^{2+}, 0.9 mM NAD^{+}, and 10 mM DTT with the crystal screening kits HR2-110 containing 50 conditions and HR2-112 containing 48 conditions (Hampton Research) at room temperature and 4°C. In the Linbro plates, the well solution had 700 µl of the crystal screen solution while the hanging drop contained 2 µl of the well solution and 2 µl of the protein solution.

With the ammonium sulfate as the precipitating agent, the initial investigations for the effect of % salt concentration at 15%, 20%,25%,30%,35% and 40% were checked in the pH range of 3.5- 9.0 at 0.5 increments.

2.2 Results and Discussion

After failing many times with the pET100 D-TOPO vector system (Invitrogen), the construction of the recombinant molecule with α-galactosidase insert was successfully obtained using the pET-46 EK/LIC vector system (Novagen). The initial failure with the Invitrogen system was attributed to the fact; as the company explained, "the presence of some background" in the particular batch that the first vector was produced. However,

the correct recombinant molecule was never obtained after many repeated trials with newly bought champion pET expression system. The failure to produce the correct recombinant molecule for α-galactosidase with TOPO system could be attributed to the fact that earlier mentioned insertion mechanism was not successful with the *melA* gene insert. A use of an exonuclease to produce 3' overhangs on the PCR product might have been helpful with using the TOPO system for the construction of the recombinant molecule.

The PCR fragment of the *melA* gene is ~ 1356 bp in length. (Figure 2-5). After the gel extraction of the PCR fragment the concentration of the purified PCR insert was found to be 4 ng/µl. (Figure 2-6)

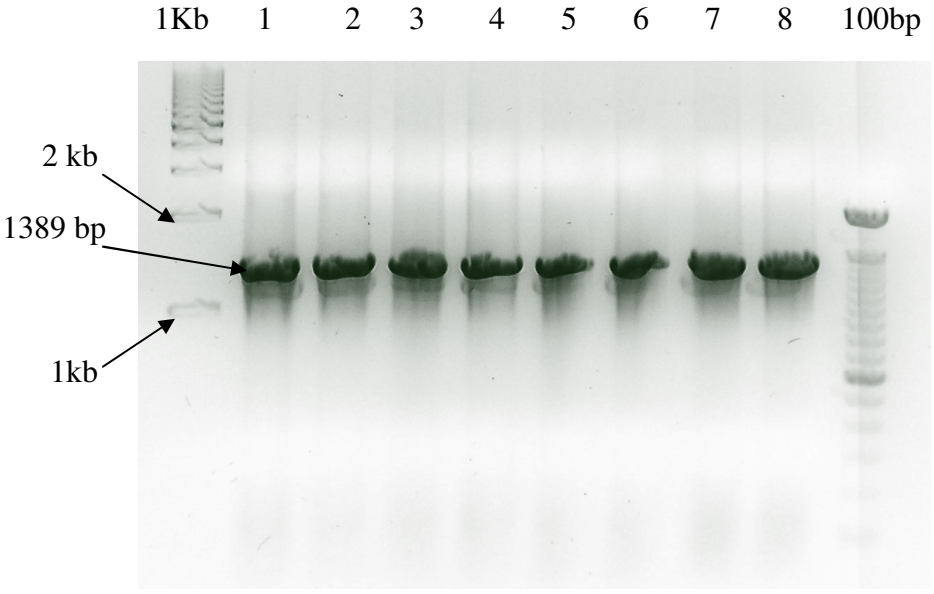

Figure 2-5. Size verification of the PCR products using 1% agarose gel stained with ethidium bromide. First and the last lanes show the size markers.

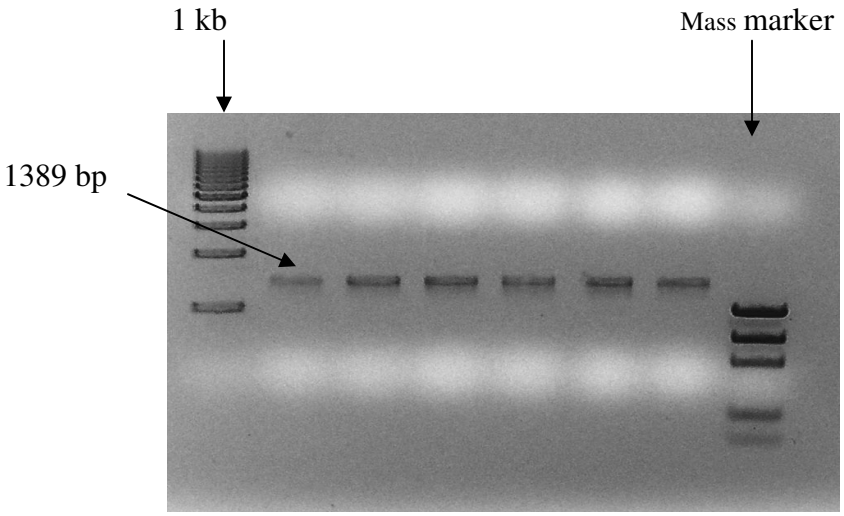

Figure 2-6. Verification of the insert after gel extraction and determination of the concentration using a 1% agarose gel. First lane shows the 1 kb ladder and the last lane shows the mass-marker used for the intensity based concentration determination.

The pET-46 EK/LIC vector size is ~5200 bp and the PCR insert is ~1389 bp. Therefore the size of the extracted plasmids from the NovaBlue GigaSingles Competent Cells is ~6589 bp. The concentration of the recombinant plasmid is found to be 20 ng/µl by comparison to the band intensities of the molecular mass standards. (Figure 2-7). The isolated plasmids did not show the expected size (6589 bp) due to the supercoiled nature of the circular plasmids. The restriction enzyme AccI cut the plasmid twice. The plasmid with the insert will give two band fragments with the longer one being 4004 bp and the shorter one being 2585 bp, while the plasmids without the insert will give two bands of 2615 bp and 2585 bp, which are very close in size and might be difficult to separately identify in a 0.9% agarose gel (Figure 2-8) .

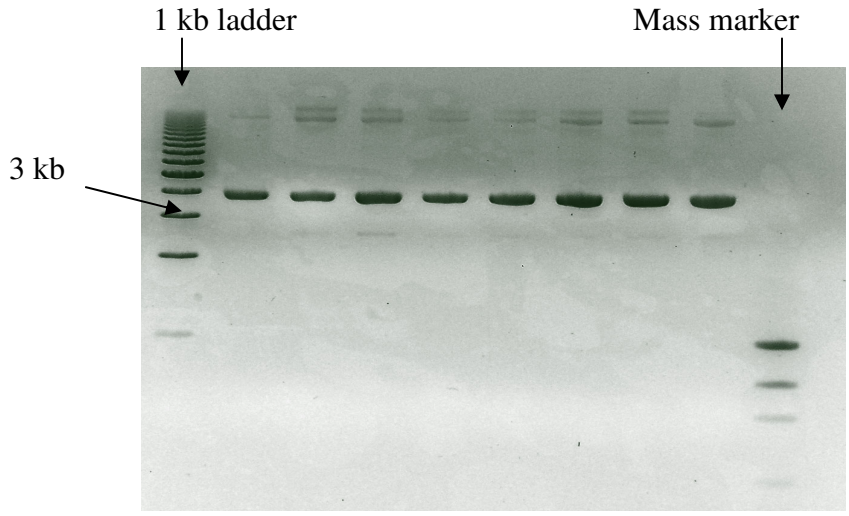

Figure 2-7 Isolated recombinant plasmid samples from NovaBlue GigaSingles cells on a 1% agarose gel. The concentrations of the plasmids were determined using the mass marker in the last lane.

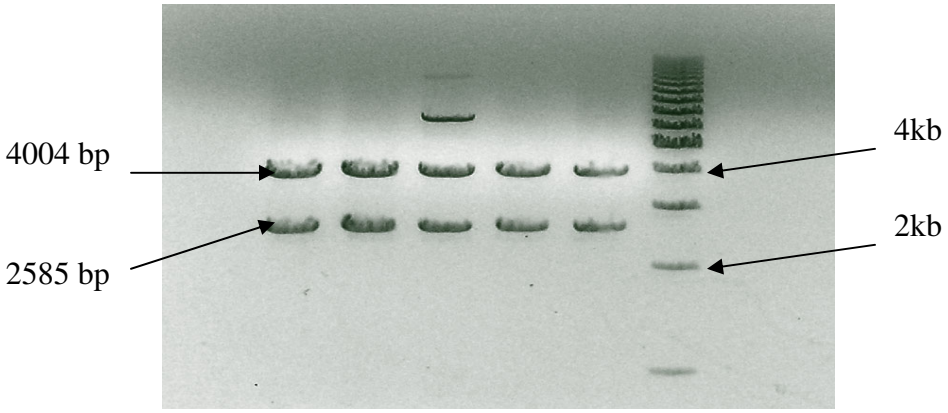

Figure 2-8 Verification of the presence of the insert in the vector on a 0.9% agarose gel stained with ethidium bromide. Last lane indicates the 1 kb size marker.

The restriction enzyme digestion of almost all the isolated plasmid samples gave two different bands with the expected sizes except in the 3rd lane where an extra band with a size ~6500 bp is also present. This extra band could be the linearlized plasmid

with the insert (6589bp) which is due to a single cut by the AccI restriction enzyme. The DNA sequencing results of the construct melA pET-46 EK/LIC matched the known DNA sequence for the *melA* gene from the NCBI database (GeneID: 948636).

In the expression of the α-glactosidase using the *E. coli* BL21 Star DE3 cells, the temperature of the growing culture should be reduced to 25°C from 37°C after addition of IPTG in order to get the protein in the soluble fraction. Temperatures above 30°C tend to produce the protein in large scale but the protein ends up in the inclusion bodies. Addition of sugar to the growing culture does not seem to affect the protein production as expected and therefore all cultures were grown without any sugar in the medium. According to literature adding sugar to the bacterial culture may help to repress basal expression of T7 RNA polymerase and stabilize the pET product.

The molecular weight of the α-galactosidase protein fused with the His tag is about 53.6 kDa. The following chromatogram shows the protein purification using a nickel(II) column (Figure 2-9). In each chromatogram the relationship between absorbance (280 nm - blue line) and the time in minutes is shown. The top horizontal line shows the collected fraction numbers of the eluted proteins and the black line indicates the concentration of imidazole while red and purple lines indicates the conductivity and the pH values, respectively.

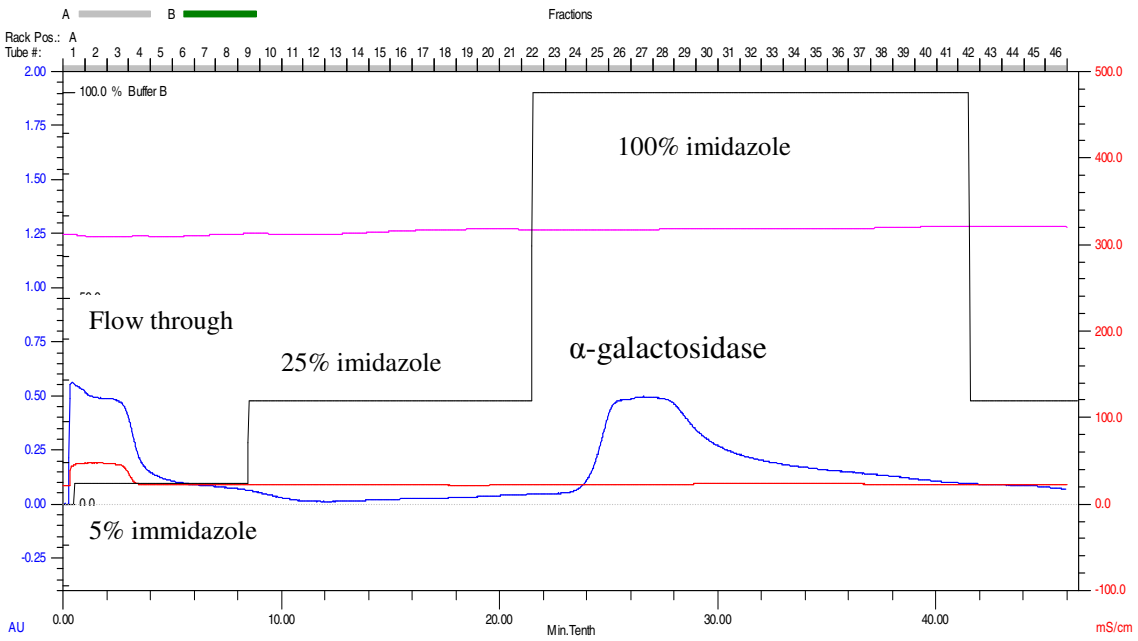

Figure 2-9. Chromatogram obtained for the purification of α-galactosidase using nickel(II) HisTrap™ HP column chromatography

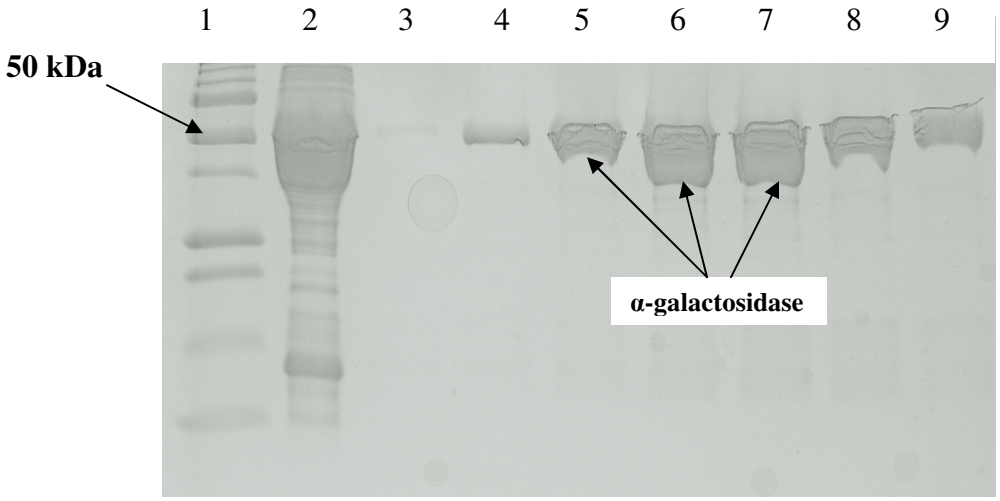

Figure 2-10. A 15% SDS PAGE gel obtained after the nickel(II) column purification of the α-galactosidase protein. The 1st lane shows the protein standards. The 2nd lane shows the components of the flow through and lanes 3-9 shows the α-galactosidase corresponding to the labeled peak in the chromatogram. Due to over loading of the column some of the α-galactosidase were eluted with the flow through.

The chromatogram obtained from the nickel(II) column chromatography indicates two major peaks: one arising until about 10 minutes into the purification and the second from 25–35 minutes (Figure 2-8). The peak around 10 minutes indicates the non-specifically bound proteins to the Nickel(II) column that elutes at a low imidazole concentration. The second peak indicates the α-galactosidase protein which would only elute at a very high imidazole concentration due to the specific interactions between the nickel(II) ions and the poly-histidine tags of the protein.

The 15% SDS PAGE gel with the precision plus protein standards (Bio Rad) of the purified α-galactosidase verified the size of the protein and the level of purification. (Figure 2-10)

The fractions containing the purified α-galactosidase protein were collected and desalted with a desalting column (Figures 2-11 and 2-12). The protein analysis through MALDI-TOF mass spectroscopy confirmed that the desired protein was obtained (Figure 2-13).

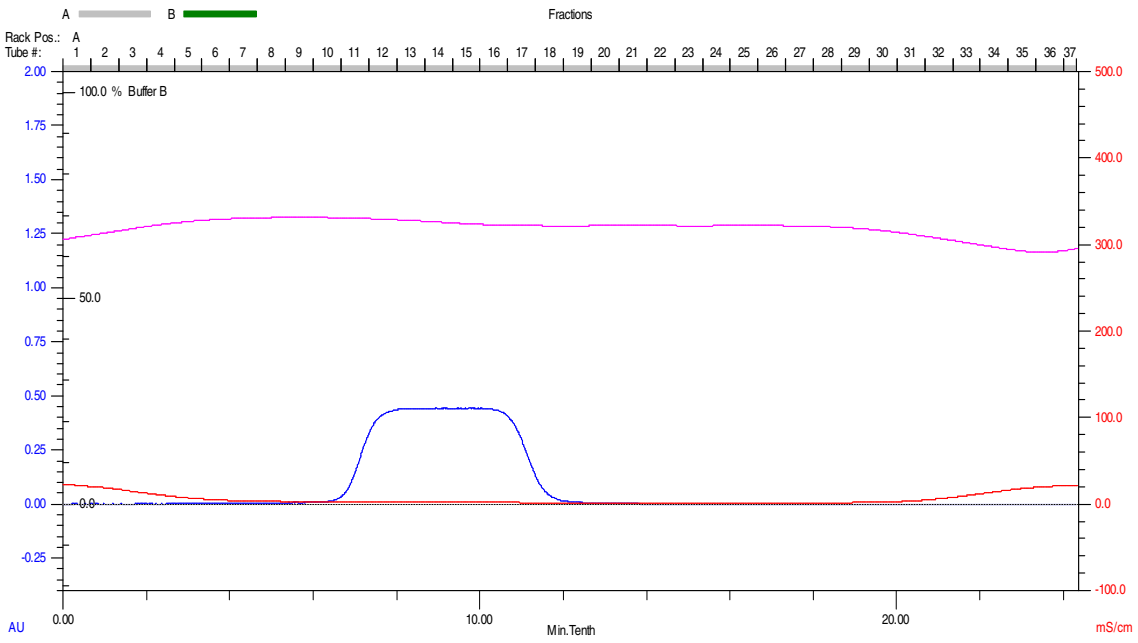

Figure 2-11. Chromatogram of the α-galactosidase obtained after desalting using HiPrep 26/10 Desalting (GE Healthcare) column.

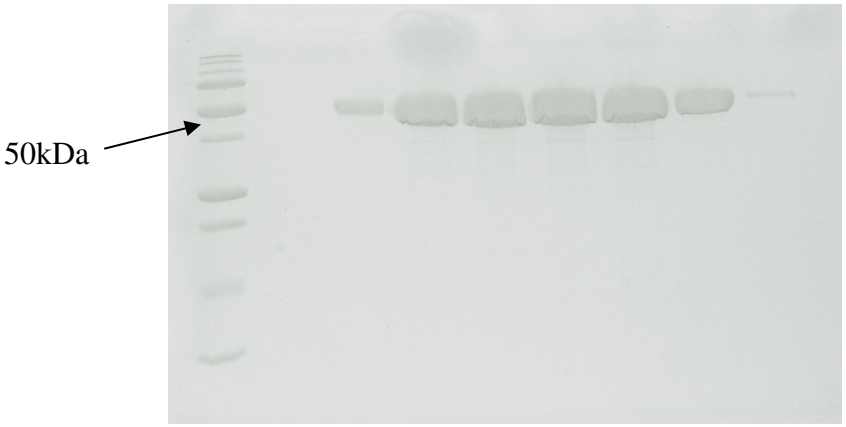

Figure 2-12. A 15% SDS PAGE gel obtained after desalting the α-galactosidase using desalting column.

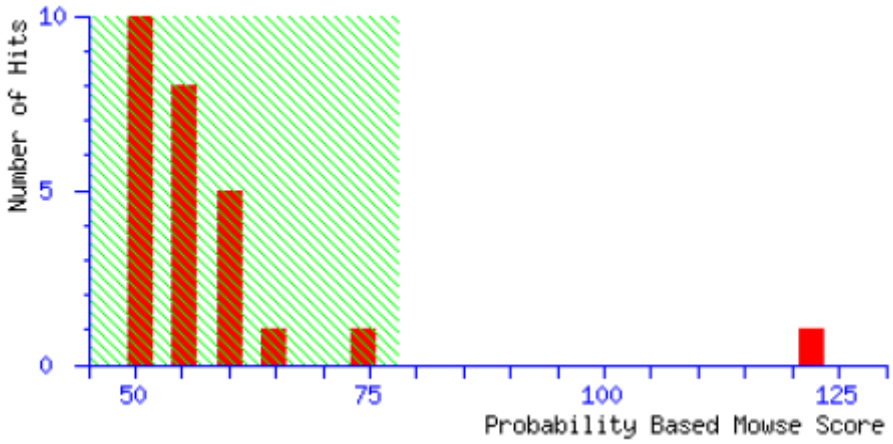

Figure 2-13. Result from the MALDI-TOF mass spectroscopic analysis of the recombinant α-galactosidase. The top score was 104 for *Escherichia coli* α-galactosidase for. Protein scores greater than 64 are significant (p<0.05).

The yield of the purified protein is measured by the Bradford assay, and from 500 ml of LB/ampicillin media, ~30 mg of α-galactosidase was produced. When the purified α-galactosidase was stored in 20 mM Tris HCl pH 7, the protein started degrading after 2 weeks at 4°C and the protein did not respond to the activity assay. The freshly purified protein samples treated with NAD^+, Mn^{2+} and DTT were stable and active for more than eight weeks. However the activity of the protein sample reduced after eight weeks of storage in 4°C.

In the crystal screening process using the Hampton Research crystal screening kits, a few small crystals of α-galactosidase were observed using two different crystallizing conditions at room temperature within 6-7 days. One of the crystallizing conditions was a well solution containing 0.01 M Iron(III) chloride hexahydrate, 0.1 M sodium citrate tribasic dehydrate (pH 5.6), 10% v/v Jeffamine M-600 (No.18, Crystal Screen 2, HR2-112) and the other is 0.1 M HEPES (pH 7.5), 20% v/v Jeffamine M-600 (No. 31, Crystal

Screen 2, HR2-112). Microseeding of the crystal produced droplets were done using a cat whisker but did not produce any results. None of the obtained small crystals diffracted X-rays to medium or high resolution and no data were collected to find the structure or the space group.

One of the major problems encountered with the crystal screening process was the instant precipitate formation on the droplet. In some of the cases this was due to the presence of Mn^{2+} as a cofactor in the protein solution that would lead to precipitate when combined with solutions from the crystal screens. A higher concentration of the α-galactosidase (9.2 mg/ml in 20 mM Tris HCl pH 8) seems to precipitate the protein instantly as well.

CHAPTER III

CONCLUSION

α-Galactosidase is reported to occur widely in animals, plants and micro-organisms[6] and has shown various potential industrial and medicinal applications. X-ray crystal structure of the glycosylated, human α-galactosidase A, which in the defective form causes Fabry disease, has already determined[64] and structural anomalies are well studied.[65,66] Even though the eukaryotic α-galactosidase structures are well studied, the information about prokaryotic α-galactosidases are seldom available. These facts led us to investigate α-galactosidase structure from an abundant micro-organism *Escherichia coli*.

In the research α-galactosidase producing gene (*melA*) from *E. coli* (~1356 bp) was successfully cloned onto a pET-46 EK/LIC vector (Novagen). This His tag bearing Ligation independent cloning vector of Novagen was extremely efficient in the cloning process and the resulting was nearly ~100%. The protein was over expressed using BL21 Star DE3 (Invitrogen) cell line which utilizes a highly efficient T7 promoter based expression mechanism to over produce the targeted protein from the recombinant molecule. The α-galactosidase was purified using metal ion affinity chromatography which utilizes nickel(II) containing His Trap columns. The initial crystallization trials were carried out using the purified α-galactosidase.

However, well grown, single crystals that would diffract X-ray were not obtained during this research. In the future, crystallization procedures of α-galactosidase could be tried using different desalting buffers in different pHs, with varying protein concentrations, micro-seeding of the precipitates in droplets, varying the pH of the salt or the precipitant and optimizing the cofactor requirements in order to obtain a well stabilized active α-galactosidase that might enhance the crystal formation. Another future consideration would be to study the enzyme kinetics of purified α-galactosidase of *E.coli*, with the cofactors.

Both structural and enzyme kinetic studies on α-galactosidase will help in the better understanding of the enzyme's biological role and mechanism that would lead to future potential applications.

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APPENDICES

1. DNA sequence of *E. coli* K-12 substr. MG1655 *melA* gene

ATGATGTCTGCACCCAAAATTACATTTATCGGCGCTGGTTCGACGATTTTCGTTAAAAATATTCTTGGTGATGTGTTCCA
TCGCGAGGCGCTGAAAACGGCGCATATTGCCCTGATGGACATTGACCCCACCCGCCTGGAAGAGTCGCATATTGTGGTGC
GTAAGCTGATGGATTCAGCAGGGGCCAGCGGCAAAATCACCTGCCACACCCAACAGAAAGAAGCCTTAGAGGATGCCGAT
TTTGTCGTGGTGGCATTTCAGATTGGCGGTTATGAACCTTGCACGGTGACTGATTTCGAGGTCTGTAAGCGGCATGGTCT
GGAACAAACCATTGCCGATACGTTGGGGCCGGGCGGTATTATGCGCGCGCTACGTACCATTCCGCATCTGTGGCAAATTT
GCGAGGACATGACGGAAGTCTGCCCCGATGCCACCATGCTCAACTATGTTAACCCAATGGCGATGAATACCTGGGCGATG
TATGCCCGCTATCCGCATATCAAACAGGTCGGGCTGTGCCATTCGGTGCAGGGAACGGCGGAAGAGTTGGCGCGTGACCT
CAATATCGACCCAGCTACGCTGCGTTACCGTTGCGCAGGTATCAACCATATGGCGTTTTACCTGGAGCTGGAGCGCAAAA
CCGCCGACGGCAGTTATGTGAATCTCTACCCGGAACTGCTGGCGGCTTATGAAGCAGGGCAGGCACCGAAGCCGAATATT
CATGGCAATACTCGCTGCCAGAATATTGTGCGCTACGAAATGTTCAAAAAGCTGGGCTATTTCGTCACGGAATCGTCAGA
ACATTTTGCTGAGTACACACCGTGGTTTATTAAGCCAGGTCGTGAGGATTTGATTGAGCGTTATAAAGTACCGCTGGATG
AGTACCCGAAACGCTGCGTCGAGCAGCTGGCGAACTGGCATAAAGAGCTGGAGGAGTATAAAAAAGCCTCCCGGATTGAT
ATTAAACCGTCACGGGAATATGCCAGCACAATCATGAACGCTATCTGGACTGGCGAGCCGAGTGTGATTTACGGCAACGT
CCGTAACGATGGTTTGATTGATAACCTGCCACAAGGATGTTGCGTGGAAGTAGCCTGTCTGGTTGATGCTAATGGCATTC
AGCCGACCAAAGTCGGTACGCTACCTTCGCATCTGGCCGCCCTGATGCAAACCAACATCAACGTACAGACGCTGCTGACC
GAAGCTATTCTTACGGAAAATCGCGACCGTGTTTACCACGCCGCGATGATGGACCCGCATACTGCCGCCGTGCTGGGCAT
TGACGAAATATATGCTCTTGTTGACGACCTGATTGCCGCCCACGGCGACTGGCTGCCAGGCTGGTTGCACCGTTAA

Forward primer
Reverse primer with stop codon in blue

2. Amino acid sequence of α-galactosidase from *E.coli*

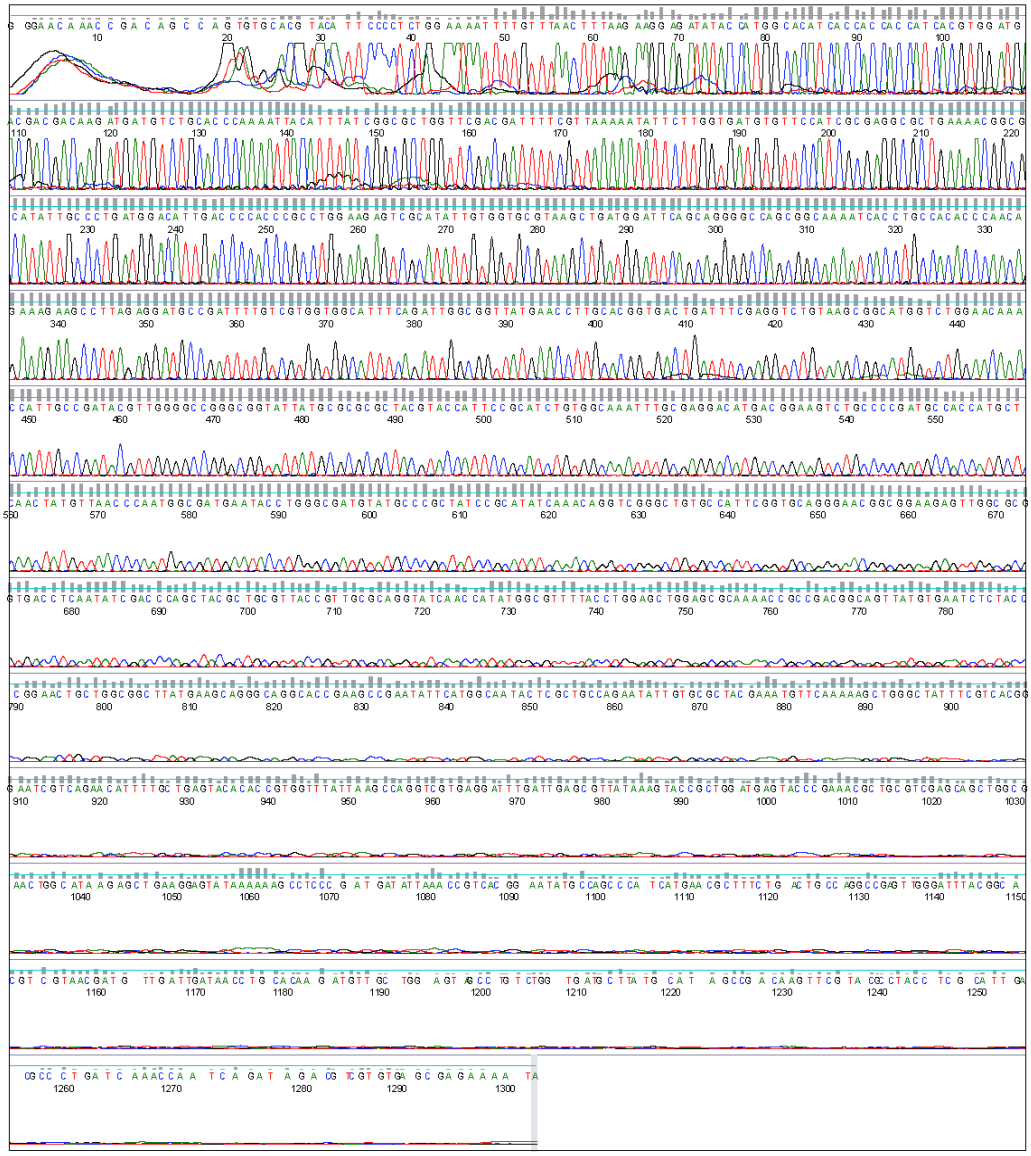
MMSAPKITFI GAGSTIFVKN ILGDVFHREA LKTAHIALMD IDPTRLEESH 50
IVVRKLMDSA GASGKITCHT QQKEALEDAD FVVVAFQIGG YEPCTVTDFE 100
VCKRHGLEQT IADTLGPGGI MRALRTIPHL WQICEDMTEV CPDATMLNYV 150
NPMAMNTWAM YARYPHIKQV GLCHSVQGTA EELARDLNID PATLRYRCAG 200
INHMAFYLEL ERKTADGSYV NLYPELLAAY EAGQAPKPNI HGNTRCQNIV 250
RYEMFKKLGY FVTESSEHFA EYTPWFIKPG REDLIERYKV PLDEYPKRCV 300
EQLANWHKEL EEYKKASRID IKPSREYAST IMNAIWTGEP SVIYGNVRND 350
GLIDNLPQGC CVEVACLVDA NGIQPTKVGT LPSHLAALMQ TNINVQTLLT 400
EAILTENRDR VYHAAMMDPH TAAVLGIDEI YALVDDLIAA HGDWLPGWLH 450
R 451

File: F11_melA-T7Pro.ab1

Geospiza
www.geospiza.com

Sample Name: melA-T7Pro
Mobility: KB_3730_POP7_BDTv1.mob
Spacing: 16.0933
Comment: Radika

Signal Strengths: A = 825, C = 546, G = 1108, T = 800
Lane/Cap#: 43
Matrix: n/a
Direction: Native

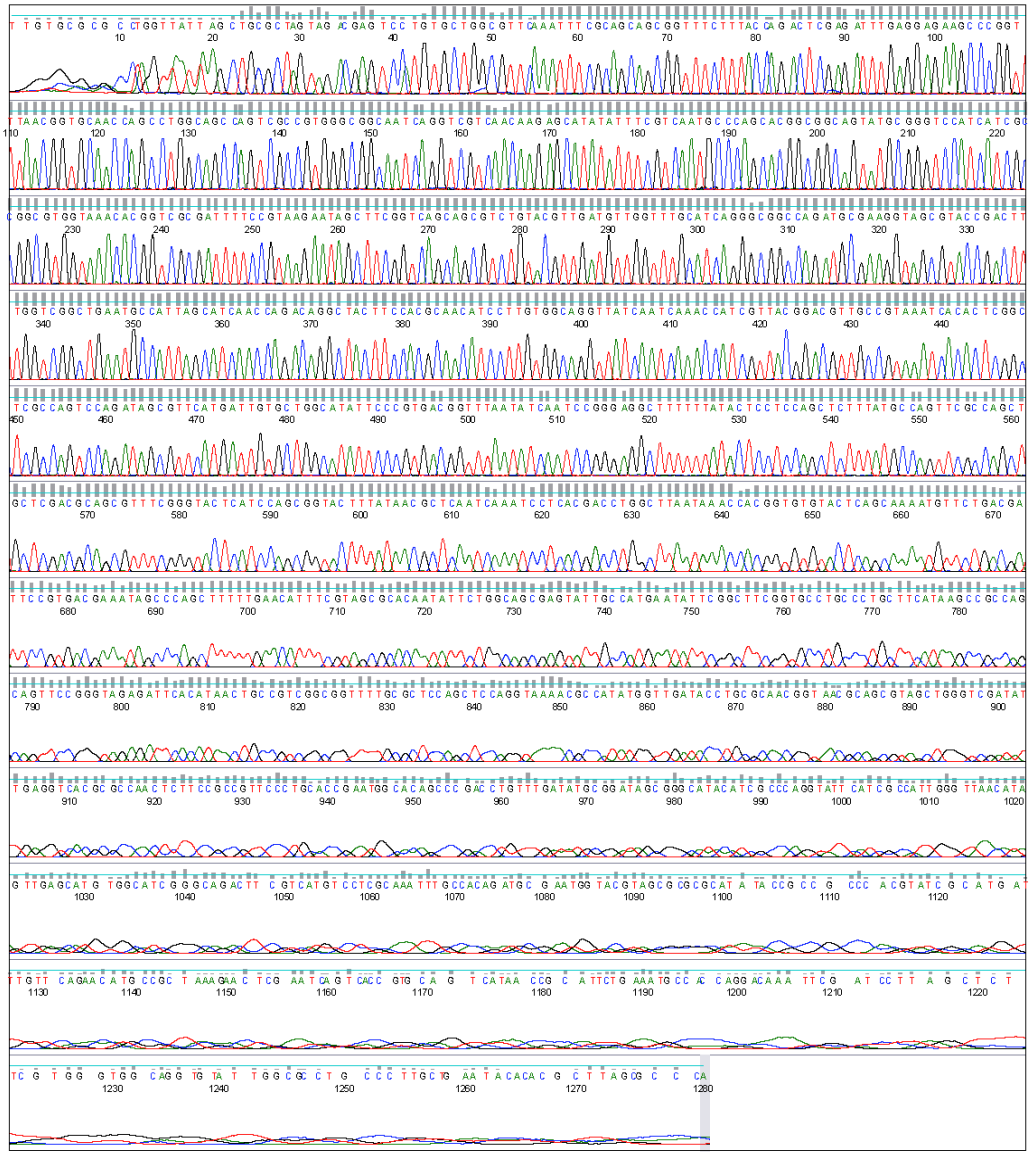


File: G01_melA-T7Term.ab1

Geospiza
www.geospiza.com

Sample Name: melA-T7Term
Mobility: KB_3730_POP7_BDTv1.mob
Spacing: 16.2249
Comment: Radika

Signal Strengths: A = 2384, C = 1899, G = 3333, T = 2605
Lane/Cap#: 2
Matrix: n/a
Direction: Native



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VITA

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Candidate for the Degree of

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Major Field: Chemistry

Scope and Method of Study: In *Escherichia coli,* the 451-residue α-galactosidase protein is produced by the gene *melA,* which is located on the chromosomal DNA. This protein is responsible for the melibiose hydrolysis in *E. coli*; whereas, in eukaryotes the gene product is involved in various other functions. The goal of this project is to express, purify and find the crystal structure of α-galactosidase from *E. coli* and compare the prokaryotic protein structure with known structures of eukaryotic originated α-galactosidases.

Findings and Conclusions: The *E. coli melA* gene has been cloned into a pET-46 EK/LIC plasmid vector that contains a 6-residue *N*-terminal histidine tag and transformed for over-expression in a BL21* DE3 *E. coli* system. The protein is isolated and purified using a HisTrap HP-Ni column and concentrated. The purified protein has been subjected to initial crystallization trials and will be used to discover its enzymatic function.

ADVISER'S APPROVAL: Dr.Stacy D. Benson