Intracellular Expression of the H15S Hen Egg White Lysozyme Mutant and the Generation of the Extracellular H15S Mutant and Transformation into *Pichia pastoris*

By

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Thesis Abstract

Systems utilizing transition metal ions such as iron and copper are responsible for the production of free radicals such as hydroxyl ions via the Fenton reaction. Free radicals and the resulting oxidative damage have been shown to be involved in over 100 human diseases. This thesis is about the investigation of the site specificity of metal catalyzed oxidation of the H15S site directed mutant of hen egg white lysozyme. Histidine at position 15 was converted to serine by site directed mutagenesis, the mutant was then cloned into pCR®4 - TOPO®. Top 10 E. *coli* was then tansformed with the H15S/pCR®4 - TOPO® plasmid. The mutant gene was then ligated into the yeast expression vector, pPICZ B, designed for intracellular expression, and pPICZα A designed for the extracellular expression of protein in the methylotropic yeast, *Pichia pastoris*.

The intracellular expression of protein was carried with three different types of media. Large scale expression was carried out in BMMH media, as this media gave the best evidence for the small scale expression of the H15S mutant lysozyme. Faint bands were seen for BMMH with SDS – PAGE, but no protein was detected with an enzyme assay or the ELISA.

An extracellular mutant was generated using the PCR overlap extension method. The mutant was cloned into the pCR®4 - TOPO® vector. Sequencing showed the successful generation of an H15S lysozyme mutant capable of extracellular expression in a pPICZα plasmid. The H15S insert was digested out of pCR®4 - TOPO® and ligated into the pPICZα A vector. The pPICZα A was linearized and transformed into X – 33 competent cells.

Future work requires the expression of mg quantities of the mutant protein. This will be followed by metal catalyzed oxidation experiments. Finally the oxidation pattern of the H15S mutant will be compared to that of the native lysozyme.

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List of Symbols and Abbreviations

α	Alpha
β	Beta
γ	Gamma
ε	Epsilon
μ	Micro
μg	Microgram
μl	Microliter
μM	Micromolar
®	Registered
TM	Trademark
%	Percent
Abs	Absorbance
bp	Base pair
BSA	Bovine serum albumin
Cu	Copper
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
Fe	Iron
hr	Hour
His	Histidine
LDL	Low density lipoprotein
Lys	Lysine
kb	Kilobase
kDa	Kilodalton
LB	Luria – Bertini
M	Molar
MCO	Metal catalyzed oxidation
Met	Methionine
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MS	Mass spectrometry
MW	Molecular weight
ng	Nanogram
O_2	Oxygen
PCR	Polymerase chain reaction
pH	Hydrogen ion activity
Rpm	Revolution per minute
s	Second
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulfate

SDS - PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
Tris	Trizma buffer
UV	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume
Zn	Zinc

CHAPTER 1: INTRODUCTION

Reactive Oxygen Species

Reactive oxygen species (ROS) are the molecules or ions formed by the incomplete reduction of oxygen. Reactive oxygen species are very small molecules and are reactive with adjacent molecules due to the presence of unpaired valence shell electrons. Reactive oxygen species include the superoxide anion radical ($^{\bullet}O_2^-$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^{\bullet}$). In cells the major types of endogenous ROS are hydrogen peroxide and the superoxide anion, which are generated as byproducts of cellular metabolism such as mitochondrial respiration.[1]

Reactive oxygen species are produced by a wide variety of different processes in biological system. They range from physiological pathways (endogenous) to environmental factors (exogenous). Physiological pathways include a range of enzymes (e.g. nitric oxide synthases, NADPH oxidases), electron transport chains of mitochondria, and the cytochrome P_{450} system. Exogenous agents include X – ray, gamma, UV, or visible radiation in the presence of sensitizer, metal ions, and atmospheric pollutants like asbestos. The primary source of ROS in the cell is by the reaction between transition metal ions, i.e. copper, and iron with internally produced H_2O_2 (eq 1).

(1) $$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^{\bullet} + OH^-$$

Free Radicals and Reactive Oxygen Species

Free radicals play an important role in both chemistry and biology, and the past twenty years has seen an explosion of interest in free radical chemistry. A free radical is

a species with one unpaired electron and it is chemically quite reactive as it looks for another electron to pair up with to satisfy the octet rule.

Mitochondria, which consume more than 90% of the oxygen in aerobic living organisms, are the main source of reactive oxygen species and free radicals. Oxygen, because of its bi - radical nature, readily accepts the electrons and gets reduced to water. This process of reduction takes place by 4 sequential steps (eq 2).

(2) $O_2 \xrightarrow{e^-} {}^{\bullet}O_2^- \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^-} OH^{\bullet} + OH^- \xrightarrow{e^- + H^+} H_2O$

Superoxide anion — Hydrogen peroxide — Hydroxyl radical

In this sequential, univalent process, several reactive intermediates such as perhydroxyl radical ($HO_2^{\bullet}$) or its ionized form, superoxide anion (${}^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and the extremely reactive hydroxyl radical ($OH^{\bullet}$), are formed.[1]

In the electron transport chain of mitochondria O_2 is reduced by four electrons to H_2O without releasing either ${}^{\bullet}O_2^-$ or H_2O_2. The reduction of O_2 to ${}^{\bullet}O_2^-$ is restricted by cytochrome oxidase, however, ${}^{\bullet}O_2^-$ is produced in living cells invariably due to leakage of single electrons at specific sites of the mitochondrial transport chain. When the electron transport chain is highly reduced, and the respiratory rate is dependent on ADP availability; 'leakage' of electrons at ubiquinone and semiubiquinone sites increases resulting in the production of ${}^{\bullet}O_2^-$ or H_2O_2.[1]

Flavoproteins and peroxisomal oxidases as well as L-hydroxy acid oxidase, D-amino acid oxidase, and fatty acyl oxidase participate in the production of ${}^{\bullet}O_2^-$ or H_2O_2. Cytochrome P-450, P-450 reductase and cytochrome *b*-5 reductase of endoplamic reticulum generates ${}^{\bullet}O_2^-$ or H_2O_2 during their catalytic cycles under certain conditions.

During ischemia, xanthine oxidase is produced from the proteolytic cleavage of xanthine dehydrogenase. On reperfusion, xanthine oxidase, in the presence of O_2 acts on xanthine or hypoxanthine to produce $^{\bullet}O_2^-$ or H_2O_2.[1]

Sources of Free Radicals

Endogenous free radicals are formed within the cell and act intracellularly. They are generated from the autooxidation and consequent inactivation of small molecules such as reduced flavins and thiols and from the action of certain oxidases, peroxidases cyclooxygenases, lipooxygenases, and dehydrogenases. Electron transfer from transition metals such as iron to oxygen containing molecules can initiate free radical reactions. Exogenous sources of free radicals include tobacco smoke, certain pollutants and organic solvents, anesthetics, hyperoxic environments and pesticides.[2]

In vivo generation of $OH^{\bullet}$ requires the presence of trace amounts of transition metals ions like iron and copper. In the presence of Fe^{2+} metal ions, H_2O_2 produces the extremely reactive $OH^{\bullet}$ radical in a process known as the Fenton reaction[1] (eq 3).

(3) $$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

$OH^{\bullet}$ is extremely reactive and it attacks and damages every molecule in its vicinity. It has a very short half life and reacts with molecules at essentially diffusion controlled rates. The extent of damage caused by $^{\bullet}O_2^-$ and H_2O_2 increases in the presence of the transition metal ions due to the generation of the more powerful $OH^{\bullet}$. This is known as the metal catalyzed Haber–Weiss reaction.[1] (eq 4).

$$Fe^{3+} + {}^{\bullet}O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

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(4) $$^{\bullet}O_2^- + H_2O_2 \rightarrow OH^- + OH^{\bullet} + O_2$$

Effect of Free Radicals on Proteins

Proteins are a major target for oxidants, as a result of their abundance in biological systems and their high rate constants of reaction. All amino acid residues of a protein are subject to attack by oxygen radicals produced during radiolysis. Tyrosine, phenylalanine, tryptophan, histidine, methionine and cysteine residues are the preferred targets. Exposure of the proteins to radiolysis in the absence of oxygen leads to appreciable protein aggregation due to $OH^{\bullet}$ facilitated tyrosine-tyrosine and –S-S- cross - linking reactions. In the presence of O_2, radiolysis leads to appreciable fragmentation of the polypeptide chain via a peroxyl radical mediated α – amidation mechanism. In contrast, only one or at most only a few amino acid residues in a protein are modified by metal catalyzed oxidation systems (MCO).[3]

The rate constants for the reaction of a range of reactive radicals, with biological macromolecules tells us the potential importance of proteins as a targets for oxidants. The rate constant for the reaction of ($HO^{\bullet}$) radical with macromolecules varies over a relatively small range. The rate constant for the reaction of $HO^{\bullet}$ with DNA is 8×10^8 $dm^3mol^{-1}s^{-1}$, with RNA is 1×10^9 $dm^3mol^{-1}s^{-1}$, and with collagen and albumin is 4×10^{11} $dm^3mol^{-1}s^{-1}$and 8×10^8 $dm^3mol^{-1}s^{-1}$ respectively. In the main, the overall rate of reaction will be driven by the concentration of the target. Rate constants of the target are in milli molar concentration and that of antioxidants in micromolar concentrations. This difference underscores the difficulty in designing antioxidant stratagies.[2]

There is large variation in the selectivity of damage between different oxidants. The most reactive radicals tend to be the least selective. The difference in variation in the rate constants for the reaction of $HO^{\bullet}$ with different side chains is relatively small. As a

result all the side chain sites are oxidized to a greater or lesser extent, though the aromatic and sulfur containing side chains would be expected to be depleted most rapidly. Less reactive oxidants are much more selective for the site that they oxidize, and the rate constants for the reaction of substituted peroxyl radical $CCl_3OO^{\bullet}$ with amino acid side - chains vary from ca. 9×10^7 $dm^3mol^{-1}s^{-1}$ for trytophan residues to below the measurable detection limit.[2]

Site Specific Oxidation

Recent studies have shown that metals such as copper, iron, cadmium, chromium, lead, mercury, nickel, and vanadium exhibit the ability to produce reactive oxygen species. These metal ions, as a result, cause protein oxidation, lipid peroxidation, DNA damage, depletion of sulfhydryls and altered calcium homeostasis. The two most commonly studied transition metal ions are copper and iron and their catalytic role in free radical reactions was implicated in the late 1970's. These metal ions are insoluble under physiological conditions and remain in solution only by becoming complexed to low and high molecular weight cellular components and serve as catalytic centers for free radical production. The metal ions that are bound to cellular components can undergo cyclic reduction and reoxidation.[4]

Iron makes stable complexes with nucleotide diphosphates and triphosphates; it must be in free form or a catalytically active form to facilitate the formation of reactive oxygen species via the Fenton reaction. An adult human being contains about 4 grams of iron. About two thirds of it is present in hemoglobin and a further ten percent in myoglobin and a very small amount of iron is either oxidized or stored in the iron storage protein ferritin, or associates with the iron transport protein transferrin. A variety of

xenobiotics have been shown to facilitate the release of iron from ferritin, including paraquat, diquat, nitrofurantoin, adriamycin, daunomycin, and diaziquone.[4]

The small pool of non-protein-bound iron moving between transferrin, cell cytoplasm, mitochondria and ferritin could provide iron for the Fenton reaction. H_2O_2 and iron salts are available *in vivo* and if they come into contact, the Fenton reaction will occur. Iron produces reactive oxygen species that causes lipid peroxidation by catalyzing the reaction between oxygen and biological macromolecules. Iron also produces reactive oxygen species by complexing with adenosine 5'-diphosphate, histidine, ethylenediaminetertaacetic acid and other chelators to facilitate the formation of reactive oxygen species and enhancing the production of lipid peroxidation.[4]

Copper is an essential trace element, widely distributed in nature and forms stable complexes with many proteins. Copper is a common cofactor for many enzymes including oxidases and oxygenases. Similar to iron, copper acts as a catalyst in the formation of reactive oxygen species and catalyzes the peroxidation of lipid membranes. In addition to generation of reactive oxygen species, Cu manifests its toxicity by displacing other metal cofactors from their natural ligands. Copper (I) salts react with H_2O_2 to make $^{\bullet}OH$ with a much greater rate constant than do Fe (II) salts. Copper was shown to accelerate the oxidation of hydroquinone to benzoquinone in a concentration – dependant manner, and contributed to the cyotoxicity of hydroquinone. Copper also enhanced DNA strand breaks in the presence of hydroquinone.[5]

An adult human body contains about 80mg of copper, and it complexes with amino acids, such as histidine, or small peptides. These copper complexes enter the blood and most of the copper binds tightly to the serum albumin with the help of three

amino acids at its N – terminus (including a histidine in the third position) and in equilibrium with a small pool of copper complexes. In liver about 95% of copper is incorporated in ceruloplasmin, a glycoprotein, with the rest being attached to albumin which is then released into circulation. Cu is delivered to specific molecules by forming complexes with small cytosolic proteins known as copper chaperone proteins.[5]

Metal Catalyzed Oxidation

Chemical reaction mixtures utilizing O_2 or H_2O_2, and often some type of reducing agent are capable of producing $OH^{\bullet}$ and are refered to as metal catalyzed oxidation (MCO) systems. Metal catalyzed oxidation systems are needed to reduce iron, copper and other metals and to generate H_2O_2. Oxidation reactions are significantly affected by protein sequence and structure and additional complexities are introduced when oxidation reactions are catalyzed by transition metals.[6]

Enzymic MCO systems that have been shown to catalyze the oxidative modification of proteins are widely distributed in nature and include NADPH and NADPH oxidases, systems comprised of NAD(P)H, cytochrome P-450, and cytochrome P-450 reducatse; xanthine oxidase and either hypoxanthine or acetaldehyde; horseradish peroxidase, H_2O_2 and ferredoxin; glucose, glucose oxidase, and ferredoxin; nicotinate hydroxylase and NADPH. All of these enzymic MCO systems require O_2 as a source of oxidizing equivalents. Non - enzymic MCO system that catalyses the inactivation of enzymes are systems comprised of ascorbate, O_2 , Fe(III) or Fe(II)and sulfhydryl compound such as GSH, dithiothreitol or β – mercaptoethanol.[7]

The rate of inactivation of enzymes by a MCO system is greatly enhanced by the presence of catalytic levels of one electron carrier's such as menadione or proteins having

nonheme iron sulfur centers. As metal catalyzed oxidation of proteins leads, in some cases to the generation of carbonyl derivatives, the level of protein carbonyl groups can be used as a measure of oxygen radical-mediated protein damage under various physiological conditions.[3]

The selective nature of metal ion catalyzed oxidation may be because of the localization of the metal ions at a particular site on the protein at residues such as His, Arg, Pro, Cys, Lys, and Met. A factor influencing the susceptibility of these amino acids residues to metal catalyzed oxidation is their ability to form complexes with metal such as Cu (II) and Fe (III). Thus, during metal catalyzed oxidation the most oxidation-sensitive residues do not necessarily have to be present on the surface of the protein.[6]

Oxidative Damage Caused by Reactive Oxygen Species

The targets of the reactive oxygen species mainly include vital cell components like proteins, polyunsaturated fatty acids and, to a lesser extent, nucleic acids and carbohydrates. The reactions produced by ROS can alter intrinsic membrane properties like fluidity, ion transport, and protein cross-linking. ROS also causes loss of enzyme activity, inhibition of protein synthesis, and DNA damage ultimately resulting in cell death. DNA strand scission, DNA –protein cross links, nucleic acid base modification, protein oxidation, and lipid peroxidation are some of the well known consequences of free radicals *in vivo*.[1]

Oxidative Damage to Proteins

ROS cause oxidative damage to proteins through the production of free radicals, during mitochondrial electron transport chain that lead to structural changes and the loss of enzyme activity that stimulates protein degradation.[1] Oxidation can result in major

physical changes in protein structure ranging from the fragmentation of the backbone to the oxidation of amino acid side chains that result in the formation of further reactive species which include the formation of hydroperoxides or peroxides, chloramines /chloramides, and bromamines/bromamides, and 3, 4 – dihydroxy – L – phenylalanine (DOPA). Enzymes that show an increase in oxidation based on an increase in protein carbonyls during aging are glutamine synthase, mitochondrial aconitase, and adenine nucleotide translocase.[2]

Modification of structural proteins leads to loss of function. When the plasma protein fibrinogen is oxidized, it loses the ability to form a solid clot and the degree of clotting inhibition correlates with the extent of carbonyl formation. Rheumatoid arthritis is due to the aggregation of oxidized synovial fluid immunoglobulins. Oxidation of a methionine residue in α-1 antitrypsin is responsible for the tissue destruction seen in emphysema. Oxidation of LDL plays a significant role in the etiology of atherosclerosis; while oxidation of crystalline proteins in the lens of eye plays a role in cataractogenesis.

The oxidation of the side chains of lysine, proline, arginine and threonine residues have been shown to yield carbonyl derivatives. Carbonyl groups may also be introduced into proteins by the interaction of reducing sugars or their oxidation products with the ε-amino groups of lysine residues, by mechanisms referred to as glycation and glycoxidaion.[9] It is well established that oxidation of protein side chain can give rise to unfolding and conformational changes in proteins that have consequential effects on their biological function. A number of studies have shown that oxidation of surface -exposed residues has less influence on protein conformation than oxidation of buried residues,

though the buried residues are less rapidly oxidized than surface – exposed residues when the initiating oxidant is present in bulk of aqueous phase.[2]

Oxidation of Lipids and Nucleic acids

Lipid peroxidation is initiated by hydroxyl radical ($^{\bullet}OH$), alkoxyl radicals ($RO^{\bullet}$), and peroxy radicals ($ROO^{\bullet}$) that leads to a self – perpetuating process, as the peroxy radicals are both initiators and the products of lipid peroxidation. Lipid peroxy radicals, thereby, enhance reaction by transferring electrons and bringing about oxidation of the substrate. [1]

Among many ROS, $^{\bullet}OH$ generates various products from the DNA bases which mainly include C-8 hydroxylation of guanine to 8-oxo-7, 8 dehydro-2'-deoxyguanosine, a ring-opened product, 2, 6-diamino-4-hydroxy-5-formamimodipyrimidine, 8-OH-adenine, thymine, glycol, cytosine glycol. It has been estimated that the DNA in each cell of our body suffers 10,000 "oxidative hits" per day, leading to the formation of more than twenty different oxidative DNA lesions. Many of these lesions are known to cause mutations. While oxidative damage to DNA is implicated in cancer, oxidative damage to lipids in low-density lipoprotein (LDL, the "bad" cholesterol) plays an important role in atherosclerosis.[1]

Antioxidant Defense against Reactive Oxygen Species

Cellular antioxidant enzymes and free radical scavengers normally protect a cell from the toxic effects of reactive oxygen species. However, when production of reactive oxygen species overtakes the antioxidant defenses of cells, oxidative damage of cellular macromolecules such as proteins, lipids and nucleic acids occurs, leading to various pathological conditions.[1] They include atherosclerosis, adult respiratory distress

syndrome, ischemia-reperfusion injury, atherosclerosis, neurodegenerative diseases, cancer, arthritis, cataractogenesis, and age related diseases such as progeria and Werner's syndrome.[3, 10]

To minimize cellular damage caused by reactive oxygen species, mammalian cells are equipped with enzymatic and non - enzymatic antioxidant defense mechanisms. Antioxidants minimize the generation and counteract the damaging effects of reactive oxygen species produced within the organism from molecular oxygen. These include primary and secondary defense mechanisms. Primary defense mechanisms include enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidases (GSHPx). Glutathione reductase and glucose 6-phosphate dehydrogenase are the enzymes involved in the recycling of oxidized glutathione and forms a mutually supportive team of defenses against reactive oxygen species. Superoxide dismutase (SOD) lowers the steady-state level of $^{\bullet}O_2^-$, catalase and peroxidases lowers the level of H_2O_2[1].

In addition to primary defenses, the secondary defense against ROS includes "scavengers". Scavengers react with radicals to produce another radical compound. Examples include tocopherol, ascorbate, and reduced glutathione. Once the tocopherol radical is formed, it migrates to the membrane's surface and reacts with ascorbate or glutathione (GSH) and is reconverted back into tocopherol. The resulting ascorbate radical undergoes reduction with GSH and reverts back to ascorbate. Glutathione disulfide (GSSG) reduces the radical form of GSH through the glutathione reductase system which in turn functions as a scavenger[1].

Methionine, like cysteine, can function as an antioxidant and as a key component of a system for regulation of cellular metabolism. Methionine is readily oxidized to methionine sulfoxide by reactive oxygen and nitrogen species, but methionine sulfoxide reductase has the potential to reduce the residue back to methionine. Surface exposed methionines thus serve to protect other functional residues from oxidative damage, increasing the scavenging efficiency of the system.[9]

Modification of Proteins by Reactive Oxygen Species

Free amino acids and their residues in proteins are highly susceptible to oxidation by reactive oxygen species. The $^{\bullet}OH$ radical generated in the presence of high, nonphysiological concentrations of Fe (II) and H_2O_2 is similar to ionizing radiation and all residues are susceptible to attack resulting in peptide bond cleavage and protein – protein cross linkage.

Peptide Bond Cleavage

In addition to the hydroxylation of proteins, an alkoxyl radical sets the stage for the cleavage of proteins by either of two mechanisms: The diamide or α – amidation pathway. Cleavage by the diamide pathway leads to two peptides, a C – terminal amino acid residue of the peptide derived from the N – terminal portion exists as a diamide derivative (II), whereas the N – terminal amino acid of the peptide derived from C – terminal portion exists as an isocyanate derivative (III) (Fig 1 – 1)

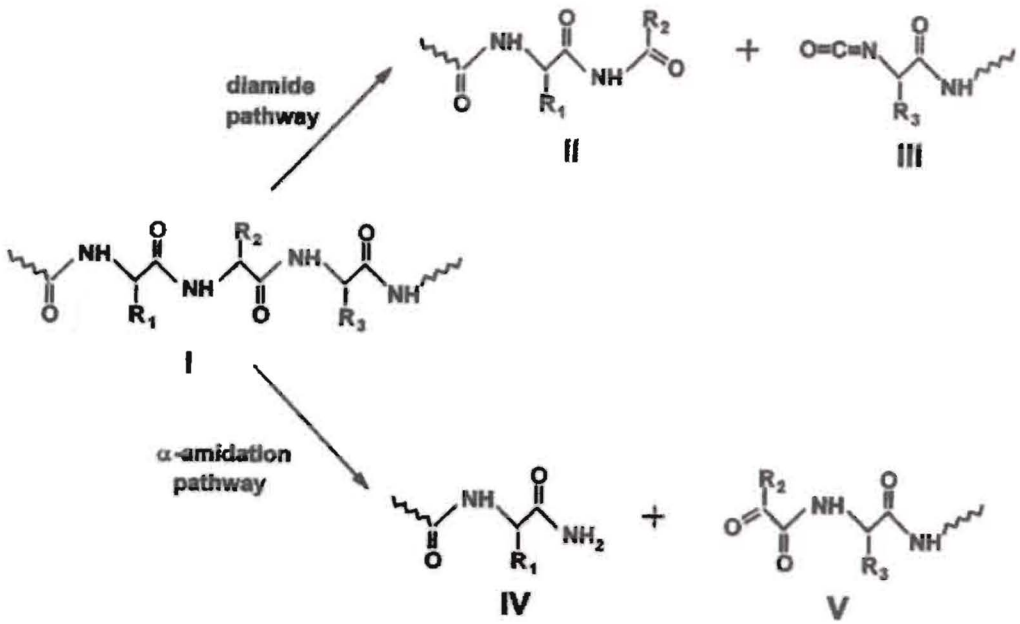

Figure 1 - 1: The α – amidation and diamide pathways

In the α – amidation pathway, the C-terminal amino acid residue of the peptide derived from the N-terminal portion of the protein exists as an amide derivative (IV) and the N – terminal amino acid residue of the peptide derived from C – terminal portion of the protein exists as an N - α - ketoacyl derivative (V). Hydroxyl radical mediated attack of glutamyl and prolyl residues of the protein, also leads to peptide bond cleavage resulting in a mixture of products.[9, 11]

Acid hydrolysis of the fragment obtained by the α – amidation pathway will yield CO_2, NH_3 and a free α – keto carboxylic acid, whereas acid hydrolysis of the peptide fragment obtained by diamide pathway will yield CO_2, NH_3 and a free carboxylic acid.[9, 11] Free radical cleavage of glutamyl and aspartyl residues of proteins leads to peptide bond cleavage. It occurs by hydroxyl radical - initiated attack and forms mixture of products as shown in Figure 1 – 2.

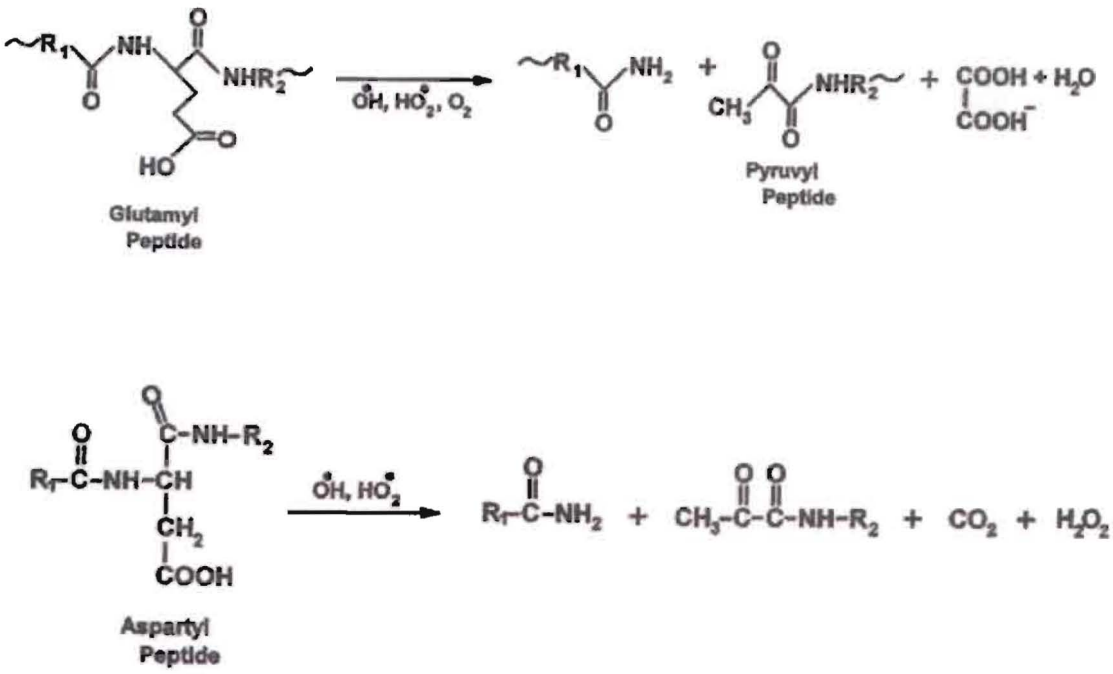

Figure 1 – 2: Peptide bond cleavage of glutamyl and aspartyl residues

Protein Carbonylation

Proteins can be modified by a large number of reactions involving ROS, among them carbonylation is irreversible and unrepairable and has attracted a great deal of attention. Carbonylated proteins undergo proteolysis but escape degradation by forming high molecular weight aggregates that are cytotoxic, and they have been responsible for a large number of age related changes such as Alzheimer's disease, Parkinson's disease, and cancer.

In the case of amino acids such as arginine, proline, lysine and threonine, carbonyl derivatives are formed by direct metal catalyzed oxidation of their side chains. Other methods of introduction of a carbonyl group into the protein is by a Michael addition reaction of 4-hydroxy-2-nonenal, which is a product of lipid peroxidation, with

either the imidazole moiety of histidine, with the ϵ-amino group of lysine, or the sulfhydryl group of cysteine residues. Aminoadipic semialdehyde from lysine and glutamic semialdehyde from arginine and proline are quantitatively the most important products of the carbonylation reaction.[9, 11]

Protein-protein Cross Linkage

$^{\bullet}OH$ radicals, generated by metal catalyzed oxidation or radiolysis of water, can abstract hydrogen atoms from the α-CH(R) - group of the polypeptide backbone forming an alkyl radical. The alkyl radical in turn reacts with oxygen to form an alkylperoxy radical or with another alkyl radical to form inter - or intraprotein cross linkages.[9]

ROS mediated protein - protein cross links can be formed by at least six different mechanisms: 1) by the oxidation of cysteine sulfhydryl groups to form disulfide cross links, 2) by addition of ϵ - NH_2 of lysine of one protein to the carbonyl group of another glycated protein/gloxylated protein, 3) by Michael additions of histidine, lysine, and cysteine side chains to the double bonds of unsaturated aldehydes, of polyunsaturated fatty acids formed during oxidation, 4) the oxidation of tyrosine residues to form Tyr-Tyr cross-links, 5) by addition of lysine amino groups to the carbonyl group of an oxidized protein, and 6) by formation of carbon-carbon covalent linkages by the interaction of carbon centered radicals in two different protein molecules.

Cross linked proteins are often resistant to proteolytic degradation by the proteasome, and have important implications in the accumulation of oxidized proteins as occurs in aging and some diseases. In addition, cross linked proteins are also potent inhibitors of degradation of other oxidatively modified proteins by the proteasome.[12]

Oxidation of Amino acid Residue Side Chains

The 20 different types of amino acid side chains results in a huge variety of potential reaction sites and products on reaction with oxidants. With highly reactive radical such as •OH, reaction occurs at all available sites resulting in the formation of a range of radicals. The majority of radical mediated reactions with aliphatic residues involve the extraction of a hydrogen atom that gives rise to carbon centered radicals. These carbon - centered radicals further reacts in three other ways: 1) by dimerization with other radicals that plays a major role in aggregation, 2) getting repaired by thiols at a slow rate with resulting formation of a thiyl radical, and 3) by reacting with O_2 at a diffusion controlled limit (i.e. k ca. 10^9 dm^3 mol^{-1} s^{-1}) to give a peroxyl radical. The peroxyl radical undergoes radical-radical mediated termination reactions that generate alcohols and carbonyl compounds or alternatively alkoxyl radicals and O_2.[2]

The aromatic amino acid residues are the prime targets of oxidation by various forms of reactive oxygen species. For example 5 - phenylalanine residues when oxidized are converted to *ortho*- and *meta*-tyrosine derivatives.[9]

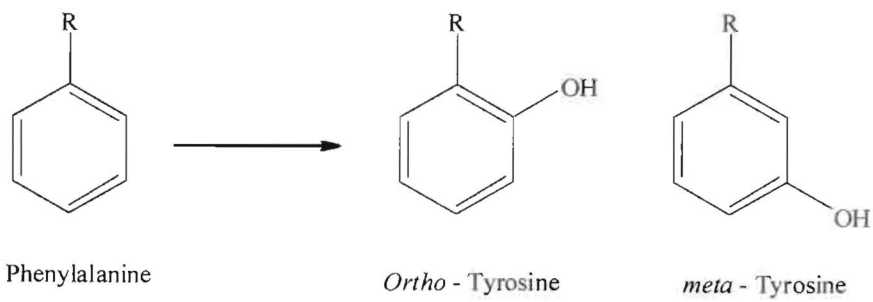

Figure 1 - 2: Oxidation of phenylalanine residue

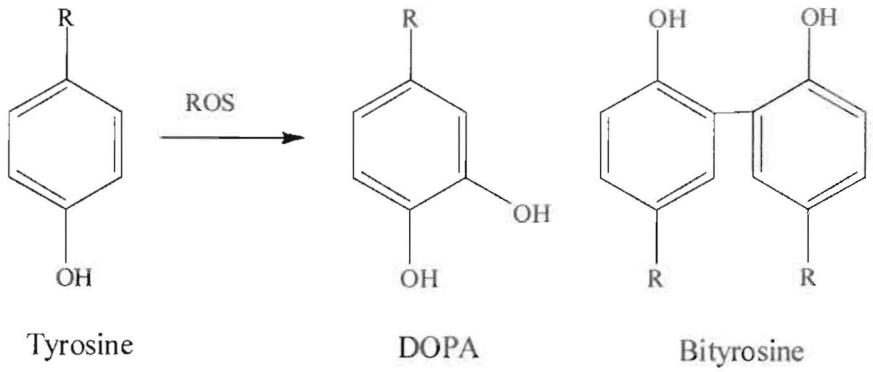

Figure 1 - 3: Oxidation of tyrosine residue

Tyrosine residues are converted to 3, 4-dihydroxy derivatives and to bi-tyrosine cross linked derivatives. This can occur via either direct oxidation of the ring and subsequent deprotonation at the hydroxyl ring or via addition-elimination reactions.

Tryptophan residues are converted to either the 2-, 4-, 5-, 6-, or 7-, hydroxyl derivatives and also to kynurenine and N-formylkynurenine and to various hydroxyl derivatines. Histidine residues are converted to 2 –oxohistidine, asparagine, and aspartic acid residues.[7, 9]

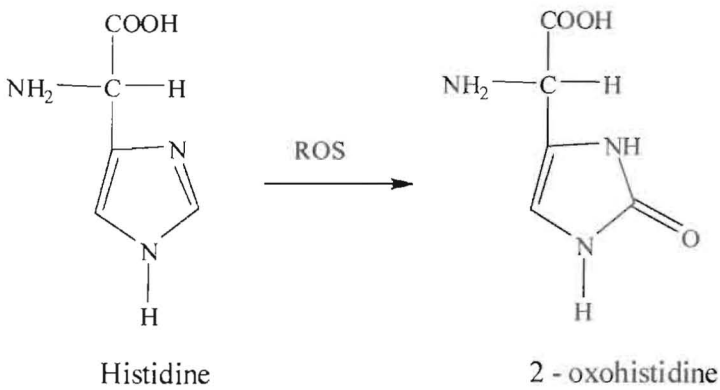

Figure 1 - 4: Oxidation of histidine to 2 – oxohistidine

Accumulation of Oxidized Proteins

The level of intracellular, oxidized proteins reflects the balance between protein oxidation and the degradation of oxidized protein. For example, oxidized forms of some "cross – linked" proteins and proteins modified by glycation/lipid peroxidation are not only resistant to proteolysis but also inhibit the ability of other proteases from degrading the other forms of the oxidized proteins.[9, 11]

The ability of reactive oxygen species to modify proteins depends on the concentration of a large number of enzymatic and nonenzymatic factors, which can either inhibit or facilitate the formation of reactive oxygen species or their conversion to inactive derivatives. For example $^{\bullet}O_2^-$ formed by several pro-oxidant systems is converted into H_2O_2 by superoxide dismutase. The H_2O_2 is then readily degraded by antioxidant enzymes such as catalase, glutathione peroxidase, and other thiol specific antioxidants and peroxidases. If these antioxidants are insufficient, some amount of H_2O_2 undergoes metal catalyzed oxidation to generate even more toxic $^{\bullet}OH$, which depends on the availability of metal ions such as iron and copper. Metal ion chelators can either suppress reactive oxygen species by forming complexes or enhance it through production of radical species.[11]

The level of production of reactive oxygen species also depends on the concentration of vitamins such as A, C and E and on metabolites such as bilirubin and uric acid, that scavenge free radicals either directly or in the form of their metabolites. Divalent cations such as Mg^{2+}, Mn^{2+}, and Zn^{2+}, compete with Fe(II) and Cu(I) for metal

binding sites and prevent the site specific production of $^{\bullet}OH$ and protects from protein damage.[11]

Conformational Changes of Proteins and their Effects

Interaction of proteins with reactive oxygen species results in covalent modifications of amino acid residues and alteration in protein conformation. Protein side chains upon oxidation give rise to conformational changes and unfolding in protein, that has consequential effects on biological function of the proteins. *In vitro* and *in vivo* studies have shown that alteration of protein size occurs only to a limited extent during oxidation. A number of studies have shown that surface exposed residues of proteins have less influence on the protein conformation when compared to the buried residues, though the buried residues are less rapidly oxidized in an aqueous phase.[8] For example, solvent accessible Met residues are more readily oxidized than buried species, which does not appear to have marked effects on the conformation of proteins, unless this exposed residue is in a binding pocket or within the active site of the enzyme.[2]

Oxidation of side chains of amino acids results in an increase in hydrophilicity, Many aliphatic side chains are converted to functional groups such as peroxide, carbonyls and hydroxyls that can participate in hydrogen bonding and have large dipole moments. Aromatic residues also become more polarized upon oxidation with introduction of polar or charged groups such as nitro, bromo, or chloro substituents, which is the driving force for residues to be present on water accessible, external surface.

The methionine side chain is nonpolar and is buried within the hydrophobic regions of the protein structure, but upon oxidation and its conversion to more polar compound have significant effect on conformation. For example, oxidation of one of the

eight methionine residue at position 385 in α1- proteinase inhibitor results in loss of inhibition of elastase activity by this enzyme. According to other studies it was observed that efficiency of binding of calcium to the C-terminal domain of calmodium was decreased by 30 fold with oxidation of Methionine at position 146 or 147.[2]

Research Objective

From studies of Stadtman, Levine and collaborators using glutamine synthase from *E.coli*, metal catalysed oxidation of proteins is a mechanism of post translational modification that binds a cation capable of redox cycling such as Fe^{2+}/ Fe^{3+} to a metal binding site on the protein. Active oxygen species are produced upon the reaction of metal ions with O_2/H_2O_2, which oxidize amino acid residues at or near that cation-binding site. Evidence suggests that these products react in a site-specific manner, and the reaction is viewed as a "caged" process in which the active oxygen species is not released into the surrounding medium. The metal ion binds in a pocket on the proteins surface residues at the metal-binding site.

This site specific mechanism is supported by the demonstration that the metal catalyzed reactions are inhibited by catalase but not by $^{\bullet}OH$ scavengers, presumably because the scavengers cannot compete with caged reactions of $^{\bullet}OH$ with amino acids at the metal binding site. Histidine has been identified as one of the amino acid residues particularly susceptible to metal catalyzed oxidation *in vitro* and *in vivo*. When glutamine synthetase was exposed to metal catalyzed oxidation, the chemical modification of one mol His/ mol residues such as Tyr, Trp, Cys and Met remained unchanged, this suggests the chemical selectivity of the site specific oxidation mechanism. From experimental observation it was found that histidine oxidation is

controlled in part, by the sequence and structure of a protein as well as the geometry of the protein / peptide metal complex.[6]

The main objective of the thesis is to better understand what level of a protein structure makes a protein most susceptible to oxidative damage caused by reactive oxygen species. Is site specific damage more dependent on a protein three dimensional structure, as suggested by the "caged" reaction concept or is the availability of metal ion binding residues such as histidine sufficient i.e, is it more dependent on the primary sequence. These questions will be investigated by creating a series of site directed mutants of hen egg white lysozyme. The first mutant will change histidine at position 15 (H15) to serine. By changing the histidine, which is more susceptible to oxidation, a different pattern of oxidation is expected when compared to native lysozyme.

The experimental techniques used in this project include site directed mutagenesis, expression of the mutant protein, and detection of the protein by ELISA assay (Enzyme-Linked ImmunoSorbent Assay)

CHAPTER 2: MATERIALS AND METHODS

The chapter consists of 2 sections. Section I describes the experiments for intracellular expression. Section II describes the generation of an extracellular mutant and transformation into *Pichia pastoris* strain for extracellular expression.

Section I: Intracellular Protein Expression of H15S Mutant

An H15S site – directed mutant of hen egg white lysozyme was created by Nanci Billock in the summer of 2003. This site – directed mutant was designed to be expressed intracellularly. The mutant was housed in the pCR®4 - TOPO® (Figure 2 – 3 on page 42) vector and was used to transform Top 10 *E. coli* cells. The following summer another student, Justin Pruneski, transformed the GS115 *Pichia pastoris* strain with the single mutant housed in a pPICZ B (Figure 2 – 1, page 25) yeast plasmid.

Site directed mutagenesis and creation of H15S mutant

Site directed mutagenesis is a technique in which a mutation is created at a defined site in a circular DNA molecule known as a plasmid. Using PCR (polymerase chain reaction) and oligonucleotides with the altered sequence allows us to generate a mutant of the native enzyme. Site – directed mutagenesis technique is used for studying structure – function relationships, gene expression and vector modification.

Using this technique the histidyl codon (CAC) in hen egg white lysozyme at position 15 was converted to a serinyl residue (AGC). This mutation was created by having a mismatch between the parental template and oligonucleotide primers that are designed to introduce a point mutation at a codon on the template. "Front" and the "back" end fragments of the gene were generated about the site of mutation and annealed using the PCR overlap extension method. Two differently designed oligonucleotides

containing the mutant sequence that attach themselves to the specific parts of DNA template were used in the polymerase chain reaction along with polymerase enzyme

Generation of intracellular H15S mutant front and back end

The front end of the mutated gene was created using the H15S Reverse (H15S R) oligonucleotide and Lyz - F primer and the back end was generated using the H15S Forward (H15S F) and Lyz - R primers. The gene sequences of the designed oligonucleotides that are used to introduce the point mutation are as follows:

H15S F: 5'- GGCTATGAAGCGTAGCGGACTTGATAACTA - 3'

H15S R: 5'- TAGTTATCAAGTCCGCTACGCTTCATAGCC - 3'

The Lyz - F primer contains a yeast consensus sequence and the initiating methionine codon (ATG). The Lyz – R primer contains an XbaI restriction (CTAGA) site. The gene sequences of the Lyz - F primer and the Lyz – R primer are as follows:

Lyz – F: 5'- ATAATAATGAAAGTCTTTGGACGATGT – 3'

Lyz – R: 5'- CTCTAGAGCCGGCAGCCTC- 3'

The PCR reaction was performed in two PCR tubes, one for the front end and the second for the back end. The front end reaction mixture contained the following reagents: 5 µl of 10x thermo polymerase buffer from Biolabs, 6 µl of $MgCl_2$, 1 µl of dNTP's from Applied Biosystems, 0.5 µl of Vent polymerase from Biolabs, 0.5 µl of H15S R primer (100 µM), 0.5 µl of Lyz – F primer (100 µM), and 2 µl (120 ng) of native lysozyme in pCR®4 - TOPO® isolated from TOP10 competent *E. coli* cells as template and 34 µl of sterile water to make a total volume of 50 µl. The back end was generated using the following reagents: 5 µl of 10x thermo polymerase buffer from Biolabs, 6 µl of $MgCl_2$, 1 µl of dNTP's from Applied Biosystems, 0.5 µl of Vent

polymerase from Biolabs, 0.5 µl of H15S F primer (100 µM), 0.5 µl of Lys – R primer (100 µM) and 2 µl native lysozyme (120 ng) in pCR®4 - TOPO® isolated from TOP10 competent *E.coli* cells as template and 34 µl of sterile water to make a total volume of 50 µl.

The PCR reaction was performed using a Peltier thermal cycler, PTC-200 and includes three major cycling reactions that are repeated for 30 – 40 cycles. The first step is the *denaturation or melting step* at 95°C for 30 sec, during which the hydrogen bonds of the double stranded DNA are broken to give a single stranded DNA template for DNA synthesis. Next was the *annealing* step, during which the temperature was reduced to 55°C for 30 sec, so that the primers form hydrogen bonds with the complementary sequence of the template DNA. This step was followed by an *elongation* step at 72°C, an ideal working temperature for the Vent polymerase enzyme. The three steps were repeated for 35 cycles followed by 72°C for 7 minutes to complete the elongation.

The PCR products were analyzed by running 1% agarose gel at 80 V for 1 hour 10 minutes in 1x TAE buffer (40 mM tris – acetate, 1 mM EDTA, and pH 8.0). The gel was stained for 15 minutes in a 1x TAE buffer containing 2 µg/mL ethidium bromide solution. Two fragments, one of about 250 bp and one of about 150 bp were eluted from the agarose gel using the AMRESCO® CYCLO – PURE Gel Extraction Kit. The front and back end fragments that were excised from the gel were annealed using the overlap extension method.

Overlap extension method

The PCR mix for the overlap extension method contained the following reagents: 5 µl of 10x PCR buffer from Promega, 6 µl of $MgCl_2$, 1 µl of dNTP's, 0.25 µl of Taq

polymerase from Applied Biosystems, 0.5 µl of Lyz – F primer (100 µM), 0.5 µl of Lyz – R primer (100 µM) and 1µl (100 ng) of the front end fragment, 1 µl (100 ng) of the back end fragment, and 34.75 µl of sterile water to make a total volume of 50 µl. The PCR program was as follows: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. These steps were repeated for 35 cycles with a final 72°C elongation step for 10 minutes. The PCR product was analyzed by 1% agarose gel electrophoresis ran at 85 V for 1 hour 10 minutes in 1x TAE buffer. The gel was stained for 15 minutes in a 1x TAE buffer containing 2 µg/mL ethidium bromide solution.

The pPICZ A, B, C vector

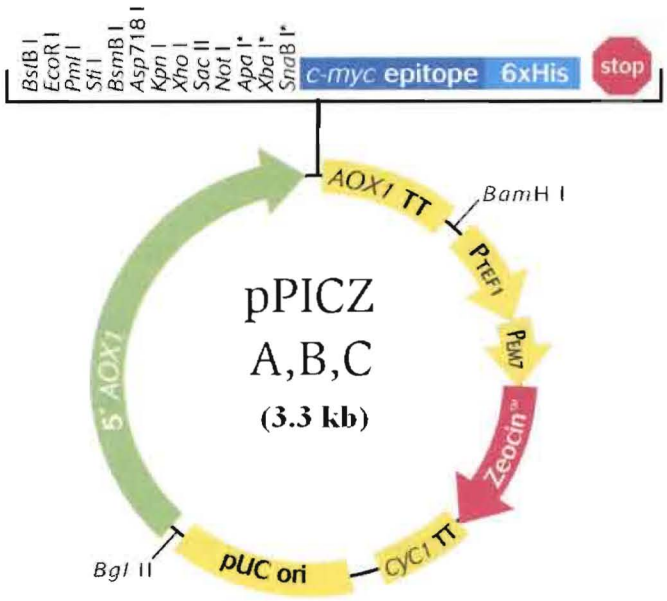

Figure 2 – 1: The pPICZ A, B, C Vector (3.3 kb)

pPICZ A, B, C vectors are used for protein expression in methylotrophic yeast, *Pichia pastoris*. pPICZ A, B, C vectors contains the following elements in which all the features have been functionally tested 1) 5' *AOX1* : a 942 bp fragment containing the

AOX1 promoter that allows methanol – inducible, high level expression in *Pichia*, 2) it contains multiple cloning sites with 10 unique restriction sites that allows insertion of the gene of interest into the expression vector, 3) a C – terminal *myc* epitope tag that permits the detection of the fusion protein by the anti – *myc* antibody, 4) a C – terminal polyhistidine tag that permits the purification of recombinant fusion protein on a metal – chelating resin such as Probond ^{TM} , 5) *AOX1* Transcription Termination (TT): A native transcription termination and polyadenylation signal from *AOX1* gene (~260bp) that permits efficient 3' mRNA processing, including polyadenylation, for increased mRNA stability, 6) a transcription elongation factor 1 (P_{TEF1}) gene promoter from *Saccharomyces cerevisiae* that drives the expression of the *shble* (*Streptoalloteichus hindustanus ble* gene) in *Pichia pastoris,* conferring Zeocin^{TM} resistance, 7) a synthetic constitutive prokaryotic promoter that drives the expression of the *sh ble* gene in *E. coli,* conferring Zeocin^{TM} resistance, 8) a Zeocin^{TM} resistance *sh ble* gene for selection in *E. coli*, 9) 3' end of the *Saccharomyces cerevisiae CYC1* (cyc1TT) gene that allows efficient 3' mRNA processing of the *sh ble* gene for increased stability, 10) a pUC origin that allows replication and maintenance of the plasmid in *E. coli*, and 11) unique restriction sites such as *Sac I, Pme I, BstX I* that permits linearization of the vectors at the *AOX1* locus for efficient integration into the *Pichia* gene.

Protein Expression in *Pichia pastoris*

Pichia pastoris is a species of methylotrophic yeast, and it has been devoleped as an expression system used frequently for the production of proteins using recombinant DNA techniques. Along with the benefits of *E. coli* expression, it also offers the advantages of expression in a eukaryotic system. *Pichia pastoris* is a highly successful

system and the factors that contributed for using this system over prokaryotic or other eukaryotic systems are 1) intracellular and secreted proteins are expressed at a high level, i.e. in the range of grams/liter, 2) it is capable of many post translational modifications performed by higher eukaryotes, such as folding, disulfide bond formation, glycosylation and proteolytic processing, 3) the techniques needed for molecular genetic manipulation of *Pichia pastoris* are similar to those of *Saccharomyces cerevisiae*, and 4) the *P. pastoris* system is generally regarded as less expensive, easier, and faster to use when compared to expression systems of higher eukaryotes such as human and mammalian tissue cultures.

Pichia pastoris is a yeast system capable of metabolizing methanol. The methanol metabolizing pathway is similar in all yeast systems that involve a unique set of pathway enzymes. In the methanol metabolization pathway, the first step is the oxidation of methanol to formaldehyde generating hydrogen peroxide. This reaction is catalyzed by the enzyme alcohol oxidase (AOX), and it degrades hydrogen peroxide to oxygen and water. The reaction takes place in the peroxisome by sequesteration of hydrogen peroxide with catalase to avoid toxicity to the rest of the cell. A portion of the formaldehyde generated in the peroxisome by AOX leaves the cell, which is further oxidized into formate and CO_2 by two dehyrogenases in the cytoplasm. The formaldehyde that remains in the peroxisome is assimilated by a cyclic pathway of condensation of formaldehyde with xylulose – 5 – monophosphate to form cellular constituents, catalyzed by dihydroxy acetone synthase (DHAS). The products of the synthase reaction are glyceraldehyde 3-phosphate and dihydroxyacetone. These two products leave the peroxisome and then enter the cytoplasm regenerating xylulose – 5 –

monophosphate and a single molecule of glyceraldehyde – 3 – phosphate for every three cycles. The two enzymes AOX and DHAS can be detected at higher levels in cells grown on methanol but not in cells grown on other carbon sources such as glycerol, glucose or ethanol.

Alcohol oxidase in *Pichia pastoris* is encoded by two genes *aox1* and *aox2*, *aox1* is responsible for most of the alcohol oxidase activity in the cell. Cells grown in media containing methanol generate ~5% of poly (A) $^{+}$ RNA from *aox1*, whereas poly (A) $^{+}$ RNA from *aox1* is undetectable in the cells grown on other carbon sources. The *aox1* gene is controlled by two mechanisms, an induction mechanism and a repression /depression mechanism. The presence of methanol is essential for high level transcription the *aox1* gene.

Techniques required for molecular genetic manipulation are similar to *S. cerevisiae*, such as DNA-mediated transformation, gene targeting, gene replacement, and cloning by functional complementation. It also exhibits a propensity for homologus recombination between artificially introduced and genomic DNA similar to that of *S. cerevisiae*. Expression of a foreign gene in *P. pastoris* requires three basic steps 1) the insertion of the gene into an expression vector, (2) introduction of the expression vector into the *P. pastoris* genome, and (3) testing of potential expression strains for the foreign gene product.

A variety of *P. pastoris* vectors and host strains are available, and the plasmid vectors designed for heterologous protein expression in *P. pastoris* have several common features. The foreign gene expression cassette is composed of DNA sequences containing the *P. pastoris aox1* promoter, followed by one or more unique restriction

sites for insertion of the foreign gene, then followed by the transcriptional termination sequence from the *P. pastoris aox1* gene that directs efficient 3' processing and polyadenylation of the mRNAs. Some vectors also contain *aox1* 3' flanking sequences that are derived from a region of the *P. pastoris* genome that lies immediately 3' of the *aox1* gene and can be used to direct fragments containing a foreign gene expression cassette to integration at the *aox1* locus by gene replacement (or gene insertion 3' to the *aox1* gene). This type of integration is responsible for the formation of Mut^S recombinant strains.

Additional features have been incorporated in *P. pastoris* for secretion of foreign proteins. *P. pastoris* α vectors have been constructed in such a way that the DNA sequence immediately following the *aox1* promoter encodes a secretion signal. The most frequently used signal for extracellular expression is the *S. cerevisiae* α factor prepro signal sequence, however, the signal sequence derived from the *P. pastoris* acid phosphatase gene (PHO1) is also available.

Three types of *picha* strains are available that vary with regard to their ability to utilize methanol due to deletions in one or both *aox* genes. Strains with deleted *aox1* are sometimes better producers of foreign protein than the wild type strain. Some have a mutation in one or more auxotrophic genes [e.g., GS115 (*his4*)] to allow for selection of expression vectors containing the complementing biosynthetic gene (e.g. *HIS4*) upon transformation. All of these strains grow on complex media but require supplementation with histidine or other appropriate nutrient for growth on minimal media. The most commonly used expression host is X – 33 (wild-type) or GS115 (*his4*), which are wild type with regard to the *aox1* and *aox2* genes and grow on methanol at the wild-type rate

(methanol utilization plus or Mut$^+$ phenotype). KM71 (*his4 arg4 aox1Δ::ARG4*) is Mut^S phenotype in which chromosomal *aox1* gene is deleted and replaced with the *S. cerevisiae ARG4* gene, due to which the strain has to rely on *aox2* gene which grows on methanol at slow rate (methanol utilization slow or Mut^S phenotype).

Transformation and protein expression in Pichia pastoris

Three *P. pastoris* strains X – 33, GS115, and KM71H provided in a kit purchased from invitrogen were made chemically competent. All three strains of bacteria were grown in 10 mL of YPD media (1% yeast extract, 2% peptone and 2% glucose) by incubating overnight at 28 - 30°C in a shaking water bath at 250 – 300 rpm. The cells from this culture were diluted to an OD_{600} of 0.1 – 0.2 in 10 mL of YPD and grown at 28 - 30°C in a shaking incubator until the OD_{600} reaches 0.6 – 1.0 (approximately 4 to 6 hours). The cells were pelleted by centrifuging at 500 x g for 5 minutes at room temperature, the supernatant was discarded and the cell pellet was resuspended in 10 mL of solution 1 (1 M sorbitol, 10 mM bicine, 3% (v/v) ethylene glycol, 5% (v/v) DMSO, pH 8.35). The solution was immediately centrifuged at 500 x g for 5 minutes, and the cell pellet was resuspended in 1 mL of solution 1 and the cells were now competent. 50 – 200 µl of competent cells were aliqouted into 1.5 mL sterile screw - cap microcentrifuge tubes. These competent cells can be directly used for transformation or can be placed directly in a styrofoam box or can be wrapped in several layers of paper towel and place in a -80°C freezer.

Confirmation of the mutant phenotype

The worked described here used the transformed yeast created by Justin Pruneski. Transformation of X – 33 or GS115 with linearized constructs favor single crossover recombination at the *aox1* locus. Most of the transformants should be Mut^+(methanol utilization plus), however, with the presence of *aox1* in the plasmid at more than one site, there is a possibility of recombination occurring at the 3' *aox1* region thereby disrupting the wild type *aox1* gene and creating Mut^S (methanol utilization slow) transformants. There is no need to test recombinants for the Mut^+ phenotype in case of KM71H, because all Zeo^R transformants will be Mut^S because of the disruption of the *aox1* gene.

The phenotype of the mutant is determined by checking growth on two media, MDH (minimal dextrose with histidine) and MMH (minimal methanol with histidine) plates. The MDH plates were prepared as follows: 1.34% YNB, 4 x 10^{-5} % biotin, 2% dextrose, 0.004% histidine with 1.5 % agar. The MMH plates contained the following reagents: 1.34% YNB, 4 x 10^{-5} % biotin, 0.5% methanol, 0.004% histidine and 1.5 % agar. Using sterile toothpicks, colonies of the GS115 *Pichia pastoris* strain containing pPICZ B vector with H15S mutant were streaked on both MMH and MDH plate in a regular pattern in the form of dots.

Mut^+ and Mut^S can be differentiated by making one patch for each of the controls GS115 albumin (Mut^S) and GS115/pPICZ/*lacZ* (Mut^+) onto the MDH and MMH plates. The plates were incubated at 30°C for 2 days. Mut^+ strains grow normally on both MMH and MDH plates and Mut^S grow normally on MDH plates but show little or no growth on the MMH plates. The plates are scored after incubating them at 28 - 30°C for 2 days.

Small scale expression of protein in different media

Mut^+ recombinant strains that have been confirmed by PCR for the insert were used for the expression of protein. Small scale expression conditions may not be optimal for some proteins, so methods like SDS – PAGE, Western Blot or functional assay are used to detect the desired protein. The expression of the mutant lysozyme protein was carried out in three types of media. The three media are as follows:

1) BMGY/BMMY (buffered complex glycerol or methanol medium)
2) BMGH/BMMH (buffered minimal glycerol or methanol medium containing histidine)
3) MGYH/MMH (minimal glycerol or minimal methanol medium containing histidine)

Expression of the protein in BMGY/ BMMY media

The composition of the BMGY media is as follows: 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 1 % glycerol. The GS115 strain with insert was grown on YPD plates containing 100 μg/mL Zeocin™ for 2 – 3 days at 30°C. A single colony was picked and inoculated into a sterile 250 mL conical flask containing 25 mL of BMGY media. The cells were grown at 28 - 30°C in a shaking incubator at 250 – 300 rpm until the culture reached an OD_{600} of 2 – 6 (approximately 16 – 18 hours, cells will be in log phase growth). The cells were harvested by centrifugation at 1500 – 3000 x g for 5 minutes at room temperature, the supernatant was discarded and the cell pellet was resuspended to an OD_{600} of 1.0 in 100 – 200 mL of BMMY media in a sterile 1 liter baffled flask (1% yeast, 2% peptone, 10% mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 0.5% methanol)

to induce the protein expression. 0.005% antifoaming agent from Sigma was added to the media to prevent frothing.

1 mL of the culture was collected at time points of 24, 48, 72, and 96 hours. The culture was made 0.5% in methanol after each time point collection. The collected samples were centrifuged, and the supernatant was discarded. The cell pellet was quickly frozen in a dry ice acetone bath and stored in a -80°C freezer.

Expression of the protein in BMGH/ BMMH media

The BMGH media was prepared as follows: 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 0.004% histidine, 1 % glycerol. A single colony was picked and inoculated into sterile 250 mL conical flask containing 25 mL of BMGH media. The cells were grown overnight at 28 - 30°C in a shaking incubator at 250 – 300 rpm until the culture reached an OD_{600} of 2 – 6 (approximately 16 – 18 hours, cells will be in log phase growth). The cells were harvested by centrifugation at 1500 – 3000 x g for 5 minutes at room temperature, the supernatant was decanted and the cell pellet was resuspended to an OD_{600} of 1.0 in 100 – 200 mL of BMMH media (10% mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 0.004 % histidine, 0.5% methanol) in a sterile 1 liter baffled flask to induce the expression. 0.005% antifoaming agent from Sigma was added to the media to prevent frothing. The culture was allowed to grow for 4 days at 30°C.

1 mL of culture was collected from the baffled flask at 24, 48, 72, and 96 hours. The culture was made 0.5% in methanol after each time point collection. The 1 mL cultures collected in microcentrifuge tubes were centrifuged in a table top centrifuge. The

supernatant was discarded and the cell pellet was quickly frozen in a dry ice acetone bath, and stored in the -80°C freezer.

Expression in MGYH/MMH media

The MGYH media was prepared as follows: 1.34% YNB, 4 x 10^{-5}% biotin, 0.004% histidine, and 1 % glycerol. The GS115 strain containing the H15S insert in pPICZ B plasmid was first grown in 25 mL of MGYH media in a 250 mL baffled flask at 30°C in a shaking water bath. The culture was grown until it reached an OD_{600} of 2 – 6. The cells were harvested by centrifugation at 1500 – 3000 x g for 5 minutes at room temperature, the supernatant was decanted and the cell pellet was resuspended to an OD_{600} of 1.0 in 100 – 200 mL of MMH media in a sterile 1 liter baffled flask. MMH media consisted of 1.34% YNB, 4 x 10^{-5}% biotin, 0.004% histidine, and 0.5 % methanol to induce the expression. 0.005% antifoaming agent from Sigma was added to prevent frothing and the culture was allowed to grow for 4 days at 30°C

1 mL of culture was collected from the baffled flask at 24, 48, 72, and 96 hours. The culture was made 0.5% in methanol after each time point collection. The 1 mL cultures collected in 1.5 mL microcentrifuge tubes were centrifuged in a table top centrifuge. The supernatant was discarded and the cell pellet was quickly frozen in a dry ice acetone bath and stored at -80°C.

Analysis of cell contents by SDS – PAGE

The cell pellets collected from the three different types of media were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE). The cell pellets collected and stored at -80^0 C were thawed and the cells were lysed using the following procedure. For each 1 mL of the cell pellet collected at time points of 24, 48,

72, and 96 hours , 100 µl of breaking buffer (50%, 50 mM sodium phosphate pH 7.4, 10% 1 mM phenyl methyl sulfonyl fluoride, 10% 1 mM EDTA, 5% glycerol (v/v)) and an equal volume of acid washed glass beads (size 0.5 mm) were added. The above mixture was vortexed with a Maxi Mix II vortexer for lysing the cells. The cells were vortexed for 30 sec kept on ice and again vortexed. This process of vortexing was done 8 times. The cells were centrifuged at 13,000 rpm for 10 min and the clear supernatant was transferred to a sterile microcentrifuge tube.

20 µl of the collected lysate was transferred to a separate sterile microcentrifuge tube. This was followed by the addition of 20 µl of 62.5 mM Tris – HCl, pH 6.8, 10 µl of 10% SDS, and 4 – 6 µl of 2 – mercaptoethanol. This mixture was placed in a boiling water bath for 3 – 4 min. The mixture was cooled, and an equal volume of 20% (v/v) glycerol was added. Finally, 10 µl of 0.05% bromophenol blue tracking dye was added. The samples were centrifuged to remove traces of insoluble material and the samples were separated by SDS – PAGE.

SDS - PAGE was performed by using 18 % mini gels and a Mini – Protease II system from Biorad. The gels were set in a SDS chamber and filled with electrophoresis running buffer (0.25 M Tris, 1.92 M glycine, 1% SDS, pH 8.3). 10µl of molecular protein marker, 20 µl of 24, 48, 72 and 96 hour samples and 10µl of native lysozyme were loaded into the wells, and electrophoresis was performed at 150 V until the dye front reached the bottom of gel. This usually required 1 to 1.5 hour. The gels were then washed with water three times for 5 min each then placed in Gelcode ® blue (Pierce) solution overnight The gels were then destained in DI water.

Large scale expression of protein in BMGH/BMMH media

Experiments involving small scale expression indicated that the BMGH/BMMH media showed promise for expression of the H15S mutant. Further work was continued on optimizing the conditions for scale up expression in BMGH/BMMH media. The cells frozen in YPD media in glycerol stock solution at -80°C freezer were thawed. 50 µl of the cells were spread on YPD plates with 100 µg/mL of Zeocin™ and kept for growth in a 30°C incubator. A single colony from this plate was picked up with a sterile tooth pick and placed into 25 mL of BMGH for large scale expression. This culture was grown at 29.8°C in a shaking incubator at 250 rpm until the OD_{600} reached 2 – 6 (approximately 16 – 18 hours). This 25 mL culture was further used to inoculate 125 mL of BMGH media containing 0.005% antifoaming agent. This media was allowed to grow at 29.8°C in a shaking incubator at 250 rpm until the OD_{600} reached 2 – 6 (approximately 16 – 18 hours). The cells were harvested by centrifugation at 4,500 rpm for 5 minutes at 4°C using 250 mL Sorval sterile centrifuge bottles. The supernatant was discarded and the cell pellet was resuspended in 250 mL of BMMH media. The cells were grown for 3 days at 29.8°C and the culture was made 0.5% in methanol every 24 hours. After 72 hours the cells were harvested by centrifugation at 4,500 rpm for 5 minutes at 4° C. The supernatant was discarded and the cell paste was weighed, frozen in liquid nitrogen and stored in the -80°C freezer.

The cell paste was thawed to room temperature and resuspended in breaking buffer. The cells were lysed with a bead beater (Biospec). The bead beater chamber was filled with 0.5 mm glass beads to ½ to ¾ of its volume. The remaining volume was filled with cell suspension and breaking buffer. The chamber was placed in a water jacket that

was filled with crushed ice and water and the whole setting was fixed onto the bead beater. The bead beater was switched on for 1 minute and off for 1 minute. This was done 5 times.

The collected lysate was ultra centrifuged at 94,000 rpm for 15 minutes to remove the cell wall material and then at 37,000 rpm at 4°C for 1 hour to remove the cell organelles and the nucleic acids such as DNA and RNA. The pure lysate was then stored in the -80°C freezer and saved for further analysis.

Enzyme assay

An enzyme assay was used to detect the presence of the H15S mutant. The assay measures the rate of lysis of a *Micrococcus lysodeikticus* suspension by the decrease in its turbidity at an absorbance of 450 nm based on assay by shugar. The reagents used for doing this assay were buffered media (39 mL of 0.1 M KH_2PO_4, pH 7.0, and 1 mL 0.1 M K_2HPO_4, pH 7.0). To 20 mL of the buffer media, 60 mg of powdered *Micrococcus lysodeikticus* was added and incubated at room temperature for 1 hour. Different concentrations of lyophilized native lysozyme i.e 1 mg/mL, 100 µg/mL, 10 µg/mL were prepared and the absorbance was measured using a Hewlett – Packard diode array spectrophotometer at 450 nm. The cell holder was maintained at a temperature of 25°C using a circulating water bath. 2.9 mL of *Micrococcus lysodeikticus* was pipetted into a 3 mL cuvette and incubated for 4 – 5 minutes in order to achieve temperature equilibrium and to establish a blank rate. 100 µl of lysate was added to the cuvette, and the absorbance was recorded every 10 seconds for a period of 4 minutes. The ΔA_{450} was caluculated using zero – order kinetics fitting data from 40 - 240 sec. The assay was

done on all the lysate samples collected at regular time points and also on the native lysozyme.

ELISA Test

The enzyme – linked immunosorbent assay (ELISA) is a sensitive immunoassay that uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein.

The ELISA test was done several times for optimization with different dilution of primary antibody (1^0 Ab and antigen) and antigen. After optimization, the ELISA was performed with a 1:30,000 dilution of biotinylated polyclonal antibody to lysozyme with antibody 1^0 Ab (Acris anribodies) and peroxidase conjugated streptavidin (Bio – Genex) as 2^0 reagent.

The test was performed in an 8 cm x 12 cm plastic plate which contains a matrix of 96 wells, each of which was about 1 cm high and 0.7 cm in diameter. The plastic plated is coated with antigen (Ag) and incubated at room temperature. A 10 µg/mL sample of native lysozyme, and 5 mg/mL of H15S mutant lysate was prepared in 50 mM sodium carbonate buffer, pH 9.6.

Dilution of primary (1^0) antibody

A first dilution of 1:100 was prepared by taking 2 µl of 1^0 Ab and 198 µl of PBS (phosphate – buffered saline, 0.01 M sodium phosphate monobasic and sodium phosphate dibasic mixed in equal volume, 0.15 M sodium, pH 7.4). A second dilution was made by mixing 2 µl of the first dilution with 198 µl of PBS (1:10,000 dilution). A 1:30,000 solution was made from the second dilution by mixing 1 mL of the 1:10,000 dilution with 3 mL of sodium carbonate buffer.

Method

The wells A1 – A12 and B1 – B12 were filled with 100 µl Na_2CO_3 buffer. A two fold serial dilution with 100 µl of native lysozyme (100 µg/mL) was made from the wells A1 - A12 and B1 – B12. C1 – C4 wells were filled with just the sodium carbonate buffer and served as a blank. Wells C5 – C8 were filled with 100 µl of 100 µg/mL of the native lysozyme (no 1^0 antibody added – negative control) and F1 – F4 wells were filled with lysate (no 1^0 antibody added – negative control). Wells C9 – C12 were filled with 1:30, 000 solution of the 1^0 antibody. D1 – D12 and E1 – E12 wells are first filled with Na_2CO_3 buffer. A 2 fold serial dilution was made from D1 - D12 and E1 – E12 with 100 µl of lysate. The plate was incubated at room temperature for 2 hours. The solutions were removed from the wells by flicking and blotting with a paper towel. The wells were then filled with 50 µl of blocking buffer (1% bovine serum albumin from Sigma in 1x PBS) and incubated for 30 min at room temperature. 100 µl of 1:30, 00 dilution of 1^0 antibody was added to all the wells except wells C5 – C8 and F1 – F4. The plate was incubated for 2 hours at room temperature. The wells were then washed three times with wash buffer (PBS with 0.05% tween – 20) and 100 µl of a 1:20 dilution of streptavidin (2^0 antibody) was added to all the wells. The plate was incubated at 37^0 C for 30 minutes, the wells were again washed with wash buffer. 100 µl of the substrate, tetramethyl benzidine from Sigma, was added to all the wells with a multichannel pipette. After devolepment of the blue color the reaction was stopped with the addition of 50 µl of 2 M sulphuric acid. The absorbance was measured at 450 nm (Bio – Tec instruments microplate auto reader EL 311).

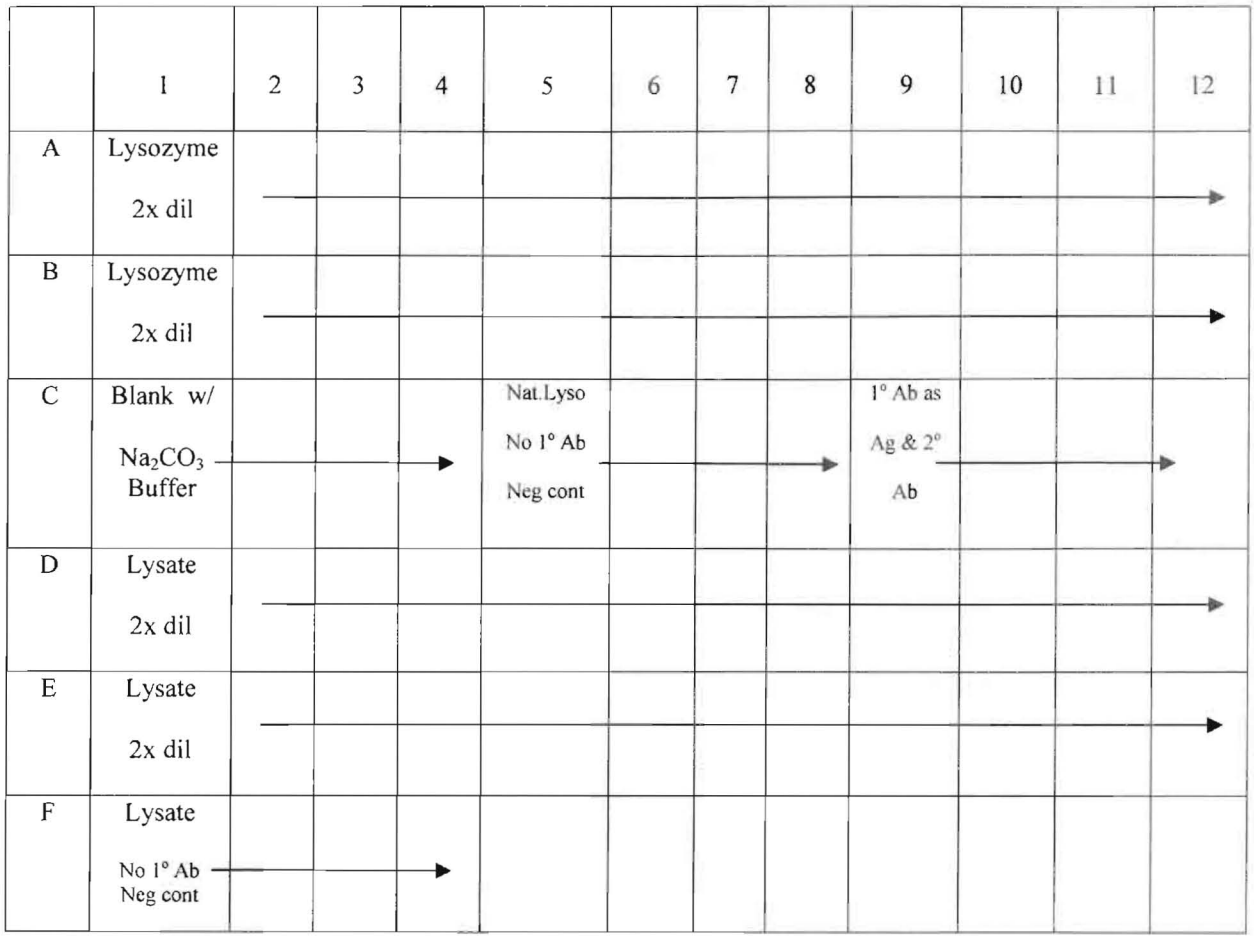

Figure 2 – 2: Preparation of the 96 well microtiter plate for ELISA

Section II: Materials and Methods for Extracellular expression

Creation of H15S mutant for extracellular expression

The H15S mutant created by Nanci Billock was used as a template for my thesis work, in which the gene designed for intracellular expression was changed to a template for extracellular expression by using the 5' Ex Lyso primer instead of the Lyz – F primer. The 5' Ex Lyso primer contains a XhoI restriction site for digestion and insertion into pCR®4 - TOPO® (Figure 2 – 3 on page 42) vector and then into pPICZα A vector (Figure 2 – 4, page 44). This primer also places the gene in the correct reading frame for α signal sequence.

5'Ex Lyso : 5'-CCCGCCTCGAGAAAAGAAAAGTCTTTGGACGATGTGAG- 3'

XhoI 1^{st} codon for HEWL

Using this primer and the H15S as template the PCR reaction mixture contained the following reagents: 5 µl of 10x PCR buffer from Biolabs, 6 µl of $MgCl_2$, 1 µl of dNTP's, 0.25 µl of Taq polymerase from Applied Biosystems, 0.5 µl of 5' Ex Lyso primer (100µM), 0.5 µl of Lyz – R primer (100µM), 2 µl (100 ng) of pure H15S mutant gene excised from gel and 34.75 µl of sterile water to make a total volume of 50 µl. The PCR program includes these steps: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. These steps were repeated for 35 cycles with a final 72°C elongation step for 10 minutes. The PCR product was analyzed by 1% agarose gel electrophoresis run at 85 V for 1 hour 10 minutes in 1 x TAE buffer. The gel was stained for 15 minutes in a 1x TAE buffer containing 2 µg/mL ethidium bromide solution.

The pCR®4 - TOPO® vector

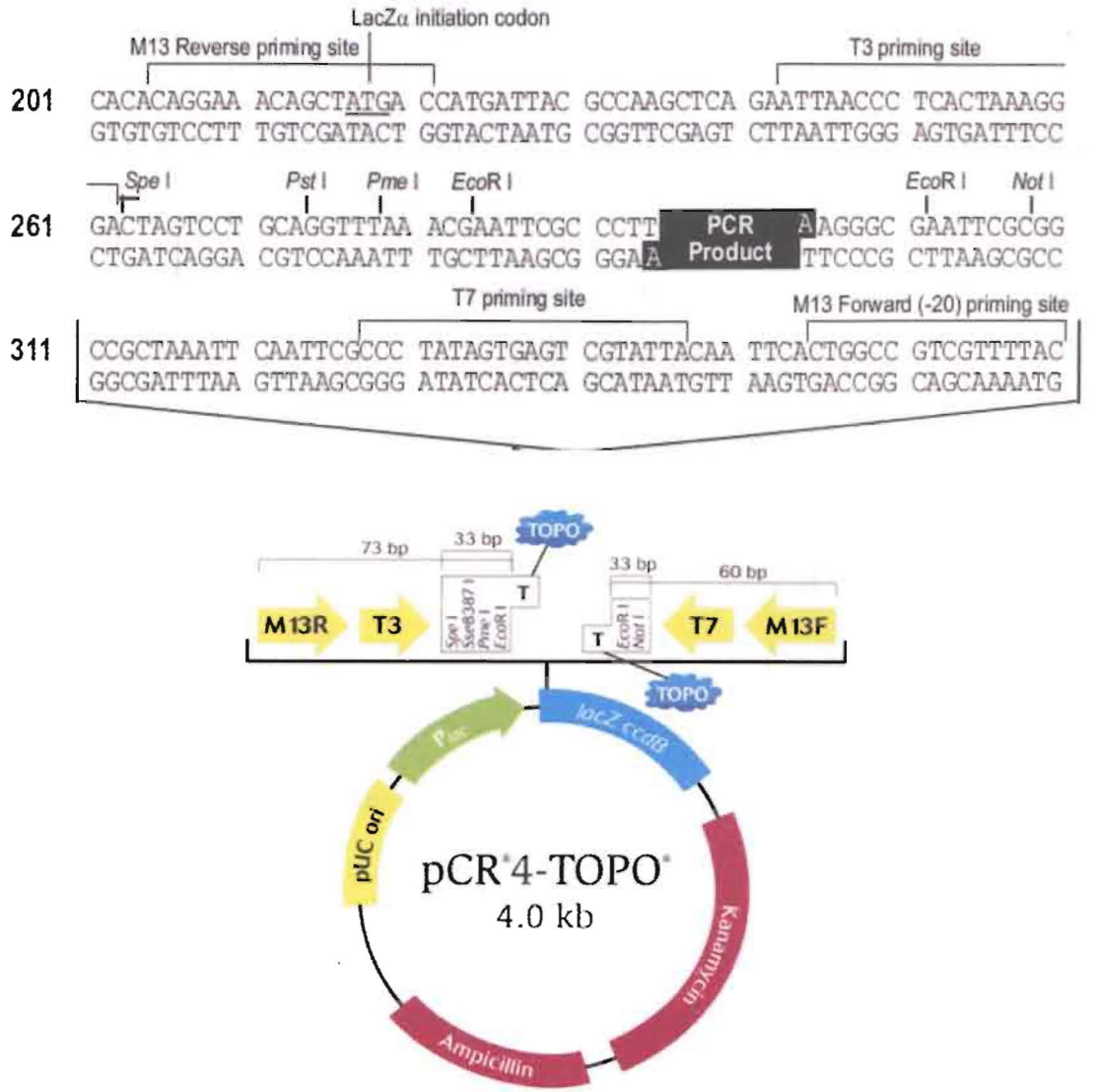

Figure 2 – 3: The pCR®4 - TOPO® vector (~ 4.0 Kb) with TOPO® cloning site and the sequencing primers.

The pCR®4 - TOPO® vector (Figure 2 – 3) enables fast and efficient cloning of *Taq*-amplified PCR products. The pCR® 4- TOPO® vector is provided with 3′-T overhangs and is topoisomerase I-activated to readily accept PCR products with 3′-A overhangs. The pCR®4 - TOPO® vectors is ideal for applications such as probe generation, *in vitro* transcription, or general subcloning. Some of their convenient

features include 1) *Eco*R I sites flanking the PCR product insertion site for easy removal of inserts, 2) kanamycin and ampicillin resistance genes with choice of selection in *E. coli*, 3) T3, T7, M13 forward (-20) and M13 reverse (-20) priming sites allows for sequencing or PCR screening, and 4) pUC origin allows replication and maintainence of the plasmid in *E. coli*.

Transformation of H15S gene into one shot® TOP10 E. coli cells

The H15S mutant was subcloned into the pCR®4 - TOPO® vector by the TOPO cloning reaction, and then transformed into competent *E. coli* cells. The reagents used in the TOPO cloning reaction were 1 µl of pCR®4 - TOPO® vector, 1 µl of salt solution, 1 µl of sterile water and 2 µl of annealed PCR product. 1 – 2 µl of the TOPO® cloning reaction was added to 50 µl of competent *E. coli* cells. The reaction mixture was incubated at room temperature for 5 minutes and then on ice for 5 minutes and then spread on LB (Luria - Bertani) with ampicillin (100 µg /mL) plates, and incubated in a 37°C incubator overnight.

Eight colonies from the plate were selected and streaked on fresh LB plates with ampicillin (100 µg/mL). One colony from each of the eight plates was selected for overnight growth in LB plus ampicillin (100 µg/mL) broth in a shaking incubator at 37°C. The plasmid was extracted from each of the eight colonies using an AMRESCO Cyclo Prep Kit. The plasmids were analyzed by running a 1 % agarose gel at 85 V for 1 hour and 10 minutes in 1x TAE buffer. The gel was stained for 15 minutes in a 1x TAE buffer containing 2 µg/mL ethidium bromide solution.

The pPICZα A, B, C vector

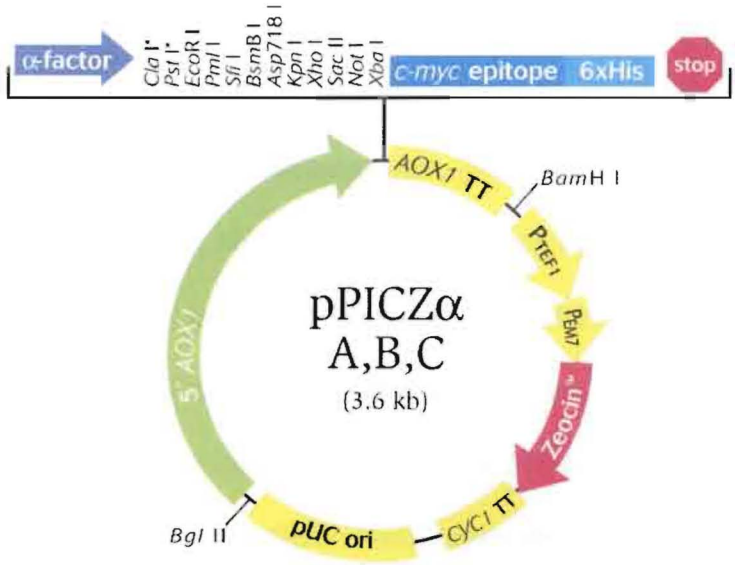

Figure 2 – 4: The pPICZα A, B, C Vector (3.6 kb)

pPICZ α A, B, C vectors contains the following elements, and all the features have been functionally tested 1) 5' *AOX1* : A 942 bp fragment containing the *AOX1* promoter that allows methanol – inducible, high level expression in *Pichia*, 2) native *Saccharomyces cerevisiae* α – factor secretion signal that allows for efficient secretion of most proteins from *Pichia*, 3) multiple cloning sites with 10 unique restriction sites that allows insertion of the gene of interest into the expression vector, 4) a C – terminal *myc* epitope tag that permits the detection of the fusion protein by the anti – *myc* antibody, 5) a C – terminal polyhistidine tag that permits the purification of recombinant fusion protein on metal – chelating resin such as Probond ™, 6) *AOX1* Transcription Termination (TT): a native transcription termination and polyadenylation signal from *AOX1* gene (~260bp) that permits efficient 3' mRNA processing, including polyadenylation, for increased mRNA stability, 7) a transcription elongation factor 1

(P_{TEF1}) gene promoter from *Saccharomyces cerevisiae* that drives the expression of the *shble* gene in *Pichia,* conferring Zeocin™ resistance, 8) a synthetic constitutive prokaryotic promoter that drives the expression of the *sh ble* gene in *E. coli,* conferring Zeocin™ resistance, 9) a Zeocin™ resistance *shble* gene (*Streptoalloteichus hindustanus ble* gene) for selection in *E. coli*, 10) 3' end of the *Saccharomyces cerevisiae CYC1* (cyc1TT) gene that allows efficient 3' mRNA processing of the *sh ble* gene for increased stability, 11) a pUC origin that allows replication and maintenance of the plasmid in *E. coli*, and 12) unique restriction sites such as *Sac I, Pme I, BstX I* that permits linearization of the vectors at the *AOX1* locus for efficient integration into the *Pichia* gene.

Transformation of JM110 *cells with pPICZαA vector*

pPICZα A, B, and C vectors in lyophilized form (2 μg) from Invitrogen were dissolved in adding 20 μl of sterile H_2O to make a final concentration of 0.1 μg/μl. 50 μl of JM110 competent cells from Stratagene were placed into three pre - chilled tubes, followed by addition of 0.85 μl of β-mercaptoethanol to each tube to increase the transformation efficiency. The tubes were incubated on ice and the contents were swirled every 2 minutes for a period of 10 minutes. 2 μl of the diluted pPICZα A, B, C plasmids were added to three different tubes containing JM110 competent cells. The tubes were incubated on ice for about 30 minutes, and the cells were heat shocked in a 42°C waterbath for 45 seconds. The cells were again incubated on ice for 2 minutes, and then 450 μl of SOC media were added. The tubes were incubated in a 37°C shaking incubator for 1 hour.

About 100 μl of transformation mixure were plated on low salt LB plates containing 25 μg/mL of Zeocin™. A single colony from each of the plates was grown in

a shaking waterbath overnight at 37°C in LB media supplemented with 25 µg/mL Zeocin™ antibiotic. Plasmid was isolated from the colonies using a QIAgen® Spin Miniprep Kit and a 1% agarose gel was run at 85 V for 1 hour 10 minutes in 1x TAE buffer. The gel was stained for 15 minutes in a 1x TAE buffer containing 2 µg/mL ethidium bromide solution.

Restriction digestion of the sample to detect the presence of the insert

The cloned pCR®4 - TOPO® vector of each of these colonies was subjected to restriction digestion using XhoI and XbaI restriction endonuclease enzymes. The double digestion reaction contained the following reagents: 10 µl of the above plasmid (150 ng) isolated from competent *E. coli*, 1 µl XhoI, 1 µl XbaI, 2 µl of 10x restriction endonuclease buffers from Invitrogen and 6 µl of sterile water to give a total volume of 20 µl. The above reaction mixture was incubated at 37°C for 1 hour and the enzymes were deactivated by incubating them at 65°C for 20 minutes. The reaction mixture was analyzed by 1% agarose gel at 85 V for 1 hour and 10 minutes.

DNA sequencing

Sequencing was performed using the Beckman Coulter (GenomeLab™ Methods Development Kit). The materials that were used for DNA sequencing are a premix (components are 200 µl 10x sequencing reaction buffer, 100 µl of dNTP mix, 200 µl ddUTP dye terminator, 100 µl ddGTP dye terminator, 200 µl ddCTP dye terminator, 200 µl ddATP dye terminator and 100 µl of polymerase enzyme to make a total volume of 100 µl) T3 and T7 primers, and the insert cloned into pCR®4 - TOPO® vector as template. The materials for the control template were pUC 18 as template, -47 sequencing primer, and the premix.

Two sets of samples were prepared for each template isolated from a single colony, one with T3 primer and other with T7 primer. For each template the reagents used were, 11 µl of premix, 2 µl of T3 primer or T7 primer (0.1 µg/mL) , 6 µl of template (180 ng), 1 µl of sterile water. A control was prepared by mixing 11 µl of premix, 2 µl of -47 sequencing primer, 0.5 µl of pUC18 control template and 6.5 µl of sterile water.

T3 primer: ATTAACCCTCACTAAAGGGA

T7 primer: CCCTATAGTGAGTCGTATTA

The PCR for the thermal cycling reaction was set at 96°C for 20 sec, 50°C for 20 sec, and 60°C for 4 min for 30 cycles followed by holding the sample at 4°C. Samples from the PCR were transferred into labeled, sterile microcentrifuge tubes, followed by addition of 5 µl of fresh stop solution/glycogen mixture (2 µl of 3M sodium acetate pH 5.2, 2 µl of 100 mM Na_2-EDTA pH 8.0 and 1 µl of 20 mg/mL of glycogen) and the contents were mixed thoroughly. To these samples 60 µl of -20°C ethanol/dH_2O (95% (v/v) was added followed by centrifugation at 14,000 rpm at 4°C for 15 minutes. The supernatant was removed and the pellet was washed 2 times with 200 µl of -20°C 70% (v/v) ethanol/dH_2O. For each rinse the pellet was centrifuged at 14,000 rpm for 2 minutes. The pellet was vacuum dried for 30 minutes followed by resuspension in 40 µl of the sample loading solution. Sequencing was performed using the CEQ 2000XL DNA analysis system.

Cloning of H15S insert into pPICZα A vector

Double digestion was performed by taking 3 µl of pPICZα A (150 ng) vector, 6 µl of H15S/ pCR®4 - TOPO® vector (300 ng) (1:2 molar ratio of vector to insert), 1 µl of XbaI, 1 µl of XhoI and 2 µl 10x endonclease buffer, 0.2 µl of BSA (10 mg/mL) from Biolabs and 5 µl of sterile water to make a total volume of 20 µl . The reaction mixture was incubated at 37°C for 1 hour followed by heat inactivation of the restriction enzymes XbaI and XhoI at 65°C for 20 minutes. To the above reaction mixture, 1 µl of T4 DNA ligase buffer with 10 mM ATP, 1 µl of T4 DNA ligase, 0.2 µl of BSA (10 mg/mL) from Invitrogen and 3 µl of sterile water were added to make a total volume of 25 µl. The ligation mixture was incubated at room temperature for 1 hour, followed by heat inactivation of the ligase enzyme at 65°C for 10 minutes.

2 µl of the ligation mixture were added to 50 µl JM110 competent cells. The transformation mixture was plated on low salt LB plates supplemented with 25 µg/mL of Zeocin™. Four colonies were picked up from the plate with sterile tooth picks, and were grown in LB broth with 25 µg/mL of Zeocin™. Plasmid was isolated from the colonies using the QIAgen® Spin Miniprep Kit. All four plasmids of the cloned H15S insert in pPICZα A were double digested using XbaI and XhoI restriction enzymes. The reagents used for double digestion were 10 µl of the above isolated plasmid (200 ng), 1 µl of XbaI, 1 µl of XhoI, 2 µl of 10x restriction endonuclease buffer, 0.2 µl of 10 mg/mL BSA and 6 µl of sterile water to make a total volume of 20 µl. The samples were incubated at 37°C for 1 hour followed by heat inactivation of the restriction enzymes at 65°C for 20 minutes. 1% agarose gel electrophoresis was run at 85 V for 1 hour 10 minutes in 1x TAE buffer with the extracted plasmids and restriction digested samples. The gel was

stained for 15 minutes in a 1x TAE buffer containing 2 μg/mL ethidium bromide solution.

Plasmid isolated from JM110 cells was transferred into competent *E. coli* cells as JM110 cells strains of bacteria have a high frequency of recombination. Plasmid isolated from *E. coli* cells were used to transform the yeast X – 33 *Pichia pastoris* strain.

Transformation into X – 33 *Pichia pastoris* strain

Linearization of pPICZα A vector

Competent *E. coli* cells containing H15S in pPICZα A vector were grown in low salt LB broth (1% tryptone, 0.5% yeast extract, and 0.5% Nacl, pH 7.5) supplemented with 25 μg/mL of Zeocin™. Plasmid was extracted from the cells by using the QIAgen® Spin Miniprep Kit with the protocol designed for preparation of up to 10 μg of high or low copy plasmid. 10 μg of the isolated plasmid was used for the linearization reaction. The reaction mixture contained 10 μg of the purified vector, 2 μl Bstx 1 buffer, 0.2 μl of 10 mg/mL BSA, 1 μl BstX 1 and sterile water to make a total volume of 20 μl. The reaction mixture was incubated at 50°C for1 hour. At the end of 1 hour, reaction mixture was incubated at 65°C to heat inactivate the enzyme. About half of the linearization reaction mixture was analyzed by running 1 % agarose gel at 85 V for 1 hour and 10 minutes and the remaining was lyophilized and used for transformation into competent yeast cells.

Transformation of Pichia pastoris

The linearized H15S in pPICZα A vector was transformed into competent X-33 *Pichia* strain. For each transformation, 50 μl of X – 33 competent cells were added to 3 μg – 5 μg of linearized DNA pellet followed by addition of 1 mL of solution II (40%

PEG 1000, 0.2 M bicine, pH 8.35). The above reaction mixture was mixed by vortexing or flicking the tube. The transformation reaction was incubated for 1 hour at 30°C in heat block, mixing every 15 minutes by vortexing or flicking the tube. After incubation, the cells were heat shocked in a 42°C heat block for 10 minutes. The reaction mixture was split into two microcentrifuge tubes (approximately 525 µl per tube) followed by addition of 1 mL of YPD medium to each tube. The cells were incubated at 30°C for 1 hour to allow expression of Zeocin™ resistance. The two tubes were pelleted by centrifuging at 3000 x g for 5 minutes at room temperature and the cell pellet was resuspended in 500 µl of solution III (0.15 M NaCl, 10 mM bicine, pH 8.35). Cells from the two tubes are combined into one tube and pelleted at 3000 x g for 5 minutes at room temperature. The cell pellet was resuspended in 100 to 150 µl of solution III. The entire transformation was plated on pre - warmed YPDS plates (1% yeast extract, 2% tryptone, 1 M sorbitol, and 2% agar) supplemented with 100 µg/mL of Zeocin™ and incubated for 3 to 10 days at 30°C.

CHAPTER 3: RESULTS

Section I: Generation and Expression of Intracellular H15S Mutant

The H15S mutant was generated using the native lysozyme as the DNA template. The front end of ~ 325 bp and back end of ~ 75 bp obtained by two PCR reactions were annealed using PCR overlap extension method. The obtained 400 bp was cloned into pCR®4 - TOPO® (Figure 2 – 3 on page 42) and sequenced. After the gene sequence was confirmed, the mutant was ligated into pPICZ B and transformed into GS115 *Pichia pastoris* yeast strain.

Small scale expression in BMMY media

Two colonies of transformed GS115 with H15S in pPICZ B were grown in three different types of media to determine the optimal conditions for the expression of the H15S lysozyme protein. The three media were BMMY, BMMH, and MMH as described in the Materials and Methods chapter for intracellular expression (pages 32 – 34).

The *aox1* promoter of the *Pichia* strain regulates the expression of the protein. Cultures were induced by transferring exponentially growing cells from 1% glycerol to 0.5% methanol. The GS115 transformants were grown in shake – flask cultures and the expression was found to be dependant on the composition of the media. 1 mL cultures were collected at 24, 48, 72, and 96 hours. The cell lysate was analyzed by SDS – PAGE. A single band migrating parallel with native lysozyme was observed in the lysate on the BMMY, and BMMH media. No bands corresponding to lysozyme were observed for the MMH media. The amounts of the protein secreted at different time intervals and in various media are shown in Figures 3 – 1, 3 – 2, and 3 - 3.

A protein with a molecular weight of approximately ~14kDa was observed in BMMY media (at 72 hours) upon induction with 0.5% methanol. SDS – PAGE of the cell lysate of the two colonies grown in BMMY media are shown in Figure 3 – 1.

(a) 1 2 3 4 5 6 (b) 7 8 9 10 11 12

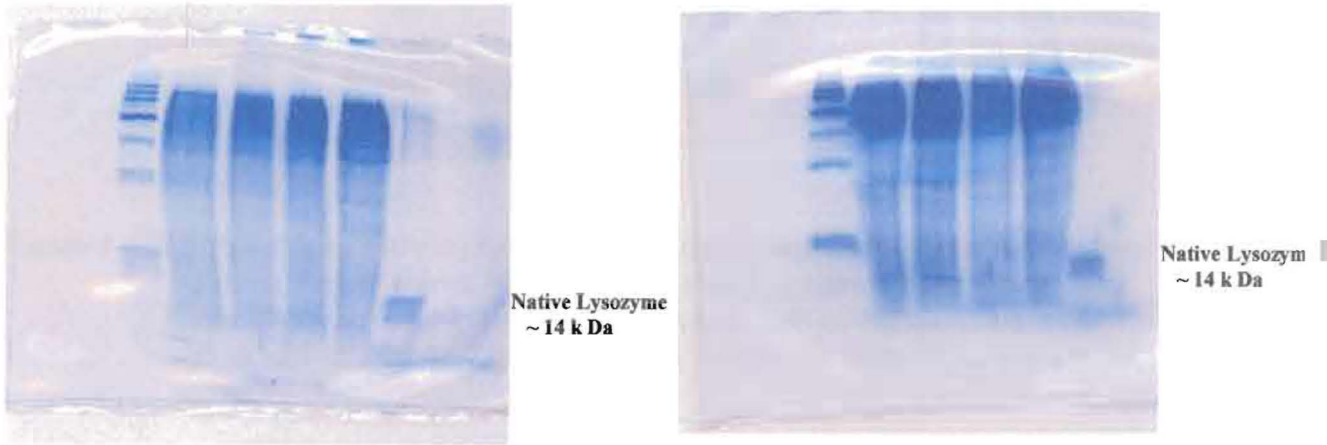

Figure 3 – 1: SDS – PAGE showing expressed protein bands in BMMY media by two colonies. Lane 1 and 7 shows MW markers. Lanes 2, 3 shows cell lysate from 24 hrs , lanes 4, 5 shows 48 hr cell lysate (a). Lanes 8, 9 shows cell lysate from 72 hr , lanes 10, 11 shows 96 hr cell lysate (b). Lanes 6 and 12 shows native lysozyme.

Small scale expression in BMMH media

Small scale expression in BMMH media was carried out as described in the materials and method chapter with two different colonies of GS115 containing the H15S mutant in pPICZ B. The cell pellet from 1 mL cell cultures were used for further analysis of the cell contents. SDS – PAGE was run with cell lysates from the two colonies collected at 24, 48, 72 and 96 hours grown in BMGH/ BMMH media. Bands of proteins with a molecular weight of approximately 14 kDa appear at 72 hours (Figure 3 – 2).

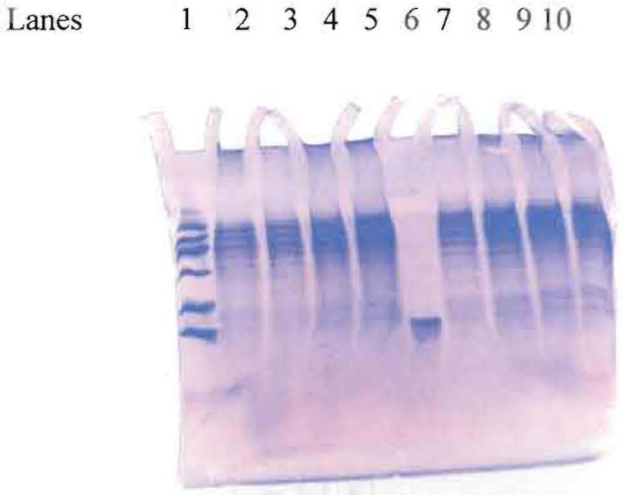

Figure 3 – 2: SDS – PAGE showing expressed protein bands in BMMH media from two colonies. Lane 1 shows MW marker. Lanes 2, 3 shows the lysate samples from 24 hours, lanes 4, 5 shows lysate samples from 48 hours. Lanes 6 shows native lysozyme, lanes 7, 8 shows cell lysate from 72 hr, lanes 9, 10 shows cell lysate from 96 hr.

Small scale expression in MMH media

The cell lysate of the two colonies grown in MMH were prepared and analyzed by SDS – PAGE. The SDS – PAGE below shows the bands of the cell lysate collected at 24, 48, 72 and 96 hours (Figure 3 – 3). No bands of ~14 kDa were observed.

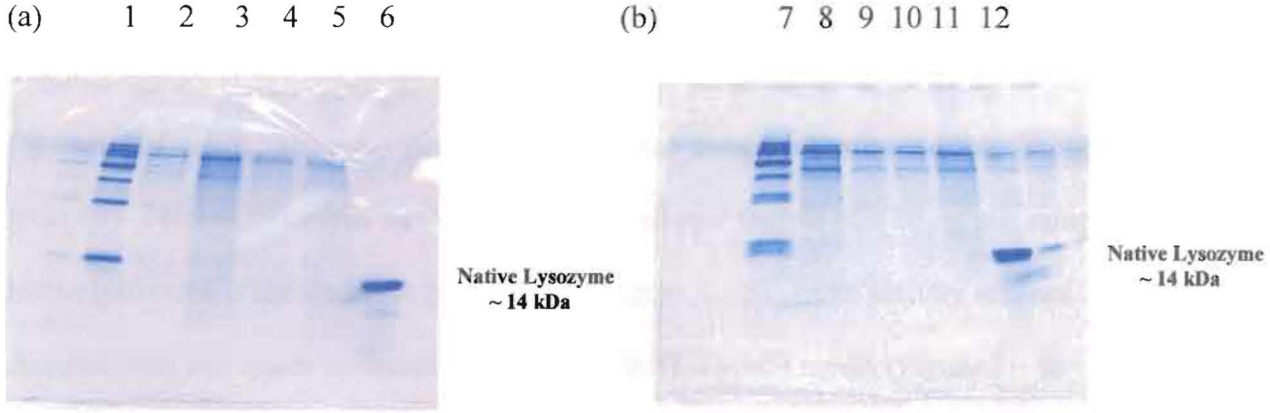

Figure 3 – 3: SDS – PAGE showing intracellular protein expression in MMH media by two colonies. Lane 1 and 7 shows MW markers. Lanes 2, 3 shows lysate from 24 hrs , lanes 4, 5 shows 48 hr cell lysate (a). Lanes 8, 9 shows cell lysate from 72 hr , lanes 10, 11 shows 96 hr cell lysate samples (b). Lanes 6 and 12 shows native lysozyme.

Scale - up expression in BMMH media

The cell paste obtained from the large scale experiment at 72 hours was lysed as described on page 36 of Materials and Methods. An SDS – PAGE was run with the collected lysate and also used for enzyme assay and an ELISA test.

Lanes 1 2 3 4 5 6 7 8 9 10

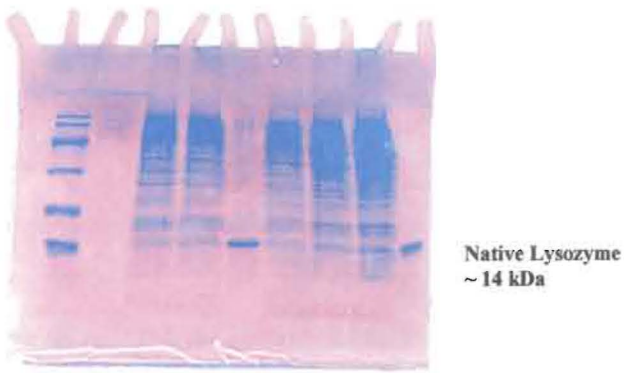

Figure 3 – 4: SDS – PAGE showing expressed protein bands at ~14kDa in BMMH media for large scale expression. Lane 2 shows MW markers, Lanes 4, 5, 7, 8, 9 shows 72 hr sample, lanes 6 and 10 shows native lysozyme.

Enzyme assay

Enzyme activity was determined by constructing a plot of absorbance versus time. The absorbance at 450 nm of the native lysozyme and lysate was recorded every 10 sec for a total of 4 min. The ΔA_{450}/minute was obtained using zero order kinetics fitting data from 40 – 240 sec. The lytic activity of a 100 µl aliquot sample of a 10 µg/mL sample of native lysozyme is shown in the graph below (Figure 3 – 5). Lytic activity was not detected with cell lysate collected at 72 hr in BMGH/BMMH media (Figure 3 – 6)

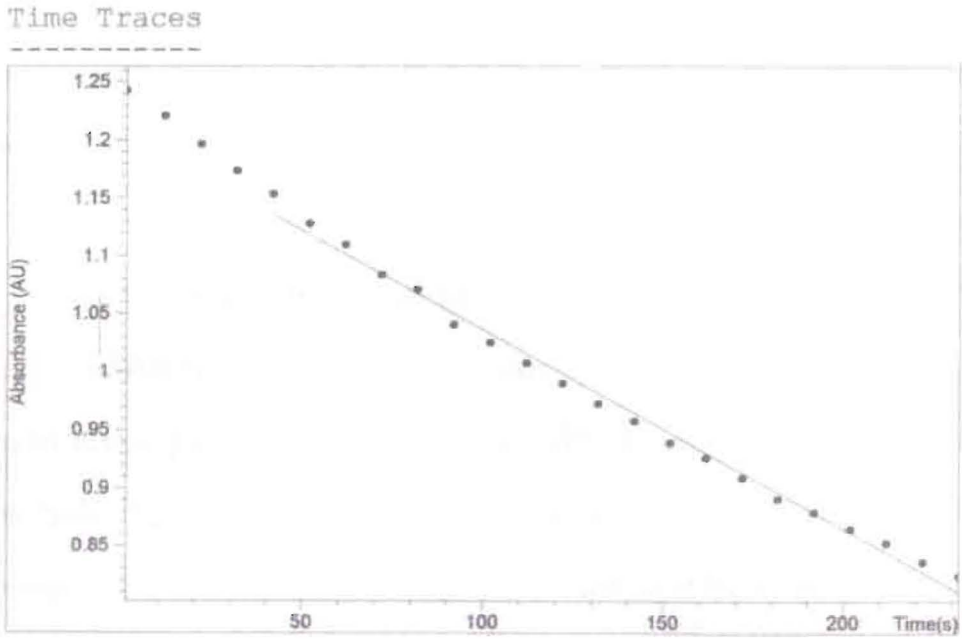

Figure 3 - 5: Graph showing lytic activity of native lysozyme

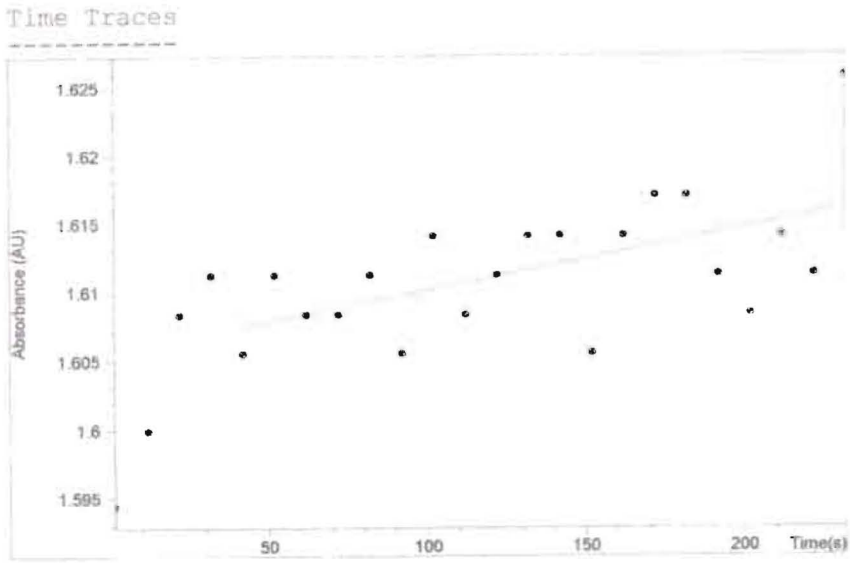

Figure 3 - 6: Graph showing lytic activity of lysate from BMMH media at 72 hrs.

Similar graphs were observed with cell lysate for different samples collected at

24, 48, 72 and 96 hours from BMMY, BMMH, and MMH media. It may be because the protein was inactive or it was produced in too low a quantity to be detected by the enzyme assay.

ELISA

An ELISA was performed to detect the presence of the mutant protein in the lysate of the BMMH media collected at 72 hours. Table 3 – 1 shows the absorbance at 450 nm for native lysozyme, and the lysate of the H15S transformed yeast *Pichia pastoris* strain. The average of the blank values from C1 – C4 was found to be 0.5575. The average of the absorbance of the 2 fold serial dilution of the native lysozymes was caluculated ((A1 + B1)/2 to (A12 + B12)/2). The average of the absorbance of the 2 fold serial dilution of the lysate was caluculated ((D1 + F1)/2 to (D12 + E12)/2). The average of the absorbance of the blank value was subtracted from the average of absorbance of native lysozyme and the lysate for each dilution (absorbace values for native lysozyme and lysate are in Table 3 – 2). A graph of the reciprocal of dilution and absorbance was plotted (Figure 3 – 7)

Table 3 – 1: Relative absorbance values at 450 nm, read by the microplate autoreader of native lysozyme, lysate, blank, positive and negative control samples.

		1	2	3	4	5	6	7	8	9	10	11	12
A	Lysozyme 100µg/mL	0.430	0.359	0.277	0.212	0.171	0.155	0.152	0.152	0.152	0.151	0.145	0.149
B	Lysozyme 100µg/mL	0.380	0.336	0.238	0.182	0.154	0.148	0.152	0.143	0.157	0.147	0.139	0.158
C	Blank	0.158	0.141	0.173	0.151	0.071	0.069	0.064	0.062	0.782	0.812	0.774	0.555
D	Lysate	0.144	0.149	0.146	0.147	0.161	0.191	0.192	0.196	0.222	0.227	0.221	0.222
E	Lysate	0.139	0.139	0.133	0.131	0.146	0.159	0.185	0.196	0.197	0.211	0.206	0.208
F	Controls	0.135	0.091	0.092	0.094								

Table 3 – 2: Table showing the reciprocal of dilution and relative absorbance values of the native lysozyme and mutant lysate BMMH sample collected at 72 hours

Reciprocal of dilution	Relative absorbance values at 450 nm after subtracting blank from average values	
	Native lysozyme	Mutant lysate
1	0.65425	-0.01425
2	0.19175	-0.01175
4	0.10175	-0.01625
8	0.04125	-0.01675
16	0.00675	-0.00225
32	-0.00425	0.01925
64	-0.00375	0.03275
128	-0.00825	0.04025
256	-0.00125	0.05375
512	-0.00675	0.06325
1024	-0.01375	0.05775
2048	-0.00225	0.05925

2 fold serial dilutions were made for both native and mutant lysate samples. To compare the lysate samples with that of native lysozyme no data points falling in the range of absorbance for lysate samples were used to plot the graph. The absorbance decreased with a decrease in the concentration for the native lysozyme whereas the absorbance was almost constant for the mutant lysate samples for various concentrations (Figure 3 – 7). The ELISA indicated that no lysozyme was detected in the mutant lysate sample.

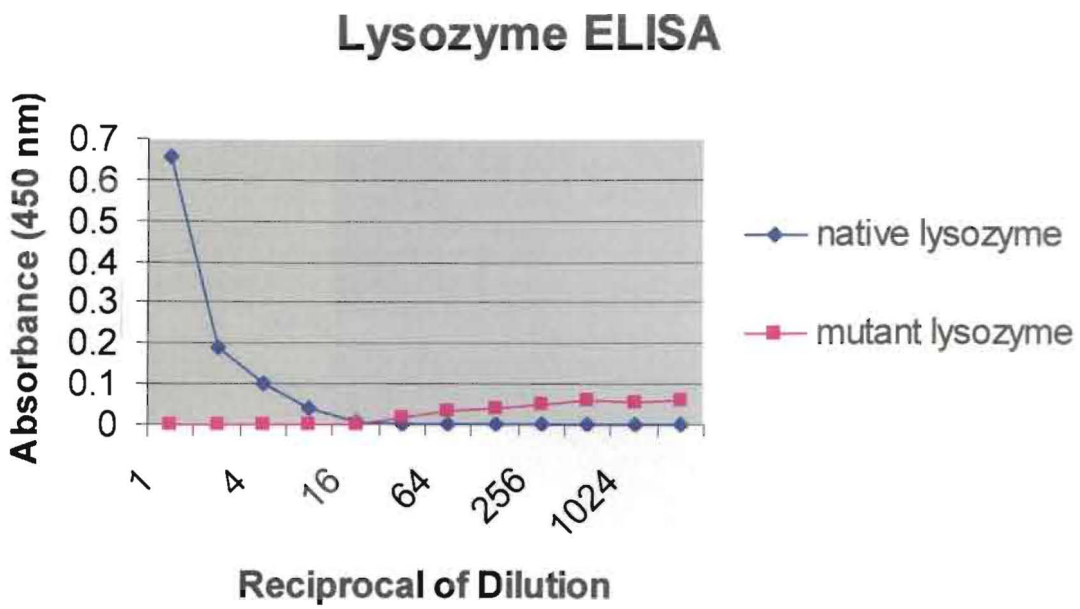

Figure 3 – 7: Graph plotted with the absorbance at 450 nm versus reciprocal of dilution for the native and the mutant lysate samples.

Section II: Generation of Extracellular H15S Mutant and Transformation of *Pichia pastoris*

The H15S mutant for intracellular expression was used as template for the creation of the H15S mutant for extracellular expression. The PCR product of the H15S template using 5′ Ex Lyso primer and Lyz – R primer was analyzed by a 1% agarose gel. The gel was visualized using the Stratagene imaging system. The gel shows a 400 bp fragment corresponding to the size of the lysozyme gene (Figure 3 – 8).

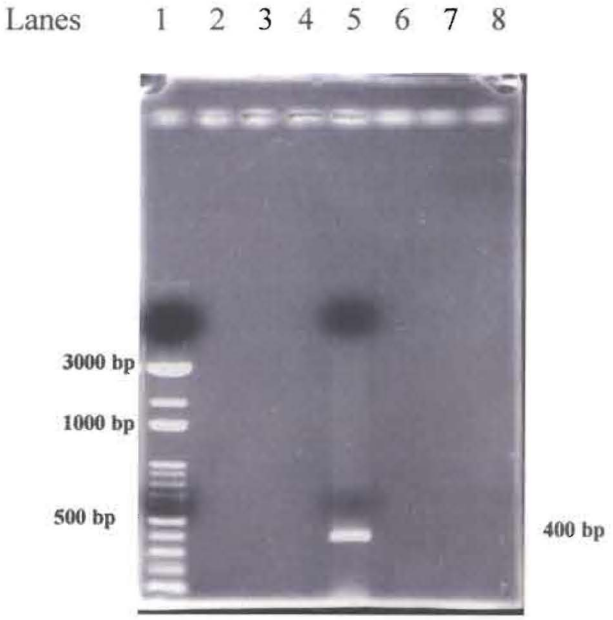

Figure 3 - 8: 1% agarose gel showing the PCR gene using 5′ – ExLyso and Lyz R primers. Lane 1 is a 100 bp DNA ladder. Lane 5 shows a 400 bp PCR product.

Restriction digestion of the cloned pCR®4 - TOPO® vector

The lysozyme H15S mutant in pCR®4 - TOPO® vector was restriction digested with XhoI and XbaI restriction enzymes. A 400 base pair fragment of the digested plasmid was seen on 1% agarose gel confirming the insertion of the gene into the pCR®4 - TOPO® vector. An agarose gel showing the bands corresponding to pCR®4 - TOPO® (~ 4.0kb) and H15S gene (~400bp) is shown in Figure 3 - 9.

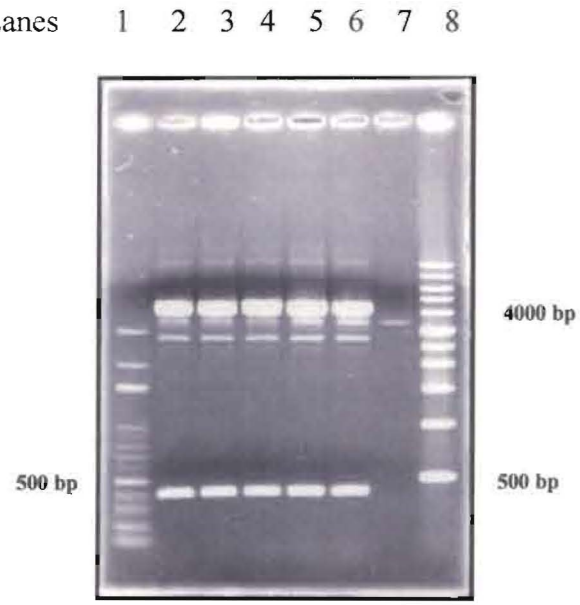

Figure 3 - 9: 1% agarose gel showing the pCR®4 - TOPO® vector and 400bp mutant Lysozyme gene. Lane 1 is 100 bp DNA ladder. Lanes 2, 3, 4, 5, 6 corresponds to pCR®4 – TOPO® vector (~ 4.0 kb) and the restriction digested product H15S lysozyme (~400 bp), lane 8 is 1 kb ladder.

DNA sequencing of the H15S mutant from transformed TOP 10 E. coli

The hen egg white lysozyme gene contains 387 nucleotides. The DNA sequencing confirmed the native lysozyme and the change of the histidine at position 15 to serine and the incorporation of α – factor signal sequence added to the front of the gene. This confirms the success of mutagenesis and the cloning experiments. A portion of the electropherogram sequence below confirms α – factor signal sequence to the front of the gene and histidine at position 15 changed to serine. The complete electropherogram is in the Appendix along with the DNA and amino acid sequence of native lysozyme (Figure 3 – 10).

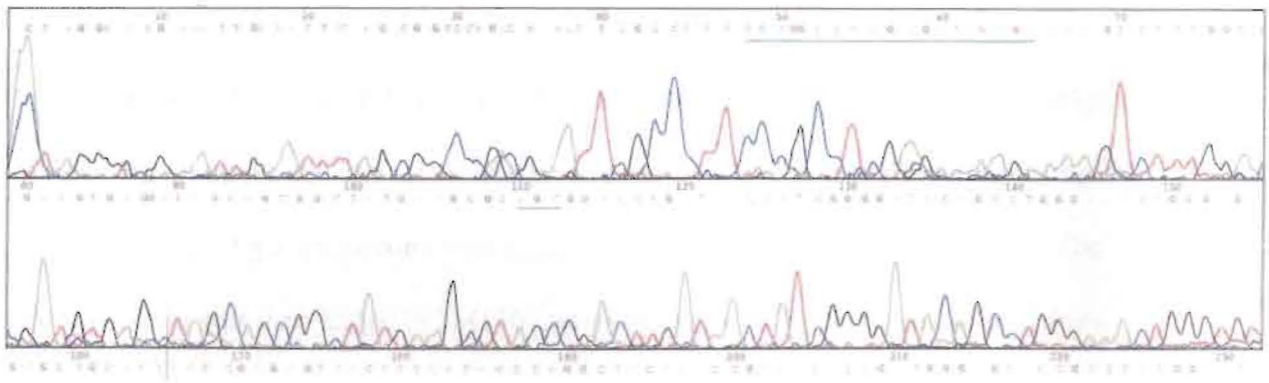

Figure 3 – 10: Sequence data from mutant lysozyme gene showing serine (AGC) at position 15 and the incorporation of the α – factor signal sequence.

Confirming the plasmid of the pPICZα A vector

The pPICZα A, B, C vectors in lyophilized form from Invitrogen were diluted to make a final concentration of 0.1 μg/μl. 1 μl of this diluted mixture was added to the JM110 competent cells from Stratagene. The transformed cells were grown on low salt LB plates containing Zeocin™. The presence of the plasmid was confirmed by 1% agarose gel electrophoresis. Bands of around 3.3 kb were seen in the gel. This corresponds to the size for the pPICZα A, B, C vectors (Figure 3 – 11).

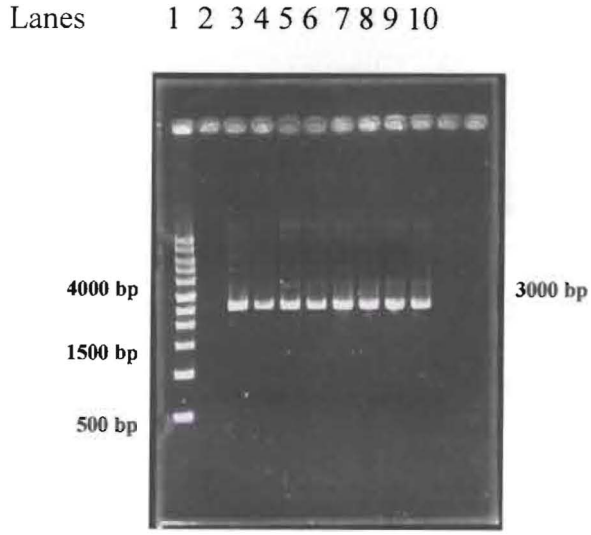

Figure 3 - 11: 1% agarose gel showing pPICZα A, B, C plasmids. Lane 1 is 1 kb DNA ladder. Lanes 3, 4, 5 shows pPICZα A vector, lanes 6, 7, 8 shows pPICZα B vector and lanes 9, 10 shows the pPICZα C vector.

Restriction digestion of the H15S mutant in pPICZαA vector

H15S/pCR®4 - TOPO® and the pPICZα A vectors were digested using restriction enzymes XhoI and XbaI. The H15S lysozyme gene was ligated into pPICZα A using T4 DNA ligase. The ligation mixture was incubated at room temperature for 1 hour. The ligated mixture was then added to JM110 competent cells. The plasmids isolated from four colonies of JM110 cell were digested with XhoI and XbaI restriction enzymes to confirm the ligation of H15S into pPICZα A vector. Bands of about 400 bp were observed in the 1% agarose gel confirm the ligation of lysozyme insert into pPICZαA vector (Figure 3 – 12).

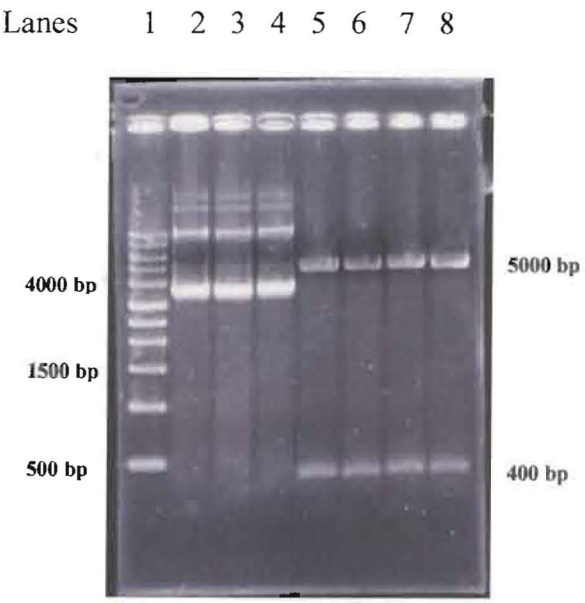

Figure 3 - 12: 1% agarose gel, Lane 1 is 1 kb DNA ladder. Lanes 2, 3, 4 shows the circular H15S/pPICZα A vector (~ 4.0 kb). Lanes 5,6,7,8 shows restriction digested sample of H15S/pPICZα A vector. A band of 400 bp corresponds to H15S and > 4 kb correspond to pPICZα A vector

Linearization of H15S/pPICZα A vector

The linearization reaction mixture mentioned in Materials and Methods chapter on page 50 was incubated at 50°C for1 hour. About half of the linearization reaction mixture was analyzed by running 1% agarose gel. The remaining half was used for transformation into the X – 33 *Pichia* strain. The result of the gel shows linearization of the complete sample by showing a single band of ~ 4.0 kb (Figure 3 – 13)

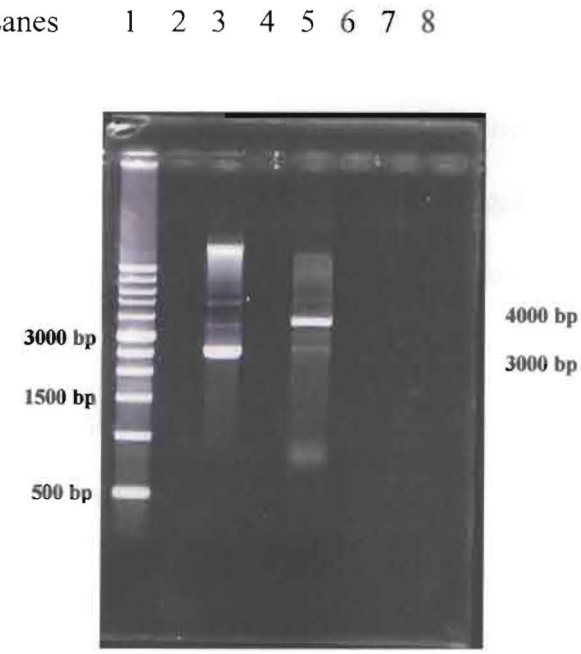

Figure 3 - 13: 1% agarose gel, Lane 1 is 100 bp ladder, lane 3 is the circular H15S/pPICZα A plasmid, lane 5 is linearized H15S/pPICZαA plasmid (~ 4 kb).

CHAPTER 4: DISCUSSION

Under normal conditions metal ions are tightly sequestered in biological systems. In certain disease states or in ischemia – reperfusion these metal ions are released. The metal ions either precipitate out of solution or form complexes with biological molecules. Reaction of the bound metal ion with H_2O_2 leads to the production of the $OH^{\cdot}$ which reacts in the immediate vicinity of its site of production. The damage has been referred to as site specific oxidation. The fact that only a few amino acid residues of a given protein are modified and that oxidation is insensitive to free radical scavengers led to the consideration of the overall process as being a "caged" reaction. This results, in the case of some enzymes, to conversion of some amino acids residues to carbonyl derivatives, to loss of catalytic activity, and to increased susceptibility of the protein to proteolytic degradation.

Selective damage to the histidine and arginine residues in glutamine synthetase is accompanied by alterations, such as protein fragmentation and changes in hydrophobicity. Exposure of lysozyme to Cu (II)/H_2O_2 resulted in initial loss of ϵ – amino groups of lysine and a subsequent increase in soluble amino groups. A number of metal catalyzed oxidation systems catalyse the inactivation of enzymes.[3, 8]

We are interested in examining what level of structure is most important in determining the site or sites of oxidation caused by the reaction between metal ions and H_2O_2. Hen egg white lysozyme (HEWL) was used in this study because it is a relatively small protein consisting of only 129 amino acid residues. It's primary and tertiary structure are known.

To examine the importance of the tertiary versus primary structure, we will generate a series of site directed mutants of HEWL. In native lysozyme histidine at position 15 is in a protective pocket (Figure 4 – 1). Histidine has been identified as one of the amino acid residue particularly susceptible to metal catalyzed oxidation. Based on an algorithm for safe substitutions developed by Bordo and Agos this histidine was changed to serine. We developed a H15S site – directed HEWL mutant that can be expressed in the yeast *Pichia pastoris*.

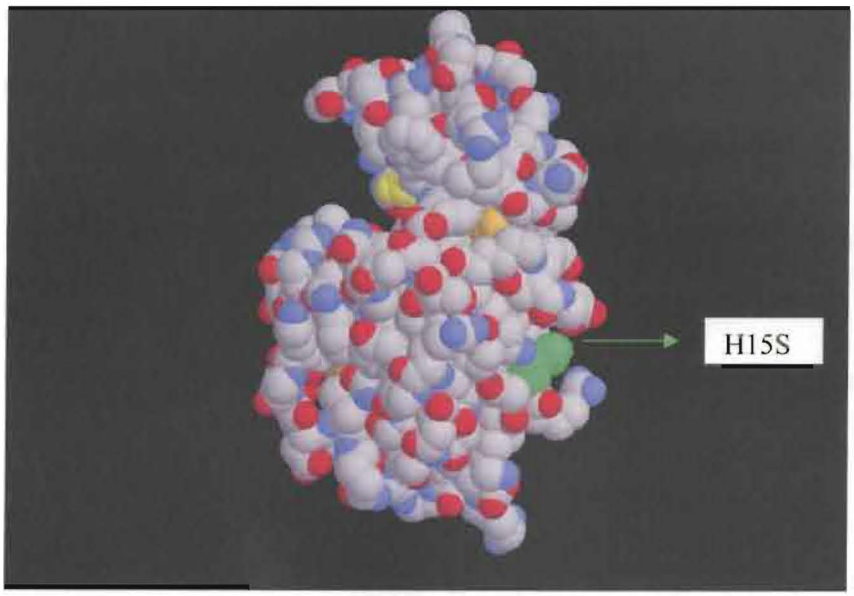

Figure 4 – 1: Tertiary structure of Hen Egg White Lysozyme showing H15S in a protective pocket

In this research, expression of the H15S mutant in pPICZ B in various media was performed. A faint protein band of ~ 14 kDa was seen on SDS – PAGE gel for the samples of the cell lysate at 24, 48, 72 and 96 hours in BMMY media and the protein produced was found to increase with time over a period of 96 hours. Bands had not appeared for BMMY and MMH media.

The large scale expression of the mutant protein was conducted using BMMH media. The culture was allowed to grow for 72 hours and was made 0.5% in methanol. The cells collected at 72 hours were lysed with breaking buffer and the collected lysate was used for further analysis of the mutant protein. Analysis of lysate by SDS – PAGE showed bands corresponding to the native lysozyme, but evidence from the enzyme assay and the more sensitive ELISA did not detect the presence of active protein. Perhaps the band corresponds to another protein of the same molecular weight. Other possibilities might be that the H15S mutant is catalytically inactive due to conformational changes or improper folding. Also, the mutant may not provide an epitope due to improper folding or the enzyme is not expressed in significant amounts. Since we didn't see protein expression with the intracellular mutant we switched to extracellular expression of the H15S mutant HEWL protein.

An H15S HEWL mutant gene capable of extracellular expression was generated by PCR and cloned into a pCR®4 - TOPO® vector. Initially, we attempted to create the extracellular H15S mutant using the intracellular H15S in the pCR®4 - TOPO® vector as template and 5` Ex lyso and Lyz – R as primers. It was found that the 5' Ex lyso primer was complimentary to a sequence on the pCR®4 - TOPO® vector upstream of the inserted gene.

To overcome this problem the H15S gene was extracted from the gel, purified and used as the template for the PCR reaction using 5` Ex lyso and Lyz – R primers. Top10 *E.coli* was used for transforming the cloned pCR®4 - TOPO® vector and the maintainence of the plasmid. The cloned gene was detected by double digestion with

XhoI and XbaI restriction enzymes. The mutant and α – factor signal sequence in front of the gene was confirmed by DNA sequencing using T3 and T7 primers.

Many attempts were made for ligating the mutant lysozyme into the XhoI and XbaI restriction sites of the pPICZα A vector isolated from *E. coli* cells. It was discovered that the XbaI restriction site of pPICZα A plasmid isolated from *E. coli* was getting methylated. Methylation inhibits cleavage by restriction enzymes. The pPICZα A vector was replicated in JM110 cells and used in the ligation experiment. JM110 competent cells do not have *dam* methylase or *dcm* methylase enzymes. The XbaI site of the plasmid isolated from JM110 cells is not methylated and can be digested using restriction enzymes XbaI and XhoI. However, propogation of vectors in these cells is only for efficient cleavage of the XbaI restriction site. Since JM110 cells have high frequency of recombination, Top10 E.coli cells were transformed with the recombinant plasmid. The mutant gene was ligated into XhoI and XbaI sites of pPICZ α A vector isolated from JM110 cells.

The pPICZα A isolated plasmid with the H15S mutant was used to transform the X – 33 competent *Pichia* cells. The isolated plasmid from X – 33 competent cells was verified by PCR and by running a 1% agarose gel. Future work includes confirming the mutant phenotype, followed by small scale expression, to determine growing conditions. Large scale expression will be carried out once the conditions have been optimized. The expressed protein will be subjected to metal catalyzed oxidation using Cu^{+2} / H_2O_2 and the oxidation pattern will be compared to native lysozyme. We also wish to develop a number of other mutants for comparison.

Conclusions:

Although the *Pichia pastoris* expression system is capable of producing protein, the expression level of the protein was not adequate to carry out metal catalyzed oxidation experiments with intracellular expression. The project was shifted to extracellular expression. The H15S HEWL mutant for intracellular expression was changed to a mutant for extracellular expression with α – factor signal sequence added to the front of the gene. This H15S mutant was ligated into pPICZα A vector and transformed into X – 33 *Pichia pastoris* strain. Further work has to be carried out on protein expression, conducting metal catalyzed oxidation experiments and comparing the oxidation pattern of the mutant H15S lysozyme with the native lysozyme.

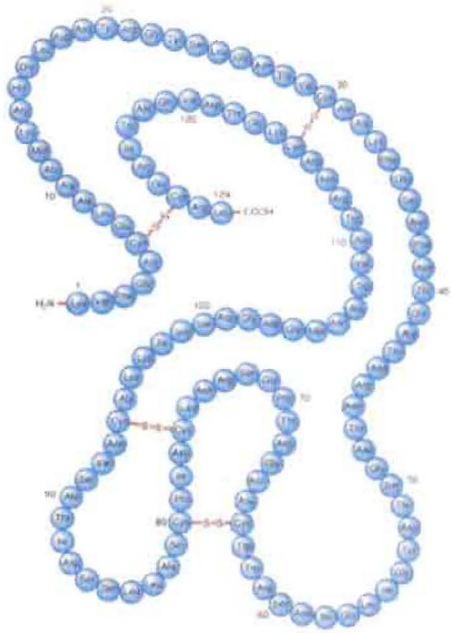

Figure 4 – 2: Primary structure of Hen Egg White Lysozyme

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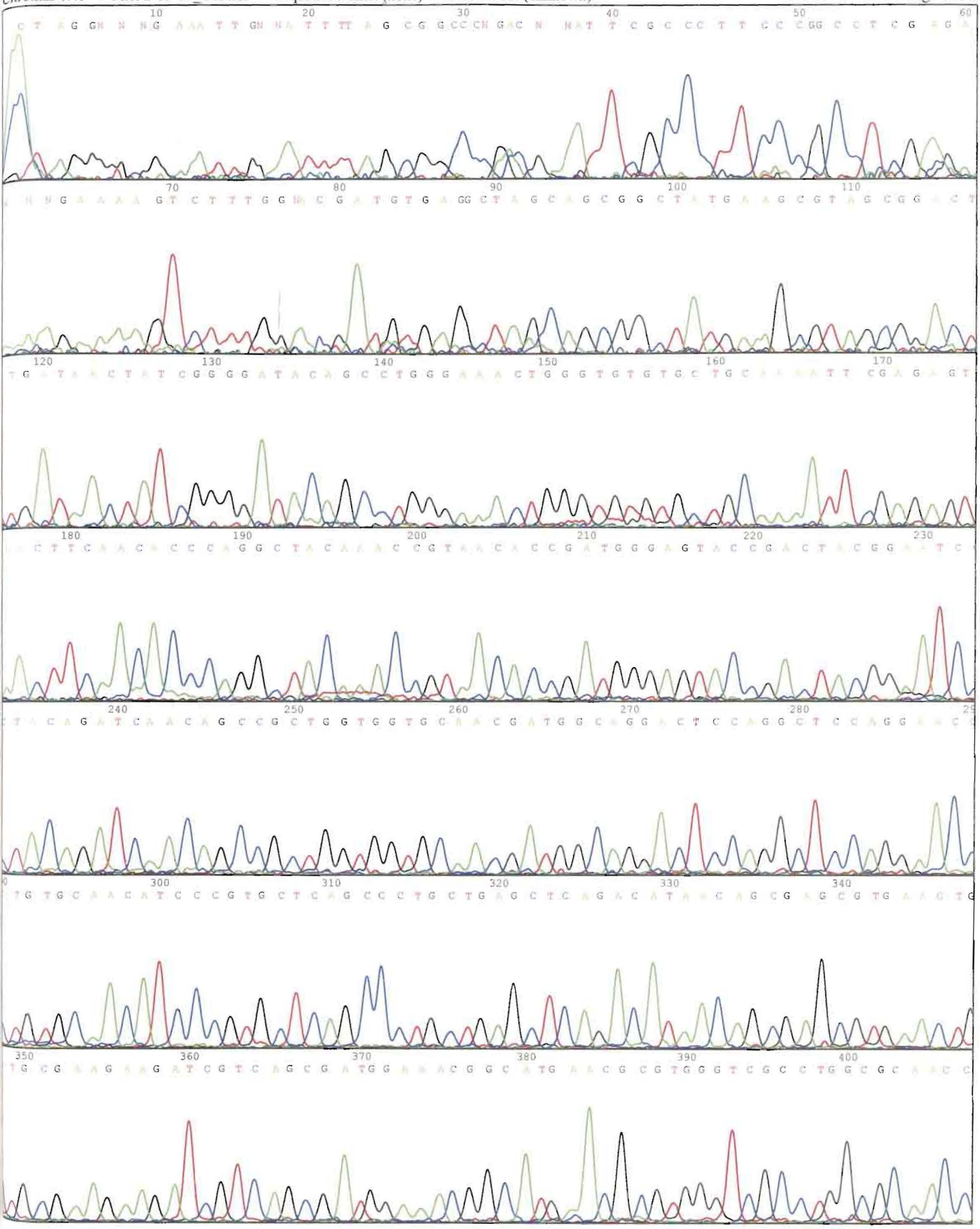
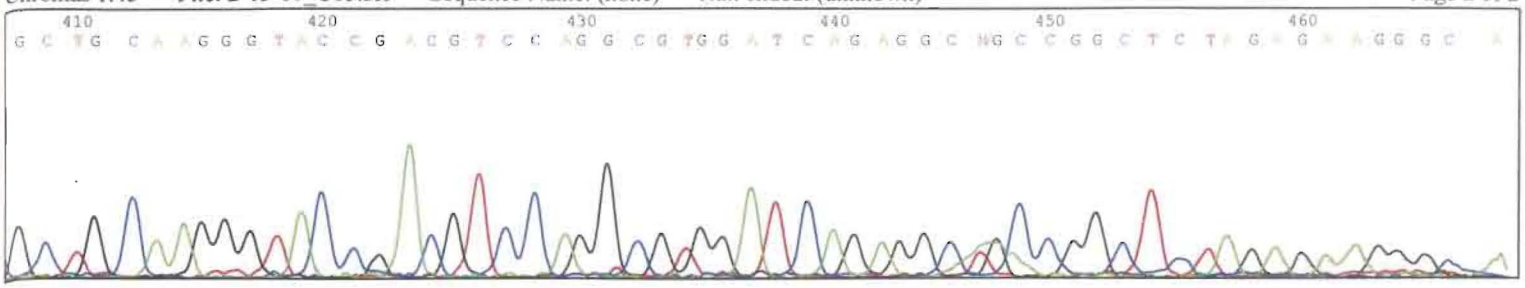
Appendix A: Hen Egg White Lysozyme Gene and Amino Acid Sequence

1-10	AAA	GTC	TTT	GGA	CGA	TGT	GAG	CTA	GCA	GCG
	Lys	Val	Phe	Gly	Arg	Cys	Glu	Leu	Ala	Ala
11-20	GCT	ATG	AAG	CGT	CAC	GGA	CTT	GAT	AAC	TAT
	Ala	Met	Lys	Arg	His	Gly	Leu	Asp	Asn	Tyr
21-30	CGG	GGA	TAC	AGC	CTG	GGA	AAC	TGG	GTG	TGT
	Arg	Gly	Tyr	Ser	Leu	Gly	Asn	Trp	Val	Cys
31-40	GCT	GCA	AAA	TTC	GAG	AGT	AAC	TTC	AAC	ACC
	Ala	Ala	Lys	Phe	Glu	Ser	Asn	Phe	Asn	Thr
41-50	CAG	GCT	ACA	AAC	CGT	AAC	ACC	GAT	GGG	AGT
	Gln	Ala	Thr	Asn	Arg	Asn	Thr	Asp	Gly	Ser
51-60	ACC	GAC	TAC	GGA	ATC	CTA	CAG	ATC	AAC	AGC
	Thr	Asp	Tyr	Gly	Ile	Leu	Gln	Ile	Asn	Ser
61-70	CGC	TGG	TGG	TGC	AAC	GAT	GGC	AGG	ACT	CCA
	Arg	Trp	Trp	Cys	Asn	Asp	Gly	Arg	Thr	Pro
71-80	GGC	TCC	AGG	AAC	CTG	TGC	AAC	ATC	CCG	TGC
	Gly	Ser	Arg	Asn	Leu	Cys	Asn	Ile	Pro	Cys
81-90	TCA	GCC	CTG	CTG	AGC	TCA	GAC	ATA	ACA	GCG
	Ser	Ala	Leu	Leu	Ser	Ser	Asp	Ile	Thr	Ala
91-100	AGC	GTG	AAC	TGT	GCG	AAG	AAG	ATC	GTC	AGC
	Ser	Val	Asn	Cys	Ala	Lys	Lys	Ile	Val	Ser
101-110	GAT	GGA	AAC	GGC	ATG	AAC	GCG	TGG	GTC	GCC
	Asp	Gly	Asn	Gly	Met	Asn	Ala	Trp	Val	Ala
111-120	TGG	CGC	AAC	CGC	TGC	AAG	GGT	ACC	GAC	GTC
	Trp	Arg	Asn	Arg	Cys	Lys	Gly	Thr	Asp	Val
121-129	CAG	GCG	TGG	ATC	AGA	GGC	TGC	CGG	CTC	TAG
	Gln	Ala	Trp	Ile	Arg	Gly	Cys	Arg	Leu	Stop

Note: The gene was mutated at the stop codon to incorporate an Xba I restriction site (T↓CTAGA). The change was as follows:

CTG	TAA	GTC	to	CT***C***	TA***G***	***AG***C
Leu	Stop	Val		Leu	Stop	Ser

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