Investigation of Site-Specific Metal Catalyzed Oxidation of the Lysozyme Double Mutant (H15S + N77H)

by

Uma Thatipally

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Uma Thatipally

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Signature:

T. Thatipally 6/15/06

Uma Thatipally, Student Date

Approvals:

Michael Serra 6/13/06

Dr. Michael A. Serra, Thesis Advisor Date

Jeffrey A. Smiley 6/14/06

Dr. Jeffrey A. Smiley, Committee Member Date

Gary Walker 6/15/06

Dr. Gary Walker, Committee Member Date

Peter J. Kasvinsky 6/16/06

Peter J. Kasvinsky, Dean of Graduate Studies Date

Thesis abstract

Although oxidative damage to proteins by reactive oxygen species is significant in many diseases, there are many open questions regarding the site –specificity of this damage by reactive oxygen species. The highly reactive hydroxyl radical reacts with the amino acids at the metal binding site or with the residues in the vicinity of metal binding site. This thesis investigates the site specificity of metal-catalyzed oxidation of a mutant hen egg white lysozyme using Cu^{+2} and hydrogen peroxide to generate the hydroxyl radical. The double mutant lysozyme gene was generated with Asn77 changed to His (N77H) and His15 changed to Ser (H15S) by site-directed mutagenesis. The lysozyme mutant gene was cloned into the yeast expression vector, pPICZ B. The methyltropic yeast *P. pastoris* was transformed with the vector to express the mutant protein. Various culture conditions were used to optimize protein production. Methods like HPLC and gel chromatography were used in an attempt to separate the mutant 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
™	Trademark
%	Percent
Abs	Absorbance
bp	Base pair
BSA	Bovine serum albumin
Cu	Copper
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
Fe	Iron
hr	Hour
His	Histidine
LDL	Low density lippoprotein
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 I: INTRODUCTION

Reactive oxygen species (ROS) include the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$) and lipid peroxides (LOOH). These species react with virtually all classes of biomolecules, including DNA, RNA, lipids, carbohydrates, proteins and antioxidants. Free radical damage and the resulting accumulation of deactivated biomolecules are significant in several disease states including neurological disorders like Parkinson's and Alzheimer's, premature aging known as progeria, as well as in the normal process of aging.[1]

ROS can be generated by different processes including both environmental factors and physiological pathways. Various exogenous sources include all ionizing radiations, or visible light in the presence of a sensitizer, pollutants like asbestos, ozone, cigarette smoke, and nitrogen oxides. Byproducts of normal metabolic processes like electron transport chains of mitochondria and cytochrome P_{450}, xenobiotics and transitional metal ions are some of the physiological sources.[1] The most significant source of ROS *in vivo* results from the reaction of transition metal ions such as copper and iron with endogenously produced H_2O_2 (eq 1).[2]

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

Oxidative degradation in the production and storage of protein pharmaceuticals can be induced by transition metals in the presence of contaminant oxidants and light, which is also a major concern.[3]

Free radicals and Reactive oxygen species

Free radical chemistry has been an extensive research topic in modern chemistry.[4] A free radical is defined as any species with an unpaired electron in the outer orbital. Consequently, they are some of the most chemically reactive molecules known. Because of the need to pair its single electron, a free radical abstracts a second electron from a neighboring molecule. This causes the formation of yet another free radical and a self-propagating chain reaction ensues.[5]

Oxygen, because of its bi-radical nature, readily accepts unpaired electrons to give rise to a series of partially reduced species collectively known as reactive oxygen species (ROS). The sequential one electron reduction of molecular oxygen results in the formation of these various ROS endogenously in biological systems and the relationships are represented below (eq 2).[6]

$$O_2 \xrightarrow{e^-} \underset{\text{superoxide anion}}{O_2^{\bullet -}} \xrightarrow{e^- + 2H^+} \underset{\text{hydrogen peroxide}}{H_2O_2} \xrightarrow{e^-} OH^- + \underset{\text{hydroxyl radical}}{OH^{\bullet}} \xrightarrow{e^- + H^+} H_2O \qquad (2)$$

The ability of transition metals to accept and donate single electrons makes them important catalysts of free radical reactions. Hydrogen peroxide, upon contact with various metals ions like Cu(I), Fe(II), and Co(II), can produce the extremely reactive $OH^{\bullet}$. Fe^{+2} ions react with H_2O_2 to form $OH^{\bullet}$. This is known as the Fenton reaction (eq 3).[2]

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^- + OH^{\bullet} \qquad (3)$$

A two step reaction known as the metal catalyzed Haber-Weiss reaction illustrates how metal ions in higher oxidation states can be reduced to catalyze the reduction of H_2O_2 (eq 4).

$$O_2^{\bullet -} + Fe(III) \longrightarrow Fe(II) + O_2$$
$$\underline{Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^- + OH^{\bullet}}$$
$$\text{Fe catalyst} \qquad (4)$$
$$\text{Sum: } O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet}$$

Copper(II) catalyzes a similar reaction with hydrogen peroxide. Moreover, the rate constant for the reaction of copper with H_2O_2 is several orders of magnitude greater than that for Fe(II). A biological reducing agent such as ascorbic acid can replace $O_2^{\bullet -}$ as a reducing agent in *in vitro* systems for the formation of hydroxyl radicals.[2]

The hydroxyl radical is the most reactive of the ROS. Hydroxyl radicals are highly reactive with short half lives and high rate constants on the order of 10^9 - 10^{10} M^{-1} sec^{-1}. As a result, hydroxyl radicals react with the biomolecules at diffusion controlled rates, which means the reaction will occur immediately at the site of formation. Therefore, if both H_2O_2 and a metal ion catalyst are available *in vivo*, then $OH^{\bullet}$ will form in biologically significant amounts. Iron and copper are the two physiologically most relevant and commonly studied transition metal ions. [2, 6]

The kinetic data of rate constants for reaction of various reactive radicals with biological molecules indicates proteins are major targets and that the $OH^{\bullet}$ is the most reactive. The rate constants for reaction of $OH^{\bullet}$ with collagen is 4 x 10^{11} dm^3 mol^{-1} s^{-1}, for DNA is 8 x 10^8 dm^3 mol^{-1} s^{-1}, and for albumin is 8 x 10^{10} dm^3 mol^{-1} s^{-1}. The difference in the rate constants of $OH^{\bullet}$ with different molecules is very small ($10^7 - 10^{10}$)

which means the hydroxyl radical is not selective in regards to its targets. Proteins are the main target of oxidation by ROS as they are the major components of most biological systems.[1]

X-rays and γ-rays can produce hydroxyl radicals by cleavage of O-H bonds in H_2O. UV ray exposure can produce singlet oxygen.[7] Singlet oxygen is formed when pigment systems are exposed to radiation in the presence of O_2. The pigments that absorb light enter a higher electronic excitation state and transfer energy onto the O_2 molecule to give singlet oxygen. One such example is the lens of the eye. A few diseases can also lead to the formation of singlet oxygen, for example patients with porphyrias are sensitive to light and produce excessive singlet oxygen.[2]

One electron reduction of oxygen produces superoxide, $O_2^{\bullet -}$. The major source of $O_2^{\bullet -}$ is leakage of electrons onto O_2 in the electron transport chain. $O_2^{\bullet -}$ production also occurs during the respiratory burst of phagocytes like monocytes and macrophages. Superoxide radical is protonated to form hydroperoxyl radical $HO_2^{\bullet}$, which is much more reactive than $O_2^{\bullet -}$ *in vitro*. $HO_2^{\bullet}$ can initiate lipid peroxidation and decompose lipid peroxides whereas $O_2^{\bullet -}$ cannot. However, with a pK_a of 4.8 only a small fraction of $O_2^{\bullet -}$ is protonated at physiological pH. $O_2^{\bullet -}$ can undergo a dismutation reaction in aqueous solution producing H_2O_2 (pH 7, K= 5 x 10^5 M^{-1} s^{-1}). With no unpaired electrons H_2O_2 is not a radical, but it can more easily cross cell membranes than can $O_2^{\bullet -}$. A high level of H_2O_2 can inactivate the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase.[2, 8]

Anti-oxidant defense mechanism

A number of antioxidant defenses have evolved in living organisms to remove ROS. Superoxide dismutase, a mitochondrial enzyme, removes $O_2^{\bullet -}$ by accelerating its conversion to H_2O_2. Catalases in peroxisomes dispose of H_2O_2 by converting it into H_2O and O_2, and glutathione peroxidases (GSHPX) removes H_2O_2 by using it to convert reduced glutathione (GSH) to oxidized glutathione (GSSG). Sequestration of transition metal ions in storage proteins that do not catalyze any free radical reactions is another important defense mechanism, especially in the extracellular environment where levels of SOD, GSHPX and catalase are very low. Although the availability of catalytically active forms of iron and copper that generate $OH^{\bullet}$ are very low *in vivo*, when free metals do become available they will not exist in the free state for long. They will bind to biological molecules or else precipitate out of solution as hydroxides or phosphates.[8]

Methionine is readily oxidized to methionine sulfoxide by ROS due to its low oxidation potential. The rate constant for reaction of $OH^{\bullet}$ with methonine is 8.5×10^9 $dm^3\ mol^{-1}\ s^{-1}$. The repair enzyme methionine sulfoxide reductase is capable of reducing oxidized methionine back to methionine. This redox reaction is believed to play a role in protecting other functional residues form oxidative damage thus functioning as an antioxidant. The antioxidant function of methionines in proteins is not limited to the protein itself. For example, high density lipoproteins reduce cholesteryl ester hydroperoxides to alcohols, with simultaneous oxidation of two methionine residues to sulfoxides. The oxidized apolipoprotein is reduced by methionine sulfoxide reductase so that it once again functions as catalyst in the reduction of the hydroperoxides.[1, 9]

Metal Catalyzed Oxidation and the Site-specific Nature of MCO

The main source of hydroxyl radicals *in vivo* is the reaction between metal ions and hydrogen peroxide. This is known as metal catalyzed oxidation (MCO). With high concentrations of Fe(II), Cu(II) and H_2O_2, which is unusual under normal physiological conditions, all amino acids are possible targets for attack but with low concentrations, protein modification occurs only at particular sites of the protein because the metals bind to specific sites on the protein.[10] Thus, it is proposed that MCO of proteins at physiological conditions is a site-specific reaction at the metal binding site on the proteins due to the high rate of reaction of $OH^{\bullet}$. The site-specific nature of the reaction is indicated by a number of characteristics. The inactivation of enzymes by MCO systems is not inhibited by free radical scavengers. One or only a few amino acids in a protein are modified by MCO, whereas almost all amino acids can be modified when proteins are exposed to free radicals generated by radiolysis. And most of the enzymes that are highly sensitive to modification by MCO usually require metal ions for catalytic activity. Therefore, they contain a metal binding site. In the case of *E.coli* glutamine synthetase, it was found that the amino acids histidine and arginine situated close to the metal binding sites on the enzyme are uniquely sensitive to MCO by a site-specific mechanism , oxidation of these residues resulted in loss of activity.[10] Mechanistic studies also identified two oxidation-sensitive His residues in human growth hormone (His18 and His21), which are located within a metal (Zn^{2+}) binding site at the interface between helix I and helix IV in the four helix bundle protein. In contrast, a surface-exposed His residue, which was not a part of metal-binding site, was resistant to metal-catalyzed oxidation. Site-specific cleavage was also observed when Cu, Zn superoxide dismutase

was exposed to hydrogen peroxide, initial cleavage occurred at Pro^{62}- His^{63} residues, followed by random degradation. Catalase inhibited both site specific and random cleavage whereas EDTA blocked only random oxidation.[12] Side chains of lysine and proline are major sites of attack in collagen by $Cu(I)/H_2O_2$ and $Cu(II)/H_2O_2$ systems. Damage by Cu(I) was much more extensive than by Cu(II) or Fe(II) and such degradation of collagen is believed to play an important role in progress of rheumatoid arthritis.[13]

MCO systems are used as a tool to identify the amino acids in the metal binding sites of certain metallo proteins. Oxidative modifications occur at the amino acids bound to the metal. Kurahashi *et al* used MCO/MS to identify the amino acids oxidized upon treatment with H_2O_2.[14] Schoneich and coworkers used MS/MCO to identify copper binding residues in bovine growth hormone.[15]

Oxidative stress

Generally ROS generation and antioxidant defenses are more or less balanced *in vivo.* Oxidative stress results when there is an imbalance in favor of ROS. Tissue injury by disease may release metal ions from their storage sites, leading to $OH^{\bullet}$ generation and oxidative stress. For example, activated phagocytes produce $O_2^{\bullet -}$ and other ROS and cause damage as in inflamed joints in rheumatoid arthritis patients.[8]

Metal catalyzed oxidation of amino acids of proteins leads to the formation of protein carbonyls. Elevated levels of protein carbonyls are found in many disease states. Levels of protein carbonyls are found to be high in the synovial fluid of patients with rheumatoid arthritis and in human lens in cataractogenesis.[10] It is believed that the MCO products of LDL are taken up by macrophages to form foam cells which may cause atherosclerosis. Iron and oxidative specific epitopes on proteins were found in the

extracts of atherosclerotic plaques.[16] Free radicals are involved in ischemia/ reperfusion injury. In neurological disorders, protein carbonyls are formed upon incubation of myelin with $Cu(II)/H_2O_2$. In individuals with the premature aging diseases, progeria and Werner's syndrome, the levels of protein carbonyl are similar to those found in 80- year-olds. Elevated levels of oxidized proteins are also found in other diseases like muscular dystrophy, respiratory distress syndrome, amyotrophic lateral sclerosis, and diabetes and in aging. Exponential increase of carbonyl content with age was observed in human brain, eye lens, erythrocytes, and the brain and kidney of mice. Interestingly, caloric restrictions in experimental mice lead to an increase in life span and a decrease in the levels of oxidized protein.[10, 17]

Since oxidative stress leads to an increase in the levels of protein carbonyls. The formation of protein carbonyls is used as a measure of oxidative damage in various pathophysiological conditions. Several sensitive carbonyl assays have been developed to measure protein oxidation, however, it is noteworthy that carbonyl moieties can be introduced onto the proteins by mechanisms other than the oxidation of amino acids.

For example, stable covalent thioether adducts carrying carbonyl groups are formed when α, β unsaturated alkenals produced during lipid peroxidation reacts with sulfhydryl groups of proteins and reaction of glucose with ε-amino group of lysine in proteins forms ketoamine protein conjugates.[18]

Giulivi and Davies, Wagley *et al.* proposed dityrosine as a biomarker for oxidative stress. Several experiments have shown that the formation of dityrosine was consistent in various proteins exposed to UV radiation, γ-radiation, and oxygen radicals and in aging.[18]

Dityrosine concentrations were found to be 100-fold higher in low-density lipoproteins isolated from atherosclerotic lesions than in normal ones.[18] Patients with systemic bacterial infections had twice the concentration of dityrosine in urine than those of healthy individuals. Moreover, no dityrosine was detectable in pronase digests of control hemoglobin which is not exposed to oxygen radicals but dityrosine was obvious in pronase digest of H_2O_2- exposed hemoglobin.[18]

The first step in the formation of dityrosine is the formation of tyrosine radical. The reduction potential of the tyrosine$O^{\bullet}$/tyrosineOH couple is 0.88 V indicating that highly reactive species like hydroxyl radical, oxoferryl radical can drive the reaction. Two monomeric molecules of tyrosine containing proteins cross link to form dityrosine.

Oxidatively modified proteins are more susceptible to degradation by proteolytic enzymes. Dityrosine is released when H_2O_2-treated oxyhemoglobin is incubated with proteases but not in control hemoglobin and its formation is increased by proteases, and inhibited by protease inhibitors. Thus, dityrosine formation is not only considered as a marker for oxidative damage but also as a specific marker for the selective degradation of oxidatively modified proteins.[18]

Methionine sulfoxide has been detected in many diseases and in *in vitro* and *in vivo* systems. Methionine sulfoxide, an oxidation product of methionine is another potential marker for oxidative damage.[1]

Modification of proteins

Exposure of proteins to oxygen free radicals may alter every level of protein structure. The modification of proteins by ROS was discovered by studying the effects of

ionizing radiation. Pioneering research done by Garrison,[19] Swallow,[20] and Schussler and Schilling [21] discovered numerous types of protein modifications.[17]

Cross-linking

Oxidative modification of proteins can lead to formation of intra or inter-peptide cross-linked derivatives by several different mechanisms. For example, carbon-carbon covalent linkages are formed by the interaction of carbon centered radicals in two different proteins (Figure 1-1). Extensive tyrosine- tyrosine (dityrosine) cross linking can be found in several proteins when exposed to UV radiation and oxygen radicals. Oxidation of cysteine's sulfhydryl groups forms disulfide cross-links.

Cross-linking of the carbonyl groups of oxidized proteins with the ε-NH_2 groups of lysine residues in the same or a different protein, interactions of glycoxidation derived protein carbonyls with either a lysine or an arginine residue of the same or a different protein molecule are few other examples of protein cross-linking. Cross-linked proteins are resistant to proteolytic degradation and can accumulate. Accumulation of cross-linked complexes is common in several disease states and in aging.[9, 17]

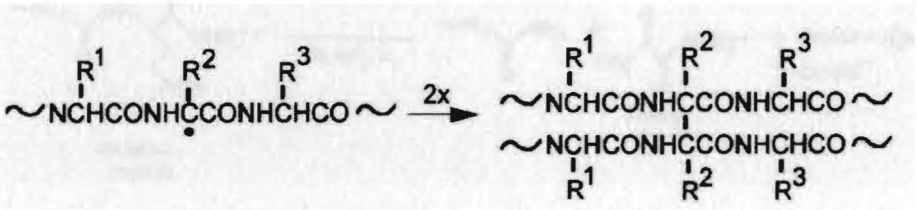


Figure 1-1: Carbon-carbon cross linking.

Peptide bond cleavage

Peptide bond cleavage of proteins can occur via the α-amidation or diamide pathway. In α-amidation the C-terminal amino acid peptide fragment derived form the N-

terminal region of a protein exists as the amide derivative and the N-terminal amino acid of the peptide fragment derived form C-terminal portion of protein exists as a α-keto-acyl derivative (Figure 1-2). Whereas in diamide pathway the C-terminal amino acid of the fragment derived from the N-terminal portion of the protein exists as the diamide derivative and the N-terminal amino acid of the peptide fragment derived from the C-terminal region of the protein exists as the isocyanate derivative.[9]

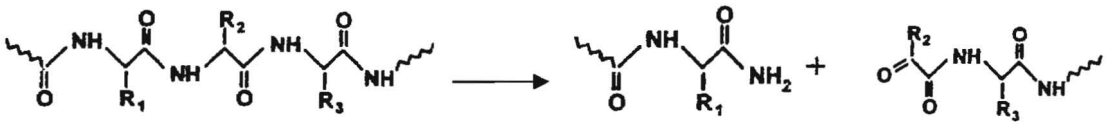


Figure 1-2: α-amidation pathway.

The oxidation of glutamyl and aspartyl residues of proteins can also lead to peptide bond cleavage in which the N-terminal amino acid of the C-terminal fragment will exist as the N-pyruvyl derivative (Figure 1-3).

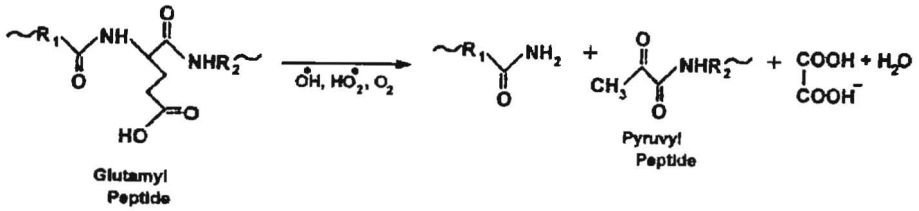


Figure 1-3: Peptide bond cleavage by oxidation of glutamyl residues.

The studies of Uchida *et al.* showed that the oxidation of proline residues of proteins can lead to peptide bond cleavage. The prolyl residue is oxidized to the 2-pyrrolidone derivative and subsequent hydrolysis yields 4-aminobutyric acid (Figure 1-

4). Therefore, the presence of 4-aminobutyric acid indicates peptide bond cleavage via prolyl oxidation pathway.[9]

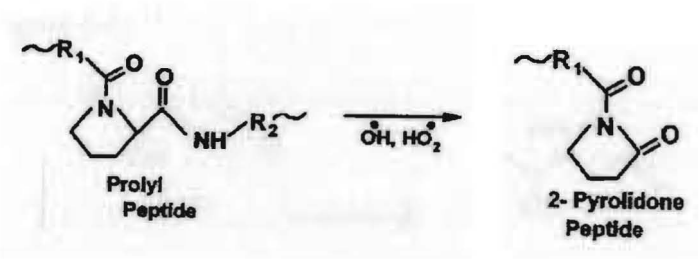


Figure 1-4: Oxidation of proline.

Oxidation of Amino Acid Residue Side Chains

The side-chains of amino acid residues in proteins are readily oxidized by metal ion-catalyzed oxidation systems. The electron rich side-chains of lysine, histidine, methionine, tryptophan, cysteine, and proline are most susceptible to oxidation. The Lysine residue is assumed to be oxidized by Fe(II), a protein chelate complex is formed by the binding of Fe(II) to the ε-NH_2 of lysine which then reacts with H_2O_2 to generate a $OH^{\bullet}$ that attacks the lysine moiety leading to its conversion to the 2-amino-adipic semi-aldehyde residue.[10, 17]

Lipid hydroperoxides can modify the imidazole side chain of histidine by a Michael addition reaction to form asparagines. 2-oxo histidine is detected in *in vitro* oxidation of many proteins such as Cu, Zn-superoxide dismutase, human growth hormone, and oxidized low density lippoproteins.[22]

Protein carbonylation

As already discussed direct oxidation of proteins with ROS yield peptide fragments possessing reactive carbonyl derivatives. Carbonyl groups can also introduced

via Michael addition reactions of 4-hydroxy-2-nonenal, a product of lipid peroxidation, with the ε-NH_2 of lysine, the imidazole moiety of histidine, or the sulfhydryl group of cysteine (Figure 1-5).[17]

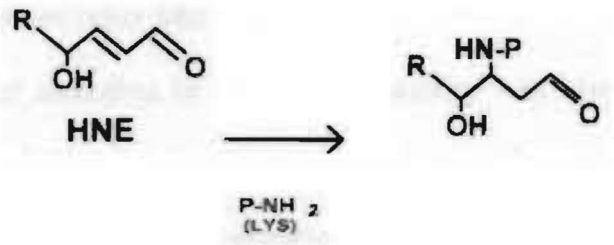


Figure 1-5: Conjugation of a lysyl residue of a protein with α, β- unsaturated aldehydes.

The process of glycoxidation may introduce carbonyl groups into the proteins by the reaction of oxidized sugars with ε-NH_2 group of lysine. The reactions of proteins with ROS lead to the conversion of some amino acid residues to aldehyde or ketone derivatives, thus level of protein carbonyls is measure of oxidative protein damage.[17]

Conformational changes and effects on structure and function

The oxidation of proteins side chains can lead to unfolding and conformational changes in proteins which consequently affects biological function.[1] Recent studies suggest that the oxidation of surface exposed residues have less effect on protein conformation than oxidation of buried residues. Solvent accessible Met residues are much more readily oxidized than buried residues, but oxidation of exposed Met does not affect the conformation of most proteins unless it is in the binding pocket. The Met side chain is relatively non polar compared to other amino acid side chains. From the calculations of free energies for transfers, with the formation of Met sulfoxide, which is more hydrophilic, there is a driving force for the alteration of the protein structure to

place this oxidized residue in a more polar environment. Thus, oxidation of initially buried Met residue may affect the protein structure. For example oxidation of surface exposed Met residue in Ca^{+2} dependent modular protein has no effect on structure whereas oxidation of other Met residues lead to detectable changes in protein structure. The role of Met oxidation in the loss of activity was also studied by site-directed mutagenesis of subtilisin. A mutant protein with Met222 changed to Ser, Ala, or Leu was resistant to oxidation by high concentration of H_2O_2.[1]

Histidine oxidation can result in loss of a basic amino acid residue which can alter the net charge on a protein which will consequently affect the hydrophobicity and the isoelectric point of the protein. It may change the conformation of protein.[3]

Research problem

Some researchers argue that site–specific damage caused by MCO systems results from a "caged" reaction in which metal ions bind in a pocket on a protein's surface. The pocket protects the ROS from the scavenging action of antioxidants. This site-specific mechanism is supported by the demonstration that the metal-catalyzed reactions are inhibited by catalase but not by $OH^{\bullet}$ scavengers, presumably because the scavengers cannot compete with the caged reaction of $OH^{\bullet}$ with amino acids at the metal binding site.[2, 14]

Evidences also suggest that protein sequence is important in determining site-specific damage. Studies showed that amino acids like histidine in the sequence significantly increased the protein oxidation which could be due to the strong affinity of

the imidazole ring of histidine for copper. Histidine is one of the most vulnerable amino acids to oxidation reactions, therefore, oxidative damage is thought to occur more

around histidyl residues in a protein. 2-oxo histidine is found to be the major oxidation product upon incubation with the Cu^{+2}/H_2O_2/ascorbate MCO systems.[3, 22]

What level of protein structure determines the pattern of site-specific oxidation? Does the free radical need to be produced in a protective pocket suggesting the importance of the tertiary structure or is the availability of a metal ion binding residue sufficient, making the primary sequence the determining factor. To investigate these questions a lysozyme double mutant (N77H + H15S) has been generated. In the native enzyme, histidine15 is in a protective pocket whereas in the mutant lysozyme histidine 77 is not in a pocket and is more accessible to the bulk of the solution. By changing histidine 15 to serine (Figure 1-6) and introducing another histidine at 77 a different pattern of oxidation is expected. Prolyl residues are more susceptible to peptide bond cleavage, oxidation is also expected at proline 79.

In this thesis, the results of expression of mutant lysozyme protein, site-directed mutagenesis experiments to generate the mutant gene, along with the attempts to detect the protein by ELISA assay and purification by HPLC and gel filtration chromatography will be discussed.

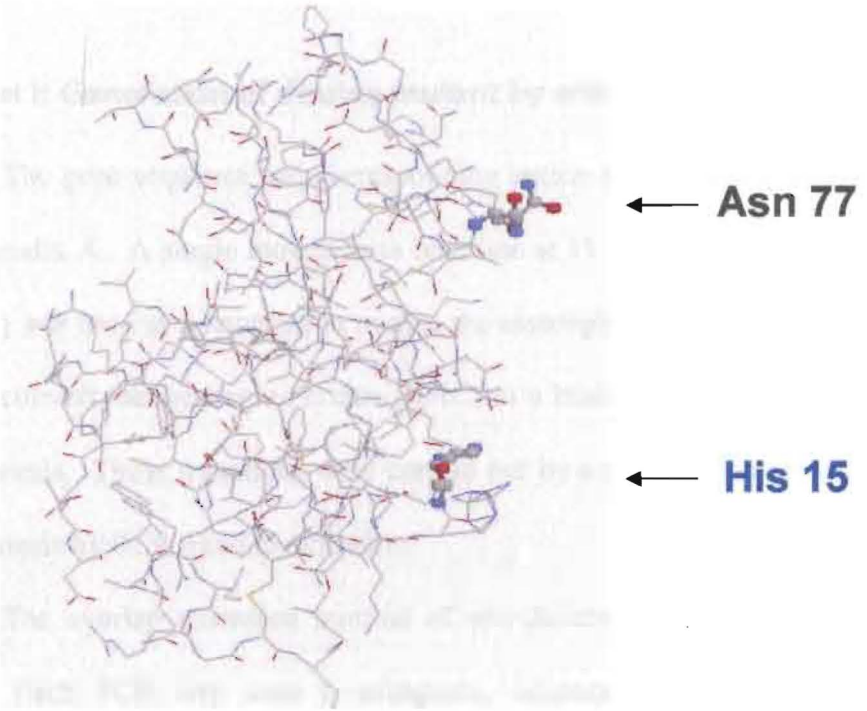


Figure 1-6: Three-Dimensional Structure Model of Hen Egg White Lysozyme.

CHAPTER II: MATERIALS AND METHODS

Section I: Generation of double mutant by site-directed mutagenesis

The gene sequence and corresponding amino acid sequence of lysozyme is given in Appendix A. A single mutant with histidine at 15 changed to serine (courtesy: Nanci Billock) was used as a template to mutate the asparagine at 77 to histidine. The objective was to convert the asparagine codon (AAC) to a histidine codon (CAC) by site-directed mutagenesis. These mutations were carried out by a method known as base substitution mutagenesis by PCR overlap extension.

The overlap extension method of site-directed mutagenesis includes two PCR steps. Each PCR step uses a mutagenic, mismatched oligonucleotide designed to introduce a point mutation at a specific point and a non-mutagenic oligonucleotide at the end of the sequence. The front end of the mutant gene was generated with N2HR oligonucleotide and Lyz-F primer and the back end of the mutant gene was generated with N2HF oligonucletide and Lyz-R primer. The synthetic oligonucleotides (Integrated DNA Technologies) designed to introduce the desired point mutations were as follows:

N2HF Forward: 5'- AAC CTG TGC CAC ATC CCG TGC- 3'

N2HR Reverse: 5'-GCA CGG GAT GTG GCA CAG GTT-3'

The Lyz-F primer used to introduce yeast consensus sequence containing initiating methionine (underlined) is as follows:

Lyz-F: 5'- ATAATAATGAAAGTCTTTGGACGATGT- 3'

The Lyz-R primer with *Xba* I with restriction site (underlined) is as follows:

Lyz-R: 5'- CTCTAGAGCCGGCAGCCTC- 3'

The mutagenic PCR reaction I contained 5 µl of 10X thermo polymerase buffer (Biolabs), 6 µl of $MgCl_2$, 1 µl of dNTPs (Applied Biosystems), 0.5 µl of Vent polymerase (Biolabs), 0.5 µl N2HR primer (100 µM), 0.5 µl Lyz-F primer (100 µM), 0.5 µl pCR®4-TOPO® plasmid (Invitrogen) with H15S mutant as template and 35 µl of sterile water to give a total volume of 50 µl. The mutagenic PCR reaction II contained 5 µl of (10X) thermo polymerase buffer, 6 µl of $MgCl_2$, 1 µl of dNTPs, 0.5 µl of Vent polymerase, 0.5 µl N2HF primer (100 µM), 0.5 µl Lyz-R primer (100 µM), 0.5 µl pCR®4-TOPO® plasmid with H15S mutant as template and 35 µl of sterile water to give a total volume of 50 µl. The DNA used as template for the PCR reactions was extracted from the TOP10 *E.coli* transformed with pCR®4-TOPO® plasmid containing the H15S lysozyme gene. The plasmid DNA was isolated by a standard alkaline lysis mini-prep method.

The thermal cycle (Peltier thermal cycler, PTC-200) included a 30 sec melting step at 95°C and 35 cycles of the following steps: annealing at 55°C for 30 sec, and an extension step at 72°C for 2 min. After 35 cycles the 72°C step was allowed to go for 7 min to complete the elongation. The PCR fragments, front end and back end were then run on a 1% agarose gel. The PCR resulted in two fragments (PCR I ~250bp, front end fragment and PCR II ~150bp, back end fragment). The two PCR products were run on a 1% agarose gel and the PCR products were eluted using Amresco's Cylco-Pure Gel Extraction Kit.

The two fragments were then annealed by PCR to reconstruct the complete, mutated gene. The PCR mix for the overlap extension reaction contained 5 µl of 10X PCR buffer (Promega), 6 µl $MgCl_2$, 1 µl dNTPs, 0.25 µl Taq polymerase (Applied Biosystems), 0.5 µl Lyz-F, 0.5 µl Lyz-R, 1 µl of the front end fragment, 1 µl of the back end fragment, and 34.75 µl of sterile water to give a total volume of 50 µl. The thermal cycle included an initial step at 95°C for 10 min was followed with 35 cycles as follows: 95°C for 1 min, 55°C for 1 min, 72°C for 1 min. after 35 cycles 72 °C for 10 min. The PCR product was run on a 1% agarose gel for verification before proceeding to the next step.

Transformation of One Shot® TOP10 E. coli cells

The suspected mutant lysozyme gene was cloned into the pCR®4-TOPO® (Figure 2-1) using the TOPO® cloning reaction. The TOPO® cloning reaction contained 3 µl of PCR product, 1 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), 1 µl of sterile water, and 1 µl of TOPO® vector. The reaction was incubated at room temperature and used to transform One Shot® competent cells (Invitrogen).

For chemical transformation, 2 µl of the TOPO® cloning reaction was added to the thawed tube of One Shot® TOP10 chemically competent *E. coli* and mixed gently. The cells were incubated on ice for 30 min then the cells were heat shocked at 42°C without shaking. The centrifuge tube was immediately transferred to ice and 250 µl of room temperature SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added. The reaction mix was incubated at 37°C while shaking at 200 rpm for 1 hr. 25 µl of reaction mix was mixed with 20 µl of SOC and plated on prewarmed LB plates containing 50 µg/ml ampicillin

and incubated at 37°C overnight. Similarly, the native gene was also cloned into the pCR®4-TOPO® and TOP10 *E. coli* competent cells were transformed according to the procedure described above. 20 µl of SOC was added to the transformation mixture. The transformed bacteria were plated on prewarmed LB plates containing 50 µg/ml ampicillin and grown overnight at 37°C.

Four double mutant *E. coli* colonies were selected from the plates grown overnight and labeled as DM1, DM2, DM3, and DM4. Four *E. coli* colonies containing native gene were selected from the plates and labeled as N1, N2, N3, and N4. PCR was performed on these colonies to verify the presence of the gene. The PCR mix contained 5 µl of 10X PCR buffer, 6 µl $MgCl_2$, 1 µl dNTPs, 0.25 µl Taq polymerase, 0.5 µl Lyz-F, 0.5 µl Lyz-R, and a small amount of culture swiped with sterile toothpick was the source of the template. The PCR thermal cycle included an initial step of inactivation at 95°C for 10 min to lyse the cells and inactivate nucleases, followed by 35 cycles of: 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 10 min. The PCR product was run on a 1% agarose gel. Plasmids containing the insert were then sequenced.

The pCR®4-TOPO® Vector

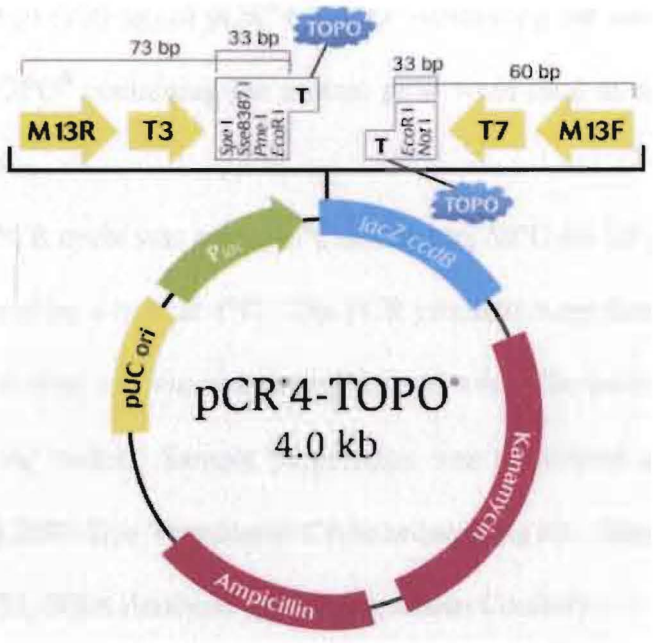


Figure 2-1: The pCR®4-TOPO® Vector (~ 4.0 Kb) with TOPO® cloning site.

Verification of native and double mutant lysozyme clones by DNA sequencing

The cloned pCR®4-TOPO® vectors were sequenced. Sequencing reactions require an oligonucleotide to use as primer, a template (mutant and wild type pCR®4-TOPO® plasmids) to synthesize a complementary strand, and dNTPs to use a substrates for synthesis. The primers (Integrated DNA Technologies) used in the sequencing reactions were:

M13 Forward: 5'-GTAAAACGACGGCCAG-3'

M13 Reverse: 5'-CAGGAAACAGCTATGAC-3'

Two sets of samples were prepared, one set using the M13 forward primer and another set with M13 reverse primer. The sequencing reactions were prepared for the native gene and the mutant lysozyme gene. Each reaction contained template, 2.0 µl of

the appropriate primer (1.6 µM), 8 µl of start mix and sterile water to make 20 µl volumes. 10 µl (200 ng) of pCR®4-TOPO® containing the native gene and 6 µl (240 ng) of pCR®4-TOPO® containing the mutant gene were used as template concentrations for each reaction.

The PCR cycle was set at 96°C for 20 sec, 50°C for 20 sec, 60°C for 4 min, for 40 cycles followed by a hold at 4°C. The PCR products were then precipitated with ethanol followed by rinsing and vacuum drying for ~40 min. The pellets were resuspended in the sample loading buffer. Sample preparation was performed according to the Beckman Coulter CEQ 2000 Dye Terminator Cycle sequencing kit. Sequencing was completed on a CEQ 2000XL DNA Analysis system (Beckman Coulter).

Ligation of native and double mutant gene into pPICZ B

Upon the verification of the sequence of the gene, the mutant gene was ligated into the pPICZ B vector. Restriction digests of both the mutant gene and the pPICZ B were performed. The resulting pieces were ligated to construct pPICZ B-lysozyme H15S+N77H.

TOP10 *E. coli* containing pPICZ B were grown in 5 ml LB broth containing 100 µg/ml Zeocin™ . The pPICZ B was isolated using Cyclo-prep kits (Amresco). The plasmid was run in 1% agarose gel for verification and the concentration was estimated by comparing the band intensity of the isolated DNA to the intensity of a known quantity of DNA.

PCR was performed on the DNA of the native (N2) and double mutant colonies (DM3, DM4) using M13 primers to obtain a purified DNA for ligation reactions. The PCR reaction included 5 µl of 10X PCR buffer, 6 µl $MgCl_2$, 1 µl dNTPs, 0.25 µl Taq

polymerase, 0.5 µl of M13 forward primer and 0.5 µl of M13 reverse primer and sterile water to make a 50 µl volume. A colony swiped with a sterile toothpick was the source of template. The thermal cycle included a heat inactivation step at 95°C for 9.30 min, followed by 34 cycles of: 95°C for 30 sec, 58°C for 30 sec, 72 °C for 1 min and a 10 min of further extension step at 72°C. The PCR products were run on 1% agarose gel. The PCR products were purified using QIA Quick PCR Purification kit. The DNA was eluted from the column with 10mM Tris-Cl pH 8.5 and concentrations of samples were measured by UV spectroscopy (Hewlett Packard 8453).

The restriction digest was performed on PCR product either the mutant gene or the native gene and the vector pPICZ B using two restriction enzymes *Eco*R I (Sigma) and *Xba* I (Sigma). The reaction mix contained 3 µl of pPICZ B plasmid (10 ng/µl), 12.6 µl of mutant gene (1 ng/µl), 2 µl of 10X multicore buffer (Sigma), 0.5 µl of *Eco*R I and 0.5 µl of *Xba* I and 1.4 µl of sterile water. The reaction mix was incubated at 37 °C. The digested products were purified using the QIA Quick PCR Purification Kit protocol before ligation.

The ligation reaction mix contained 4 µl 5X T4 DNA ligase buffer, 15 µl of purified digested DNA, and 1 µl of T4 DNA ligase (Invitrogen). The reaction mix was incubated for 1 hr at room temperature then the reaction was stopped by the addition of 1 µl of 0.5 M EDTA and stored at -20°C.

Transformation of *E. coli* with ligated pPICZ B was performed by diluting 1 µl of ligation mix with 4 µl of sterile water. 2 µl of the diluted mixture was added to a vial of TOP10 chemically competent *E. coli* cells. The cells were mixed gently and incubated on ice for 30 min. The cells were then heat shocked for 30 sec at 47°C. The heat shocked

cells were placed on ice and 250 µl of SOC media was added to each tube. The tubes were fastened to a horizontal shaker and shaken at 37°C for 1 hr. 100 µl of each transformation was spread on a low salt LB plates containing 100 µg/ml Zeocin™ and incubated overnight at 37°C.

Eight colonies from the plate labeled N2, six colonies from DM3, and three colonies from DM4 were streaked onto the fresh LB plates containing 100 µg/ml Zeocin™ and grown overnight at 37°C. The colonies from plate DM3 were labeled as DM3-1, DM3-2, DM3-3, DM3-4, DM3-5, and DM3-6. Colonies from plate DM4 were labeled as DM4-1, DM4-2, DM4-3 and colonies from the plate labeled N2 were labeled as N2-1, N2-2…..N2-8.

PCR was performed on the colonies of native (N2) and the double mutant (DM3, DM4) to determine if the *E. coli* was transformed with the pPICZ B containing the desired gene. The PCR mix contained 5 µl of 10X PCR buffer, 6 µl of $MgCl_2$, 1 µl of dNTPs, 0.25 µl of Taq polymerase, 0.5 µl of each Lyz-F and Lyz-R primer and sterile water to give a total volume of 50 µl. The thermal cycler was set to run at: 95 °C for 9.30 min, followed by 34 cycles of 95 °C for 30 sec, 58°C for 30 sec, 72 °C for 1 min and a step of 72 °C for 7 min after 34 cycles. The PCR products were analyzed on 1% agarose gel.

After verifying the transformation of *E. coli* with pPICZ B containing either the mutant or the native gene, one colony labeled as DM3-6 containing mutant gene and one colony labeled as N2-6 containing the native gene was used for the transformation of yeast (courtesy: Generation of the double mutant gene was performed by Dr. Serra).

Section II: Expression of protein in methylotrophic yeast *Pichia pastoris*

The methylotrophic yeast, *Pichia pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins.[23] The increasing popularity of this particular expression system can be attributed to several factors, most importantly: (1) the simplicity of techniques required for the molecular genetic manipulation of *P. pastoris* and its similarity to *Saccharomyces cerevisiae*, one of the most well-char-acterized experimental systems in modern biology; (2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; (3) the capability of performing many posttranslational modifications of a eukaryotic protein, such as glycosylation, disulfide bond formation and proteolytic processing; and (4) the availability of the expression system as a commercially available kit. [23]

Pichia pastoris can utilize methanol as a source of carbon and energy. Methanol utilization requires a metabolic pathway involving several unique enzymes. The enzyme alcohol oxidase (AOX) catalyzes the first step in the methanol utilization pathway, the oxidation of methanol to formaldehyde and hydrogen peroxide. AOX is sequestered within the peroxisome along with catalase, which degrades hydrogen peroxide to oxygen and water. A portion of the formaldehyde generated by AOX leaves the peroxisome and is further oxidized to formate and carbon dioxide by two cytoplasmic dehydrogenases. These reactions are a source of energy for cells growing on methanol. The remaining formaldehyde is assimilated to form cellular constituents by condensation of formaldehyde with xylulose-5-monophosphate, a reaction catalyzed by a third peroxisomal enzyme, dihydroxyacetone synthase (DHAS). The products of this reaction, glyceraldehydes-3-phosphate and dihydroxyacetone, leave the peroxisome and enter a

cytoplasmic pathway that regenerates xylulose-5-monophosphate and one molecule of glyceraldehyde-3- phosphate for every three cycles. Two of the methanol pathway enzymes, AOX and DHAS, are present at high levels in cells grown on methanol but are not detectable in cells grown on most other carbon sources like glucose, glycerol, or ethanol.

The two genes *AOX1* and *AOX2* encode alchol oxidase in *Pichia pastoris*. *AOX1* is responsible for most of the alchol oxidase activity in the cell. The *AOX1* gene is regulated by two mechanisms, a repression/de-repression mechanism plus an induction mechanism. Growth on glucose represses transcription, even in the presence of methanol so growth on glycerol is necessary for optimal induction with methanol. Expression of *AOX1* is tightly regulated and the presence of methanol is essential to induce high levels of expression of the gene of interest.

Expression of any 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) selection of potential expression strains for the gene product. A variety of *P. pastoris* expression vectors and host strains are available and all vectors are *E. coli*/*P. pastoris* shuttle vectors, functional in both organisms. Plasmid vectors designed for expression in *Pichia* have several common features. It consists of DNA sequences containing *AOX1* promoter, one or more restriction sites for gene insertion, followed by the transcription termination sequence from *AOX1* gene that directs 3' processing and polyadenyaltion of the mRNAs, and an antibiotic resistance gene for positive selection in *E. coli* and *Pichia*.

One set of vectors, the pPCIZ series, are used for intracellular expression. pPCIZ vectors contain the *sh ble* gene from *Streptoalloteichus hindustanus* which gives resistance to the antibiotic Zeocin™ in *E. coli* and *P. pastoris*. The Zoe^{R} vectors have a selectable marker for both *E. coli* and *P. pastoris*, are much smaller (3.3 kb) and easier to manipulate than other vectors.

Two different types of recombinant strains can be generated; Mut^{+} and Mut^{S}. Mut^{+} (Methanol utilization plus) phenotype is referred to as wild type because of its ability to grow and utilize methanol as sole carbon source. Mut^{S} (Methanol utilization slow) phenotype is due to loss of *AOX1* gene, this result in slow growth on methanol medium. The Mut^{+} phenotype, X-33, a wild type strain, and pPCIZ B vector containing the Zeocin™ resistance gene has been used in this research project (Figure 2-2).

The pPICZ B Vector

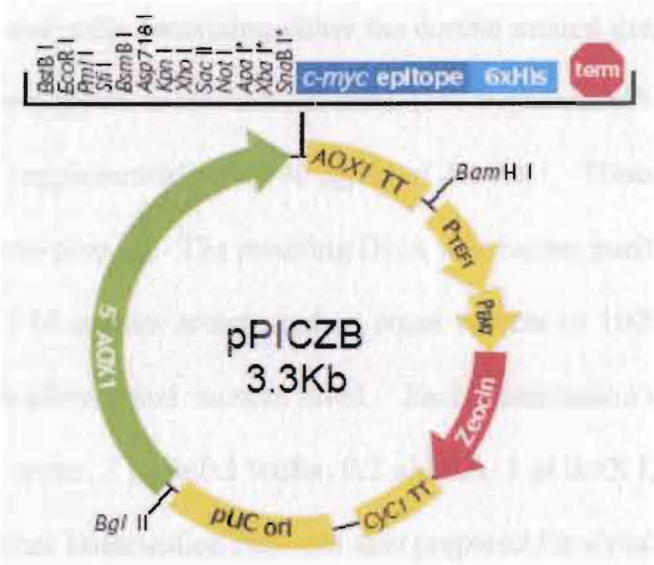


Figure 2-2: The pPICZ B plasmid (3.3 Kb) used for cloning experiments.

Transformation and expression of protein in Pichia pastoris

Three *Pichia* strains X-33, GS115, and KM71H are made competent chemically. A 10 ml YPD media (1% yeast extract, 2% peptone and 2% glucose) inoculated with a *Pichia* strain was grown overnight at 29.8°C in a shaking water bath. The cells from the overnight culture were diluted to an OD_{600} of 0.1- 0.2 in 10 ml of YPD and grown to an OD_{600} of 1.0. The cell culture was centrifuged at 2500 rpm for 5 min to collect the cell pellet which was then resuspended in 10 ml of solution I (1 M sorbitol, 10 mM bicine, 3% (v/v) ethylene glycol, 5% (v/v) DMSO, pH 8.35). The cell suspension was again centrifuged and cell pellet resuspended in 1ml of solution I. 50 µl aliquots of the competent cells were then distributed to 1.5 ml screw cap microcentrifuge tubes and wrapped in several layers of paper towel and stored at -80°C.

Linearization of pPICZ B plasmid

The *E. coli* cells containing either the double mutant gene (DM3-6) or the native gene (N2-6) were grown in low salt LB broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.5) supplemented with 100 µg/ml of Zeocin™. Plasmid DNA minipreps were made using cyclo-prep kit. The resulting DNA was further purified by precipitation with 1/10 volumes 3 M sodium acetate and an equal volume of 100% ethanol followed by a wash with 70% ethanol and vacuum dried. Each linearization reaction contained 10 µg of the purified vector, 2 µl Bsfx1 buffer, 0.2 µl BSA, 1 µl B*st*X I, and sterile H_2O in 20 µl volumes. Another linearization mix was also prepared for circular pPICZ B without any insert. The reaction mix was incubated for 1 hr at 50°C. 5 µl of samples were analyzed on 1% agarose gel. The remaining linearized samples were vacuum dried.

Transformation of Pichia pastoris

The X-33 strain of *Pichia* was transformed with plasmid containing either the double mutant or the native gene. For each transformation, 50 µl of competent cells were added to the DNA pellet. 1 ml of solution II (40% PEG 1000, 0.2 M bicine, pH 8.35) was added, vortexed, and incubated for 1 hr at 30°C. The transformation reaction was mixed every 15 min by flicking the tube to increase the transformation efficiency. Then the cells were heat shocked in a 42°C heat block for 10 min. After heat shock treatment, the cells were split into two tubes (525 µl per tube) and 1 ml of YPD was added to each tube and incubated for 1 hr to allow expression of Zeocin™ resistance. Cells were pelleted by centrifugation at 4500 rpm for 5 min at room temperature and resuspended in 500 µl of solution III (0.15 M NaCl, 10 mM bicine, pH 8.35) and combined into one tube. The cells were then centrifuged, and the cell pellet was resuspened in 150 µl of solution III. The entire transformation solution was transferred onto a pre-warmed YPDS plate (1% yeast extract, 2% tryptone, 2% glucose, 1 M sorbital, and 2% agar) containing 100 µg/ml Zeocin™ and incubated at 30°C for 4 days. Zeocin™ resistant colonies were used as expression strains.

The strain, X-33 was transformed and grown on YPDS plates. Only X-33 with the double mutant gene was successfully transformed and three colonies of X-33 were selected and grown on YPDS plates. Further research was carried out with the three colonies of X-33 strain. Hereafter, the three colonies are referred as X-33 DM3-6a, DM3-6b, and DM3-6c.

Analysis of Pichia pastoris transformants by PCR

The transformed yeast colonies were then tested for the insert. Yeast cultures were grown overnight in 10 ml YPD media containing 100 μg/ml Zeocin™. 1.5 ml of the yeast culture was spun at 13,000 rpm for 1 min to collect the cell pellet. 300 μl of acid washed 425-600 micron glass beads, 200 μl of lysis buffer (2% Triton X-100, 1% SDS, 2% 100 mM NaCl, 1% 10 mM Tris-Cl pH 8.0, 5% 10 mM EDTA) and 200 μl of PCIA (50% equilibrated phenol, 48% chloroform, 2% isoamyl alcohol) were added to the cells and vortexed to resuspend the pellet. Centrifugation for 5 min at 13,000 rpm produced a layer of beads at the bottom, an opaque PCIA layer in the middle and a clear aqueous layer on the top. The top aqueous layer is transferred into a new centrifuge tube. 200 μl 10X TE (100 mM Tris HCl pH 8.0, 10 mM EDTA) was added to the original tube, vortexed and centrifuged for 5 min at high speed. The top aqueous layer was added to the previously collected aqueous layer. 400 μl of PCIA was added to the combined aqueous layer, vortexed and centrifuged for 5 min. The aqueous layer was transferred to another tube and mixed with 400 μl of PCIA, vortexed and centrifuged. The supernatant was then transferred to a new centrifuge tube and 40 μl of 3 M sodium acetate, 1 ml of 100% ethanol was added. The samples were stored at -20°C for 30 min and then centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol, stored at -20°C for 30 min and vacuum dried. The pellet was dissolved in 20 μl 1XTE and 1 μl RNase and the solution was used for PCR.

PCR was performed on the DNA extracted from three transformed colonies of X-33 strain. The PCR mix included 5 μl PCR buffer, 6 μl $Mgcl_2$, 4 μl dNTPs, 0.5 μl 5' Lyz-F primer, 0.5 μl 3' Lyz-R primer, 0.25 μl Taq DNA polymerase, 0.5 μl DNA

template in 50 µl volumes. The mixture was subjected to 35 PCR cycles. Thermal cycles included a single 9.30 min denaturation step at 95°C, and 35 cycles of the following steps: 95°C for 30 sec, 58°C for 30 sec, 72°C for 1 min. After 35 cycles the 72°C was allowed to go for 7 min to complete transcription. PCR reactions were performed with plasmid DNA containing the native gene and native gene with *AOX* primer. The PCR mix for native gene with *AOX* primer included 5 µl PCR buffer, 6 µl $Mgcl_2$, 4 µl dNTP 0.5 µl 5'*AOX1* primer, 0.5µl 3'*AOX1* primer, 0.25 µl Taq polymerase, 0.5 µl DNA template in 50 µl volumes and PCR products were analyzed on 2% agarose gel.

Determination of mutant phenotype

Transformation of X-33 with linearized constructs favor single crossover recombination at the *AOX1* locus so the transformants are usually Mut^+ (Methanol utilization plus). However, with the presence of *AOX1* sequences in the plasmid, there is a chance of recombination occurring in the 3' *AOX1* region also, disrupting the wild type *AOX1* gene and creating the Mut^S (Methanol utilization slow) phenotype. Testing on MDH and MMH plates will allow the confirmation of the mutant phenotype.

Minimal Dextrose with Histidine (MDH) agar plates (1.34% YNB, 4 x 10^{-5}% biotin, 2% dextrose, 1% of 0.4% histidine) and minimal methanol with histidine (MMH) agar plates (1.34%YNB, 4 x 10^{-5}% biotin, 0.5% methanol, 1% 100X or 0.4% histidine) were prepared. Three MMH plates were labeled DM3-6a, DM3-6b, and DM3-6c. Using sterile toothpicks and scoring plates the transformants were streaked in a regular pattern as dots on MMH plates. To differentiate Mut^+ from Mut^s two controls, GS115/ Mut^s

Albumin and GS115/pPICZ/*lacZ* Mut^+ were also streaked as two lines on the plates. Plates were then incubated at 30°C for 2 days.

To increase the efficiency of Mut^+/ Mut^S screening the replica plating procedure was perfomed. The patches from three MMH plates were replica-plated onto three MDH plates. Plates were then incubated at 30°C for two days.

Section III: Small Scale expression

After the recombinant strain was confirmed by PCR, the optimal method and conditions for expression of the mutant gene were explored using various buffered media. Small scale expression was carried in four different media.

a) BMGY/BMMY (buffered complex glycerol or methanol medium).
b) BMG/BMM (buffered minimal glycerol or methanol medium)
c) MGYH/MMH (minimal glycerol or minimal methanol medium containing histidine).
d) Synthetic Minimal media.

Expression in BMGY/BMMY media

The transformed yeast were first grown on YPD plates containing 100 µg/ml Zeocin™ for 2-3 days at 30°C and then the three colonies of X-33 were grown in 25 ml of BMGY (1% yeast, 2% peptone, 10% 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 1% glycerol) in a 250 ml baffled flasks. The cells were grown at 28-30°C in a shaking incubator at 250 rpm until culture reaches an OD_{600} 1.5 – 2.0. The cells were harvested by centrifuging at 4500 rpm for 5 min at 4°C (SORVAL). The cell pellet was resuspended to an OD_{600} 1.5 in 120 ml of BMMY (1% yeast, 2%

peptone, 10% 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4 x 10^{-5} % biotin, 0.5% methanol) media to induce expression and 0.005% of anti-foaming agent (Sigma) was added to prevent frothing. The cultures were placed in 1 liter sterile baffled flasks and labeled as DM3-6a, DM3-6b, and DM3-6c and grown for four days. A 600 µl (0.5%) of 100% methanol was added every 24 hr to maintain induction and 1 ml of expression culture was collected in 1.5 ml centrifuge tubes at time points of 24, 48, 72, and 96 hr. The 1 ml cultures were centrifuged at maximum speed in a table top microcentrifuge for 1 min and the pellets were quickly frozen in dry ice/acetone and stored at -80°C. These samples were used to analyze expression levels and determine the optimum time to harvest the cells.

Expression in BMG/BMM media

Three 25 ml portions of BMG (10% 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4 x 10^{-5} %biotin, 1% glycerol) media were inoculated with one of each of the three colonies of X-33 and placed in a 250 ml baffled flasks. The cells were grown overnight at 28-30°C in a shaking incubator at 250 rpm. When the cultures reached an OD_{600} 1.5 – 2.0 in 12-14 hr, the cells were harvested by centrifuging at 4500 rpm for 5 min at 4°C (Sorval).

The cells were resuspended with 100 ml BMM media (10% 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4 x 10^{-5} % biotin, 0.5% methanol) to induce expression and 0.005% antifoaming agent was added to prevent frothing. The 100 ml cultures were placed in sterile 1 liter baffled flasks and allowed to grow for 4 days in a shaking incubator (250 rpm) at 29.8 °C. A 500 µl aliquot (0.5% v/v) of 100% methanol was added to the cultures every 24 hr to maintain the induction. At the time points of 24,

48, 72, and 96 hr, 1 ml cultures were transferred to 1.5 ml centrifuge tubes and centrifuged for 1 min at room temperature in a table top microcentrifuge. The supernatant was decanted and cell pellets was quickly frozen in dry ice/acetone and stored at -80°C. These cells were analyzed for expression levels by SDS-PAGE.

Expression in MGYH/MMH media

Three colonies of X-33 were grown in three 25 ml portions of MGY media (1.34% YNB, 4 x 10^{-5} % biotin, 1% glycerol, 0.004 % histidine). The cultures were placed in 250 ml sterile baffled flasks and grown at 29.8°C in a shaking incubator at 250 rpm until the culture achieved an OD_{600} of 1.5 – 2.0. Then the cells were harvested by centrifugation at 4500 rpm for 5 min at 4°C. The cell pellet was resuspended to an OD_{600} 1.0 in 110 ml of MMH (1.34% YNB, 4 x 10^{-5} % biotin, 0.5% methanol, 0.004% histidine) to induce expression and 0.005% of antifoaming agent was added to prevent frothing. The 110 ml cultures were placed in 1 liter sterile baffled flasks and grown for 4 days in a shaking incubator (250 rpm) at 29.8°C. 550 µl aliquots (0.5% v/v) of 100% methanol was added to the cultures every 24 hr to maintain the induction. At the time points of 24, 48, 72, and 96 hr, 1 ml cultures were transferred to 1.5 ml centrifuge tubes and centrifuged for 1 min at room temperature in a table top microcentrifuge. Supernatant was decanted and cell pellets was quickly frozen in dry ice/acetone and stored at -80°C. These cells were analyzed for expression levels by SDS-PAGE.

Expression in synthetic media

The transformed X-33 yeast was expressed using Synthetic Minimal (SM) media. The SM media was prepared in 100 mM potassium phosphate buffer, pH 7.5. The

composition of SM media is 0.2% ammonium sulfate, 1% potassium phosphate monobasic, 0.01% calcium chloride, 0.2% magnesium sulphate, 0.2% potassium chloride, 0.01% sodium chloride, 0.01% zinc sulphate heptahydrate, 0.0005% copper sulphate, 0.01% ferric chloride, 10^{-6}% biotin, $5x10^{-5}$% thiamine, $5x10^{-5}$ % pyridoxine, $5x10^{-3}$% sodium pantothenate, 0.002% inositol, 2% methanol. The metals were dissolved in the buffer and autoclaved. The vitamins were prepared as concentrated standards and added after the media was cooled.

A 5 ml of YPD medium containing 100 µg/ml Zeocin™ was inoculated with a single colony (DM3-6c) from the frozen stock and grown for 12-16 hr in a shake flask. The 5 ml seed culture used to inoculate 400 ml of fresh YPD medium in a 2 L flask and incubated on a rotary shaker at 29.8°C for 2 days. The cells were collected by centrifugation at 3500 rpm for 5 min at 4°C and inoculated into a baffled flask containing 500 ml of SM medium. The 500 ml of media was distributed as 125 ml in four 1000 ml flasks the culture was grown at 30°C for 4 days. A 1.24 ml (1%), 100 mM PMSF was added to flask 1 at 24 hr, 1.23 ml to flask 2 at 48 hr, 1.22 ml to flask 3 at 72 hr and 1.21 ml to flask 4 at 96 hr. And a 0.5% methanol was added to the cultures at every 24 hr to induce expression. 1 ml aliquots of samples were collected from all the flasks at 24, 48, 72, and 96 hr time points. A neutral pH was maintained by checking the media every 24 hr with broad range pH paper and adjusting with ammonium hydroxide. The samples were centrifuged and the cell pellet was frozen in dry ice /acetone and stored at -80°C.

The control, X-33 with pPICZ B without insert was also grown in SM media similarly. The cell pellets were lysed open using breaking buffer and the lysate was analyzed by SDS-PAGE.

Analysis by SDS-PAGE

The cells collected at various time points in three media were analyzed by SDS-PAGE. The cell pellets stored at -80°C were thawed to room temperature and placed on ice. For each 1 ml of cell pellet 100 µl of breaking buffer (50%, 50 mM sodium phosphate pH 7.4, 10% 1 mM phenyl methylsulfonyl fluoride, 10% 1 mM EDTA, 5% glycerol (5% v/v)) was added and the pellet was resuspended. An equal volume of 0.5 mm acid washed beads was added and vortexed for 30 sec, then incubated on ice for 30 sec. This was repeated for a total of 8 cycles. The tubes were centrifuged at 13000 rpm for 10 min on a table top microcentrifuge and the clear supernatant lysate was transferred to fresh centrifuge tubes. The lysate was analyzed by SDS-PAGE.

The samples for electrophoresis were prepared by mixing 20 µl of protein lysate, 20 µl of 62.5 mM Tris-HCl, pH 6.8, 10 µl of 10% SDS, 4-6 µl 2-mercaptoethanol. The mixture was placed in a boiling water bath for 3-4 min. After the samples had cooled an equal volume of 20% (v/v) glycerol and 10 µl of 0.05% bromophenol blue were added and any insoluble material was removed by centrifuging for several seconds on a table top centrifuge.

SDS-PAGE was performed using 18% SDS gels (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). 20 µl of each sample, 10 µl of molecular protein marker and 10 µl of native lysozyme were loaded in the wells and run at 150 V for 1.0 – 1.5 hr or until the dye front reached the bottom of the gel. The gels were then stained with Gelcode® blue (Pierce). The gels were then washed with water three times for 5 min each and placed in Gelcode® blue overnight. The gels were then destained in DI water.

Section IV: Scale-up of expression

Bands of the correct molecular weight appeared with the samples grown in BMGY/BMMY media. We explored the conditions for the scale-up of protein expression in this media. The yeast from the frozen sample was grown on YPD plates containing 100 μg/ml Zeocin™ for 3 days. Large scale expression was carried out with X-33 DM3-6c because the bands in the gel were the most intense for this colony. As a first step, 25 ml of BMGY was inoculated with the yeast grown on the plates in a 250 ml baffled flask. The culture was grown at 29.8°C in a shaking incubator (250 rpm) to an OD_{600} of 2.0. The 25 ml culture was then used to inoculate 1 L of BMGY containing 0.005% antifoaming agent and allowed to grow at 29.8°C in a shaking incubator (250 rpm) to an OD_{600} of 2.0. The cells were harvested using sterile centrifuge bottles by centrifuging at 4500 rpm for 5 min at 4°C (Sorval). The supernatant was decanted and the cell pellet was resuspended in 2 L of BMMY. The culture was distributed into 4, one liter baffled flasks and grown at 29.8°C with shaking. Every 24 hours, 0.5% methanol was added to the culture until 72 hours. 72 hrs is the optimum time of induction determined from the analysis of samples from small scale expression. After 72 hrs the cells were harvested using sterile centrifuge bottles by centrifuging at 4500 rpm at 4°C. The cell pellet was resuspended in 125 ml sterile distilled water and centrifuged at 4500 rpm for 5 min. The supernatant was discarded, and the 20 g of cell paste obtained was transferred into two, sterile 50 ml falcon tubes and quickly frozen in liquid nitrogen and stored at -80°C.

The transformed yeast was grown in large scale in BMGY/BMMY two times. Ammonium fractionation, gel filtration chromatography, and separation by HPLC

experiments were performed with the yeast obtained in the first large scale expression experiment. An ELISA was performed with control and mutant yeast grown in the second large scale expression.

Collection of protein lysate from yeast

Cell lysis was performed with a beadbeater (Biospec). The cell paste was thawed to room temperature and resuspended in breaking buffer. The chamber was filled with 0.5 mm glass beads to ½ to ¾ of its volume. The remaining volume was filled with the cell suspension and breaking buffer. The chamber was filled with breaking buffer so as to exclude air and placed in the ice water jacket filled with crushed ice and water and fixed on to the beadbeater. The beadbeater was operated for one min followed by a rest of one min. This cycle was performed for 5 min. 10 µl of lysate was removed after 3, 4, 5 min step, and 100 fold dilution of lysate was prepared in a 1.5 ml centrifuge tube, and the absorbance at 280 nm was measured.

The supernatant lysate obtained was then ultracentrifuged to remove the cell wall material. The lysate was transferred to sterile centrifuge tubes and centrifuged at 94,000 rpm for 15 min. The supernatant was then ultra centrifuged at 37,000 rpm at 4°C for 1 hr to remove organelles, nucleic acids, etc. 1 ml of the supernatant lysate was distributed into 1.5 ml micro centrifuge tubes and stored at -80°C. The total protein content in the lysate was determined with Bradford assay and used for further analysis.

Enzyme Assay

To examine whether the lysozyme mutant protein was present in an active form in the cell lysate, the enzyme assay was performed. Lysozyme activity was assayed using a

turbidimetric method. The lysozyme assay is the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp) based on assay by Sugar. One unit is equal to a decrease in turbidity of a *Micrococcus lysodeikticus* suspension of 0.001 per minute at 450 nm at pH 7.0 at 25°C. A 0.3 mg/ml suspension of lyophilized cells of *Micrococcus lysodeikticus* in 0.1 M potassium phosphate buffer pH 7.0 was prepared and allowed to incubate at room temperature for 1 hr. Concentrations of 10 mg/ml, 1 mg/ml, 100 µg/ml, and 10 µg/ml of native lysozyme were prepared using lyophilized lysozyme, and the assay was performed with a UV-VIS spectrophotometer adjusted to 450 nm. The cell holder was maintained at 25°C using a circulating water bath. 2.9 ml of a *Micrococcus lysodeikticus* cell suspension was pipetted into a 3 ml cuvette and incubated for 4-5 minutes in order to achieve temperature equilibration and to establish blank rate. 100 µl of lysate was then added to the cuvette and absorbance at 450 nm was recorded every 10 sec for a total of 4 min. The ΔA_{450}/minute was calculated using zero-order kinetics fitting data from 40-240 sec. The assay was carried with lysates collected at time points of 0, 24, 48, 72, 96 hrs grown in the BMGY/BMMY media and also with native lysozyme as a control. The fractions collected by gel filtration chromatography were also tested for lytic activity by enzyme assay.

Ammonium sulfate precipitation

Ammonium sulfate fractionation of protein lysate was performed as an initial attempt to purify the protein. A simple ratio and ammonium sulfate fractionation chart were used to calculate the amount of ammonium sulfate needed to the varying amount of lysate. A 3 ml aliquot of lysate was placed in a 10 ml beaker and 0.492 g of $(NH_4)_2SO_4$ was added slowly over 20 min to make 30% concentration. The solution was stirred for

30 min and centrifuged at 8500 rpm for 10 min. The precipitate was stored and the supernatant was transferred to another beaker. To reach the concentration of 60%, 0.543g of $(NH_4)_2SO_4$ was added slowly to the supernatant over 20 min and the solution was stirred in the cold room for 30 min then centrifuged at 8500 rpm for 10 min. The precipitate at 60% and the supernatant was also stored at -80°C.

The protein precipitate at 30% and 60% was resuspended in 4 ml of 62.5 mM Tris, pH 6.8 buffer. These protein solutions and the supernatant lysate were dialyzed using Regenerated Cellulose Dialysis Membranes with MWCO 3,500 (Spectrum) against 2 L of Tris buffer. The dialyzed protein solutions were analyzed by SDS-PAGE.

Section V: Generation of X-33 with pPICZ B containing no insert

The *Pichia pastoris* strain X-33 with pPCIZ B without any insert was generated and served as a control.

Transformation of X-33

The X-33 cells were made chemically competent according to the procedure described on page 28 and stored at -80°C.

The TOP10 *E. coli* containing pPICZ B was grown overnight in a 25 ml LB media supplemented with 100 µg/ml Zeocin™ at 37°C. Plasmid DNA minipreps were made using cyclo-prep kit. The DNA was purified by precipitation with 1/10 volumes of 3 M sodium acetate and equal volume of 100% ethanol followed by a wash with 70% ethanol then dried at 37°C. The pellet was resuspended in 100 µl sterile water. To the sample 1 µl of RNase was added and incubated for 1 hr at room temperature followed by the addition of 1/10 volume, 3 M sodium acetate and two volumes of 100% ethanol. The

sample was placed in the freezer for 1 hr then centrifuged at 14,000 rpm for 5 min. The supernatant was discarded and 200 µl of 70% cold ethanol (-20°C) was added and vortexed for few seconds and centrifuged at 14,000 rpm for 5 min. The supernatant was discarded and the DNA pellet was dried at 37°C. The pellet was resuspended in 50 µl sterile water and 20 µl was used to measure the concentration at 280nm. The remaining DNA was dried to a pellet and used for linearization.

Linearization reaction included circular DNA pellet (2.5 µg/µl), 2 µl Bsfx1 buffer, 0.2 µl BSA, 1µl B*st*X I, and sterile H_2O in 20 µl volumes and incubated for 1 hr at 50°C. The samples were analyzed on a 1% agarose gel.

The linearized plasmid was concentrated by drying, as the volume of DNA should not exceed 5 µl for transformation. For the transformation, 50 µl of competent X-33 cells were added to the DNA solution. The transformation of X-33 was carried according to the procedure described on page 29. The entire transformation solution was spread onto an YPDS plate (1% yeast extract, 2% tryptone, 2% glucose, 1 M sorbital, and 2% agar) containing 100 µg/ml Zeocin™ and incubated at 30°C for 4 days. A few colonies from the plates were streaked onto the fresh YPD+Zeocin™ plates and labeled as 1A, 1B, 1C, 1D, 1E.

The transformed yeast colonies were analyzed by PCR. The PCR mix included 5 µl PCR buffer, 6 µl $Mgcl_2$, 4 µl dNTP 0.5 µl 5' *AOX1* primer, 0.5 µl 3' *AOX1* primer, 0.25 µl Taq polymerase, 2.0 µl DNA template in 50µl volumes. The mixture was subjected to 35 PCR cycles. Thermal cycles included a single 9.30 min denaturation step at 95°C, and 35 cycles of the following steps: 95°C for 30 sec, 58°C for 30 sec, 72°C for 1 min. After 35 cycles the 72°C was allowed to go for 7 min. The PCR products were

analyzed on 2% agarose gel. The 1D colony was found to be successfully transformed and grown in BMGY/BMMY media. The lysate was collected and used in the analysis by SDS-PAGE and ELISA.

Section VI: Purification and characterization of mutant lysozyme

Gel filtration chromatography

Gel filtration chromatography was used as an initial purification step of the mutant lysozyme. 13g of sephadex G-50 (Sigma Aldrich) was soaked in 200 ml of sodium chloride-Tris buffer (0.2 M sodium chloride + 50 mM Tris, pH 9.0) overnight and packed in a 1.5 cm x 10.0 cm column. 2-3 column volumes of 0.2 M sodium chloride + 50 mM Tris, pH 9.0 buffer were passed through the column to equilibrate the stationary phase. A 1 ml of 2 mg/ml lysozyme sample was prepared and added to the column. The protein was eluted with the same buffer. The samples were collected and the absorbance of every other tube at 280 nm was measured. The column was flushed with the same buffer overnight to remove the lysozyme. To recover the mutant lysozyme from the lysate, a 700 µl sample of lysate was loaded on to the column and the proteins were eluted with the same Tris-NaCl buffer. 2.5 ml fractions were collected with an elution rate of 5 ml/hr. The absorbance at 280 nm of every other tube was measured and a graph of A_{280} vs test tube number was plotted.

Based on the graph the fractions falling under a particular peak were pooled and concentrated in Amicon stir cell using an YM3 membrane (MWCO 3000 da). The concentrated fractions were precipitated in supersaturated ammonium sulfate followed by centrifugation at 8500 rpm for 10 min. the precipitated was then resuspended in 2-3 ml of 50 mM Tris pH 9.0 buffer and dialyzed with the same buffer overnight. The various

fractions collected were analyzed for the lytic activity by enzyme assay. The fractions were also analyzed on SDS-PAGE for the presence of lysozyme.

Separation by HPLC

In an attempt to purify the protein by HPLC, 30% and 60% ammonium sulfate precipitations of the protein lysate were obtained. To 5 ml of lysate, 0.82 g of ammonium sulfate was added over 20 min and allowed to stir for another 30 min. The solution was centrifuged at 8500 rpm for 10 min to obtain a precipitate at 30% ammonium sulfate. The supernatant was transferred into a beaker to which 0.905 g of ammonium sulfate were added over 20 min, then allowed to stir for 30 min in cold room. The solution was then centrifuged to collect a precipitate at 60% ammonium sulfate. The precipitates were resuspended with 4-5 ml of HPLC buffer A (20 mM potassium phosphate, pH 6.0) and dialyzed overnight against buffer A.

The HPLC separation was performed with dialyzed 30% and 60% precipitated protein samples using HPLC buffers A and B (20 mM potassium phosphate, 0.4 M NaCl, pH 6.0). At the beginning, the column was flushed with ~8 volumes of the high salt buffer (buffer B) before equilibration with the low salt buffer. Initially, the column was equilibrated for 30 min with 20% buffer B at the flow rate of 1.00 ml/min. 1 ml of native lysozyme (1mg/ml) was loaded onto a 100 x 4.6 mm column (polyCAT A^{TM}). The adsorbed protein was then eluted for 40 min with the linear gradient to 100% B followed by 5 min with the gradient to 100% B. Data was collected from 0-40 min. sample was injected every 75 min.

For the second run, the column was equilibrated for 30 min with 20% B, and 900 µl of 60% sample was loaded onto the column. Similarly 30% sample and supernatant lysate was also applied to the column and eluted with the gradient as mentioned above.

Since lysozyme is a basic protein, the HPLC was also performed with more acidic, 2-morpholinoethane sulphonic acid. The two buffers, 2-morpholinoethane sulphonic acid buffer A (20 mM MES, pH 5.0) and Buffer B (20 mM MES, 0.4 M NaCl, pH 5.0) were used to elute 30%, 60% precipitated protein samples, supernatant lysate and the native lysozyme. The samples were dialyzed overnight with Buffer A before being loaded onto the column. The following gradient was used for the separation: An equilibration at 1.0% B for 15 min followed by 30 min gradient to 11% B then a 15 min gradient to 100% B and 5 min at 100% B.

Section VII: Quantitation of protein by Bradford Assay and ELISA

Bradford Assay

The Bradford assay is a calorimetric assay used for total protein quantitation. Coomassie dye binds protein in an acidic medium immediately shifting the absorbance from 465 nm to 595 nm with concomitant color change from brown to blue. Coomassie (Bradford) Protein Assay Kit (Pierce) was used to determine the total protein concentration using bovine serum albumin (BSA) of a known concentration as a standard. A small amount of protein sample (30µl) is mixed with the Coomassie reagent (1.5 ml), incubated for 10 min at room temperature and the absorbance measured at 595nm.

A set of protein standards were prepared using 1 ml BSA ampule (bovine serum albumin at a concentration of 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide) and DI water as diluent (Table 2-1).

Tube	Diluent volume	Volume and Source of BSA	Final BSA Conc.
A	0 µl	300 of stock	2,000 µg/ml
B	125 µl	375 of stock	1,500 µg/ml
C	325 µl	325 of stock	1,000 µg/ml
D	175 µl	175 of dilution B	750 µg/ml
E	325 µl	325 of dilution C	500 µg/ml
F	325 µl	325 of dilution E	250 µg/ml
G	325 µl	325 of dilution F	125 µg/ml
H	400 µl	100 of dilution G	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

Table 2-1: Preparation of BSA samples for the standard curve.

30 µl of each standard sample was pipetted into the appropriately labeled microcentrifuge tube and 1.5 ml of the Coomassie Reagent was added to each tube. The sample with the protein lysate was similarly prepared. The solution was mixed well and samples were incubated for 10 min at room temperature. The spectrophotometer (Hewlett Packard 8453) was set to 595 nm, the instrument was zeroed on the cuvette filled with only water. The absorbances of the samples were then measured. A standard curve of absorbance versus concentration of BSA in µg/ml was plotted. The standard curve was used to determine the protein concentration of the lysate. The total protein concentration of the lysate obtained in the first large scale expression (BMGY/BMMY) was measured.

The Bradford assay was also performed on the lysate obtained from the control and mutant yeast grown in second large scale experiment (BMGY/BMMY). The

standard solutions of BSA were prepared from a stock solution of 2.0 mg/ml. 0.2, 0.3, 0.4, 0.6, 0.8 mg/ml standards were prepared using DI water as diluent. The preparation of samples and measurement of the absorbance was similar to that described above. The standard curve of absorbance versus concentration of BSA mg/ml was constructed and the concentrations of the protein in the lysates were calculated.

ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) is a powerful method in estimating ng/ml to pg/ml ordered materials in solution such as culture supernatant.

ELISA was performed several times with different dilutions of primary antibody (1° Ab) and antigen for optimization. Finally ELISA was performed with a 1:30,000 dilution of biotinylated polyclonal antibody to lysozyme as 1°Ab (Acris Antibodies) and peroxidase conjugated streptavidin (Bio-Genex) as 2° reagent.

The assay was performed in 8x12 matrix, tissue culture coated microtiter plates (Figure 2-3). As a first step the plate is coated with antigen (Ag) and incubated at room temperature. A 10 µg/ml solution of native lysozyme, 5 mg/ml of lysate from control yeast, and 5 mg/ml of lysate from mutant yeast (DM3-6c) was prepared in 50 mM sodium carbonate buffer, pH 9.6.

100 µl aliquots of native lysozyme (10 µg/ml) were added to wells A1-A2 and B1-B2. 5-fold serial dilutions were made of lysozyme in wells A2-A12 and B2-B12 using sodium carbonate buffer. 100 µl of 5 mg/ml of lysate from control yeast was added to wells C1-C2, D1-D2 and 100 µl of sodium carbonate buffer was added to wells C2-C12 and D2-D12 and 5-fold serial dilutions were prepared of the control yeast lysate in the remaining wells of rows C and D. 100 µl of lysate from the mutant yeast was added to

E1-E2 and F1-F2. E2-E12 and F2-F12 were filled with 100 µl of sodium carbonate buffer and 5-fold serial dilutions were prepared for the remaining wells. 100 µl of carbonate buffer was added to wells G1-G4 and served as blank controls. Wells G5-G8 was used as negative controls, and contained native lysozyme without 1°Ab. Wells G9-G12 had 1°Ab as antigen and no lysozyme.

After the preparation of serial dilutions, a 1:30,000 dilution of 1°Ab was prepared in 1X PBS (phosphate-buffered saline, 0.01 M sodium phosphate monobasic and sodium phosphate dibasic mixed in equal volume, 0.15 M sodium chloride, pH 7.4). A first dilution of 1:100 was prepared by taking 2 µl of 1°Ab and 198 µl of PBS. A second dilution of 1:10,000 was prepared by taking 2 µl from the first dilution and 198 µl of PBS. Finally, a 1:30,000 dilution was made by taking 1 ml of 1:10,000 dilution and 3 ml of sodium carbonate buffer. 100 µl of 1:30,000 1°Ab solution was added to wells G9-G12. The solutions were incubated for 2 hr at room temperature. After the incubation, the solution from the wells was removed by flicking and blotting the wells with a paper towel. The wells were then filled with 50 µl of blocking buffer (1% bovine serum albumin (Sigma) in 1X PBS) and incubated for 30 min at room temperature. The solution was discarded and the wells were washed three times with wash buffer (PBS with 0.05% tween-20). 100 µl of 1:30,000 solution of 1°Ab was added to all wells except G5-G8 and incubated for 2 hr at room temperature. The wells were then washed three times with wash buffer and 100 µl of 1:20 dilution of streptavidin, a 2° reagent, was added to all wells. After incubating for 30 min at 37°C the wells were washed with wash buffer and 100 µl of the liquid substrate tetramethyl benzidine (Sigma) was added to all the wells with a multichannel pipette. After the development of blue color the reaction

was stopped with the addition of 50 µl of 2 M sulphuric acid and the absorbance was read at 450 nm (Bio-Tek Instruments microplate auto reader EL 311).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Lysozyme 10µg/ml	5x dilution	→									
B	Lysozyme 10µg/ml	5x dilution	→									
C	Control 5mg/ml	2x dilution	→									
D	Control 5mg/ml	2x dilution	→									
E	lysate 5mg/ml	2x dilution	→									
F	Lysate 5 mg/ml	2x dilution	→									
G	Blank	→	→	→	No 1° Ab	→	→	→	1°Ab as Ag	→	→	→
H												

Figure 2-3: Preparation of the 96 well microtiter plate for ELISA.

Section VIII: Expression in BMGY/BMMY with changed parameters

As the expression in various media was not high enough to give an intense band on SDS-PAGE. We decided to express in BMGY/BMMY media by changing the parameters. Three 20 ml volumes of BMGY were prepared and distributed into 250 ml

baffled flasks. Two flasks were inoculated with frozen stock of mutant yeast colony DM3-6c and one flask was inoculated with control yeast. The yeast was grown at 28°C in a shaking incubator at 300 rpm until an OD_{600} 1.5 – 2.0. The cells were harvested by centrifuging at 4500 rpm for 5 min at 4°C. The cell pellet was resuspended in 50 ml of BMMY media to induce expression and 0.005% of anti-foaming agent was added to prevent foam.

The induction was maintained with the addition of 1% and 2% methanol every 24 hr. 500 µl (1%) of 100% methanol was added to a flask containing mutant yeast and a flask with control yeast and 1 ml (2%) of 100% methanol was added to another flask containing mutant yeast. 1 ml of expression culture was collected in 1.5 ml centrifuge tubes at time points of 24, 48, 72, and 96 hr. The 1 ml cultures were centrifuged at maximum speed in a table top microcentrifuge for 1 min and the pellets were quickly frozen in dry ice/acetone and stored at -80°C. These samples were used to analyze expression levels by SDS-PAGE.

The frozen cell pellets were thawed and placed on ice. Only 100 µl of breaking buffer was used to resuspend two 1 ml sample pellets in order to concentrate the protein. The lysate was prepared as described on page 36. The samples for electrophoresis were prepared by mixing 20 µl of protein lysate, 20 µl of 62.5 mM Tris-HCl, pH 6.8, 10 µl of 10% SDS, and 6 µl 2-mercaptoethanol. The samples were then placed in a boiling water bath for 8-10 min. After the samples had cooled 40 µl 20% (v/v) glycerol and 10 µl of 0.05% bromophenol blue were added and any insoluble material was removed by centrifuging for several seconds on a table top centrifuge.

SDS-PAGE electrophoresis was performed using 15% gels (Biorad). 30 µl of each sample, 10 µl of molecular protein marker and 10 µl of native lysozyme were loaded in the wells and run initially at 80 V and 120 mA until the samples reached the separating gel and then at 140 V until the dye front reached the bottom of the gel. The gels were then stained with Gelcode® blue. The gels were washed with water three times for 5 min each and stained with Gelcode® blue overnight. The gels were then destained in DI water.

Two more SDS-PAGE gels were run with the 72 hr samples from the 1% and 2% methanol cultures. The preparation of lysate and SDS-PAGE samples were similar to as described above except that the protein lysate volume was 30 µl in the samples. One set of sample was prepared with freshly extracted lysate and another with previously prepared lysate. 30 µl of the 72 hr sample and 10 µl of native lysozyme were loaded onto the gels.

The SDS-PAGE gels were run and the gel fixing and staining were done according to the Coomassie stain protocol (OSU). After the electrophoresis, the gel was soaked in 500 ml of the gel-fixing solution (50% (v/v) ethanol in water with 10% v/v acetic acid) for 1 hr. At the end of 1 hr, the solution was removed and the gel was covered with 500 ml of gel-washing solution (50% (v/v) of methanol in water with 10% acetic acid) and fixing of the proteins in the gel was continued by incubating overnight at room temperature. The gel was then stained with Coomassie stain (0.1% (w/v) Coomassie blue R250 (Biorad), 20% (v/v methanol, and 10% (v/v) acetic acid) for 4 hr at room temperature. The gel was destained with ~250 ml of destain solution (50% (v/v) methanol in water with 10% (v/v) acetic acid). The gel was destained by changing the

destain solution until the bands are seen without the background staining. The gel was then equilibrated in 500 ml of the storage solution (5% (v/v) acetic acid) until it was sent for analysis. The bands corresponding to lysozyme were cut from the gel containing samples from 2% methanol culture and covered with 5% acetic acid in a micro centrifuge tube and sent for analysis by mass spectrometry.

CHAPTER III: RESULTS

Section I: Generation of the double mutant

The double mutant gene (H15S+N77H) was generated using an H15S lysozyme mutant gene as the DNA template. 5 µl of samples obtained by the two PCR reactions were analyzed on a 1% agarose gel electrophoresis in 1XTAE buffer. The gel was run at 96 V for 55 min, soaked in ethidium bromide for 15 min and analyzed by imaging system (Stratagene). 1% agarose gels show the front end of ~250 bp (Figure 3-1) and the back end of ~150 bp (Figure 3-2) of PCR products.

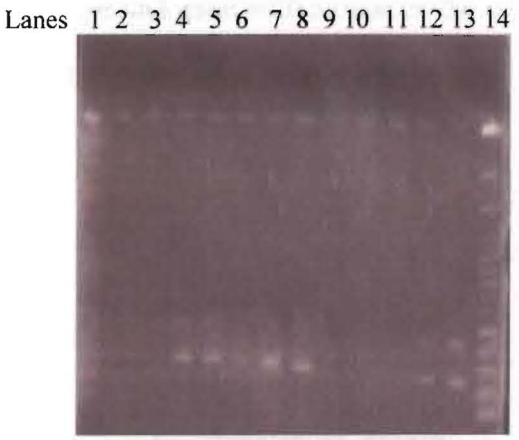


Figure 3-1: 1% agarose gel showing the front end fragments of mutant gene. Lanes 1 and 14 show 100 bp DNA ladder, Lanes # 4-8 and 12-13 show PCR I product (~250 bp).

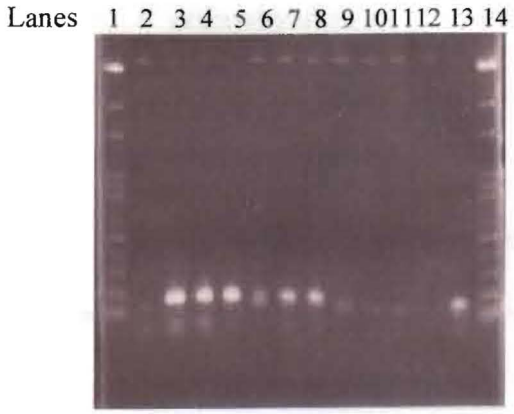


Figure 3-2: 1% agarose gel showing back end fragments of mutant gene. Lanes 1 and 14 show 100 bp DNA ladder, Lanes # 2-13 show back end of gene about ~150 bp.

The two overlapping fragments were annealed together to construct the complete gene. The PCR product was run on 1% agarose gel at 96 V for 55-60 min and stained with the ethidium bromide. Figure 3-3 shows the PCR product from overlap extension. The PCR yielded the expected gene with a band of the correct size (~400 bp).

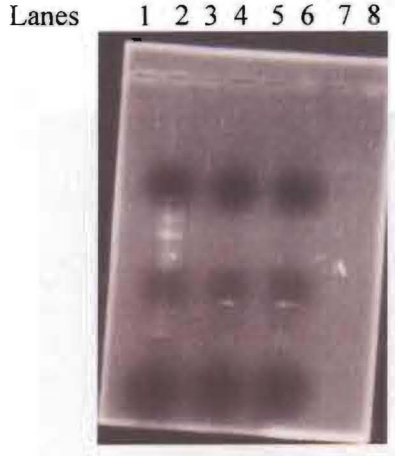


Figure 3-3: Agarose gel showing the annealed PCR product. Lane 2 shows 100 bp DNA ladder, lanes 4 and 6 shows annealed mutant gene of 400 bp.

Transformation of TOP10 E. coli cells

The mutant gene obtained by the overlap extension method was cloned into the pCR®4-TOPO® vector. The plasmid pCR®4-TOPO® supplied in the kit was linearized

with single overhanging 3' deoxythymidine (T) residues. This allows the PCR insert to ligate efficiently with the vector because use of Taq polymerase adds overhanging Adenine residues to the PCR product. After the transformation of TOP10 *E. coli* competent cells with pCR®4-TOPO® with the inserted gene, the transformations were plated on the LB plates. The LB plates incubated overnight at 37°C showed growth of ampicillin-resistant *E. coli* colonies.

The double mutant colonies that were labeled as DM1, DM2, DM3, DM4 and colonies containing native gene N1, N2, N3, and N4 were tested for the insert. The plasmid DNA of positive clones was analyzed by PCR. The PCR products were run on a 1% agarose gel at 96V until the dye migrated to the end of the gel. The gels stained with ethidium bromide indicated that the gene of right size had been successfully inserted into the vector (Figure: 3-4 and 3-5).

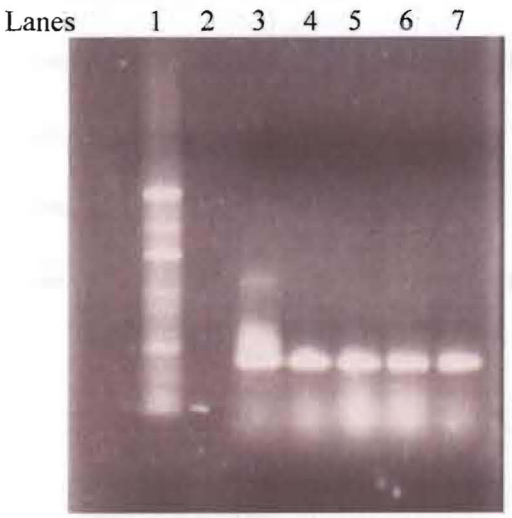
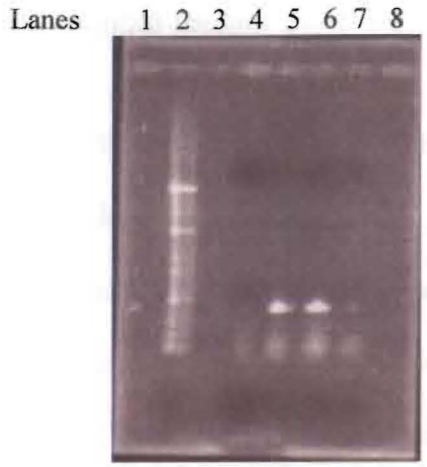


Figure 3-4: 1 % agarose gel with PCR products of plasmid from transformed *E. coli* colonies showing successful ligation of the mutant gene. Lane 1 shows 100 bp ladder, lane 3 shows native gene, lanes # 4-7 shows mutant gene (DM1, DM2, DM3, and DM4).



Figue 3-5: 1% agarose gel with PCR products of plasmid from transformed *E. coli* colonies showing successful ligation of native gene. Lane 2 shows 100 bp ladder, lane # 4-7 shows native gene (N1, N2, N3, N4).

Sequencing of native gene and double mutant gene in pCR®4-TOPO®

The gene sequence for hen egg white lysozyme contains 390 nucleotides (Appendix A). In the native sequence the codon AAC encodes for Asparagine whereas in mutant sequence CAC now codes for histidine.

The DNA sequencing reactions confirmed the identity of both the native and mutant gene sequences, thereby proving the success of the mutagenesis and cloning experiments. A portion of the sequence is shown by the electropherogram below confirming the mutation of the AAC codon for asparagine to CAC for histidine (Figure 3-6)

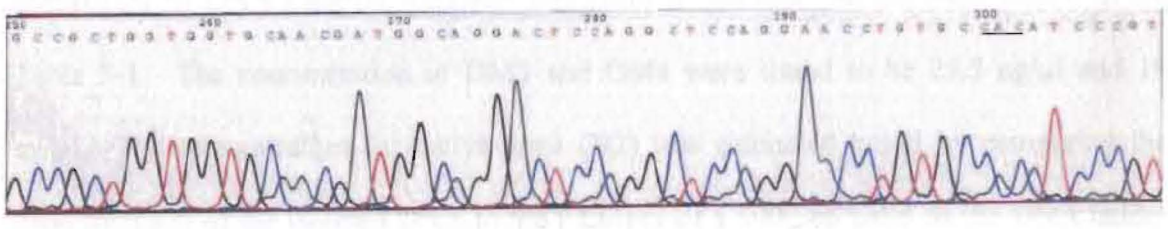


Figure 3-6: Sequence data from mutant lysozyme gene where codon CAC encodes for histidine.

Ligation of native and double mutant gene into pPICZ B and transformation of E.coli

Once the sequence of the native and mutant gene was confirmed, the desired gene and the pPICZ B were double digested with *Eco*R I and *Xba* I. A 400 bp pair DNA fragment containing mutant hen egg white lysozyme coding sequence flanked by *Eco*R I and *Xba* I sites was isolated from pCR®4-TOPO® and inserted into the pPICZ B plasmid.

As a first step, the PCR was performed on the pCR®4-TOPO® containing either the native gene (N2) or the double mutant gene (DM3 and DM4). The PCR products run on 1% agarose gel showed bands of ~400 bp (Figure 3-7).

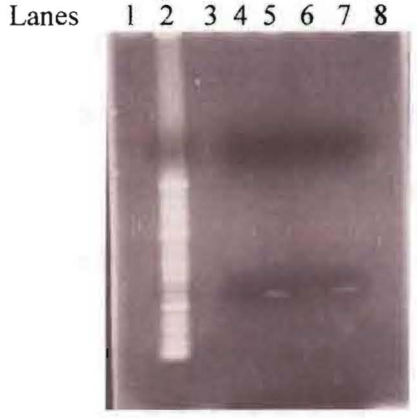


Figure 3-7: Agarose gel showing the PCR product of native and double mutant gene. Lane 2 shows 100 bp ladder, lane 5 shows native gene (N2), lane 6 and 7 shows double mutant gene (DM3 and DM4).

The absorbance of purified DNA was determined using UV spectrometer. The absorbance of DNA samples for DM3 and DM4 at 260 and 280 nm were shown in the Table 3-1. The concentration of DM3 and DM4 were found to be 25.5 ng/µl and 19 ng/µl. The concentration for native gene (N2) was estimated based by comparing the band intensity of the isolated DNA to the intensity of a 3000 bp band on the DNA ladder

and it was predicted to be ~4 ng/µl. The concentrations were found to be sufficient to perform double digestion and ligation reactions.

Sample	Abs260	Abs 280
DM3	0.05113	0.04768
DM4	0.03829	0.03072

Table 3-1: The absorbance of DNA at 260 and 280 nm.

The pPICZ B plasmid extracted from the *E. coli* was run on the 1% agarose gel. The ethidium bromide stained showed the presence of the purified plasmid pPICZ B of ~3.0 kb (Figure 3-8). The concentration was estimated to be 4 ng/µl for the band in lane 4 and 15 ng/µl for the band in lane 6.

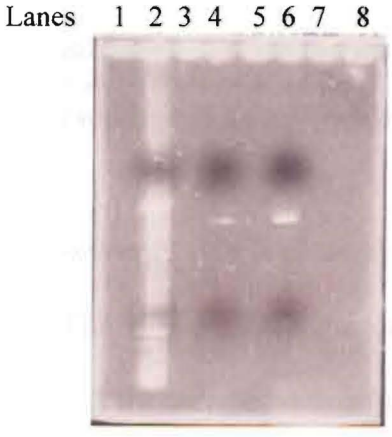


Figure 3-8: Agarose gel showing the purified pPICZ B plasmid from *E. coli*. Lane 2 shows 100 bp ladder, lane 4 and 6 shows the pPICZ B plasmid of ~ 3.0 Kb.

The TOP10 *E. coli* transformed with the pPICZ B plasmid containing either native or double mutant gene showed growth on the low salt LB plates containing

Zeocin™. The Zeocin™ resistant colonies were analyzed for the presence of the desired gene by PCR. The PCR products of the double mutant colonies of DM3 (DM3-1, DM3-2, DM3-3, DM3-4, DM3-5, and DM3-6) and colonies of DM4 (DM4-1, DM4-2, DM4-3) were run on a 1% agarose gel. The gel run at 96 V for 55-60 min and stained with ethidium bromide is showed below (Figure 3-9). The colony labeled DM3-6 showed an intense band of ~400 bp, therefore DM3-6 was used to transform the yeast.

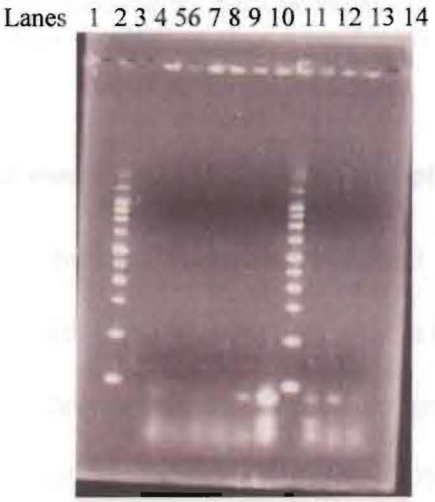


Figure 3-9: Agarose gel showing the positive transformations of *E. coli*. Lanes 2 and 10 show 100 bp ladder, lanes # 4-9 show PCR product from DM3 colonies (DM3-1, DM3-2, DM3-3, DM3-4, DM3-5, and DM3-6) and lanes # 11- 13 show PCR product of DM4 colonies (DM4-1, DM4-2, DM4-3).

PCR was performed on the plasmid containing the native gene, N2 colonies N2-1, N2-2…..N2-8. The PCR products run on a1% agarose gel showed bands of correct size (Figure 3-10). All the colonies showed a band of ~400 bp. The colony N2-6 showed an intense band, therefore, this colony was used for the transformation of *Pichia*.

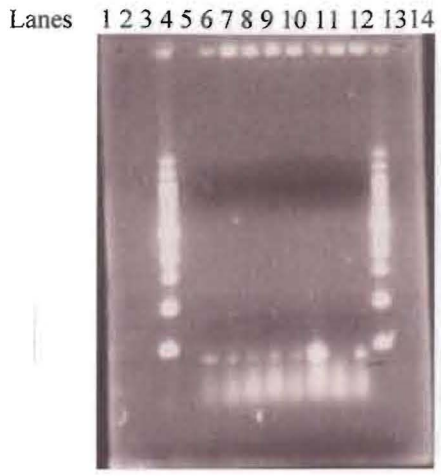


Figure 3-10: Agarose gel showing the positive transformants of *E. coli*. Lane 3 and 13 show 1 kb ladder, lanes # 5-12 show the PCR product from the colonies containing native gene (N2-1, N2-2…..N2-8).

Section II: Transformation and expression of protein in the *Pichia pastoris*

The desired gene was successfully cloned behind the *AOX1* promoter, in the multiple cloning site with unique restriction sites in the pPICZ B plasmid. The pPICZ B plasmid containing either mutant gene or native gene and pPICZ B without any insert were then linearized with *Bst*X I. The linearized DNA samples were run on 1% agarose gel. The ethidium bromide stained gel showed bands of correct size (Figure 3-11). Lane 5 and 6 show ~4000 bp of pPICZ B plasmid containing gene.

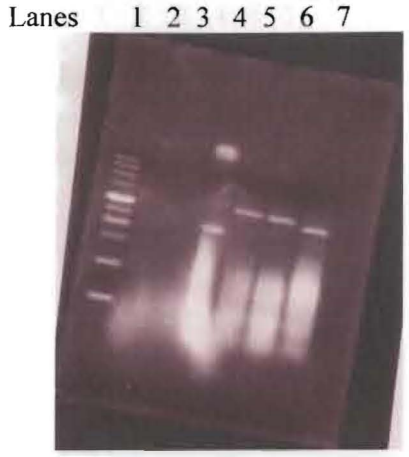


Figure 3-11: Agarose gel of linearized pPICZ B vector. Lane 1 is 1 Kb DNA ladder, lane 4 is uncut pPICZ B plasmid, lane 5 is linearized pPICZ B with native gene, lane 6 is linearized pPICZ B with double mutant and lane 7 is linearized pPICZ B without insert.

X-33, GS115, and KM71H competent cells were transformed with *Bst*X I digested pPICZ B plasmid with the double mutant gene. Only one strain, X-33, was transformed with the native gene. The YPD plates containing 100 μg/ml Zeocin™ incubated at 30°C for 2 days showed growth of Zeocin™ resistant colonies in the plate labeled as X-33 with pPICZ B plasmid containing the mutant gene. The colonies that showed Zeocin™ resistance were grown in 10 ml YPD media containing 100 μg/ml Zeocin™. PCR analysis on the plasmid extracted from the X-33 strain colonies (DM3-6a, DM3-6b, and DM3-6c) confirmed the presence of a ~400 bp insert. A 2% agarose gel was run with the samples prepared by mixing 10 μl of PCR product and 2 μl of dye at 96V for 55mins. Fig 3-12 is a picture of a gel which shows positive integrants in lanes 2, 3, 4, and 5 representing a band of DNA corresponding to ~400 base pairs. Lane 6 shows a ~700 bp product with *AOX* primer.

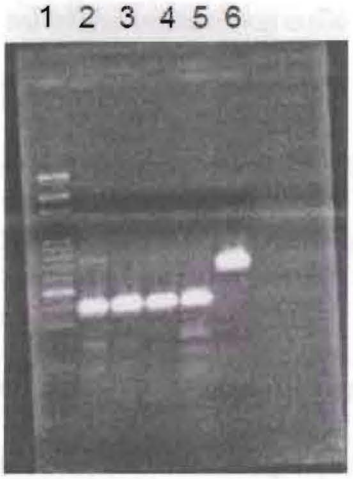


Figure 3-12: Agarose gel with PCR product of colonies that showed positive transformation. Lane 1 is the 100bp ladder, lanes 2, 3, 4 are from three colonies Dm3-6a, Dm3-6b, Dm3-6c, lane 5 is pPICZ B with native gene, lane 6 is pPICZ B with AOX primer.

Transformations of X-33 with plasmid DNA linearized in the 5'*AOX1* region yielded Mut^+ transformants. After the single colonies were grown for 2 days on the MMH plates, the patches were replica-plated onto the MDH plates. The X-33 colonies were scored as Mut^+ (Methanol utilization plus) based on the growth on both MMH and MDH plates.

Section III: Small scale expression

Three colonies of transformed X-33, labeled as DM3-6a, DM3-6b, and DM3-6c were grown in various media to determine the optimal conditions to express the active mutant lysozyme. The small scale expression of the mutant lysozyme was performed in BMMY, BMM, MMH and SM media as described in Materials and Methods.

Since the *AOX1* promoter regulates the expression of protein, cultures were induced by transferring exponentially growing cells from 1% glycerol to 1% methanol. The X-33 transformants were grown in shake-flask cultures and the expression of the protein was found to be dependent on the composition of the medium.

Every 24 hr, 1 ml of the culture was collected and the cell lysate was prepared and investigated by SDS-PAGE. A single band of ~14kDa migrating parallel with native lysozyme was observed in BMMY, BMM and SM media. The band density represents the secretion level. The amount of protein secreted over a period of 96 hr in various media was as shown by SDS-PAGE gels.

A protein with a molecular weight approximately ~14 kDa upon induction with 0.5% methanol was produced in BMMY by three colonies DM3-6a, DM3-6b, and DM3-6c. SDS-PAGE gels from cell lysate are shown below (Figures 3-13 and 3-14).

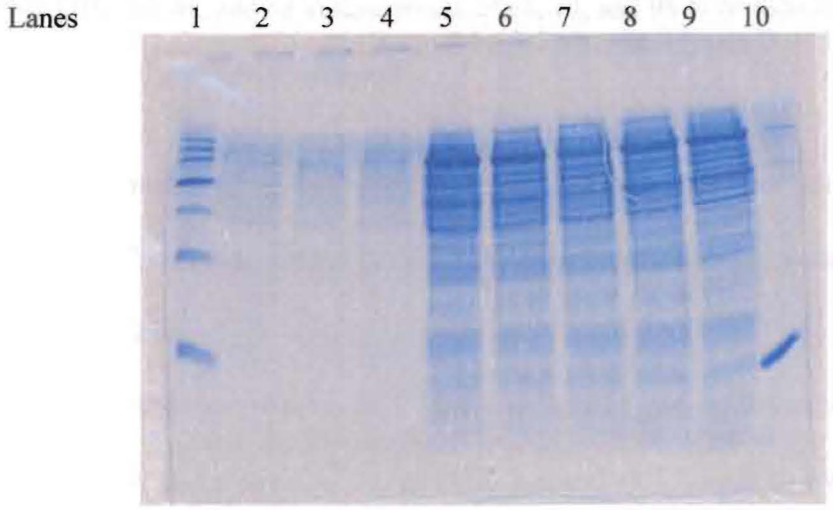


Figure 3-13: SDS-PAGE showing expressed protein bands at ~14 kDa in BMMY media by three colonies DM3-6a, 6b, and 6c at time points of 0, 24, and 48 hr of induction. Lanes 1 show MW marker, Lanes # 2-4 show cell lysate from 0 hr, Lanes # 5-7 from 24 hr, Lanes #8-9 from 48 hr and Lane 10 show native lysozyme.

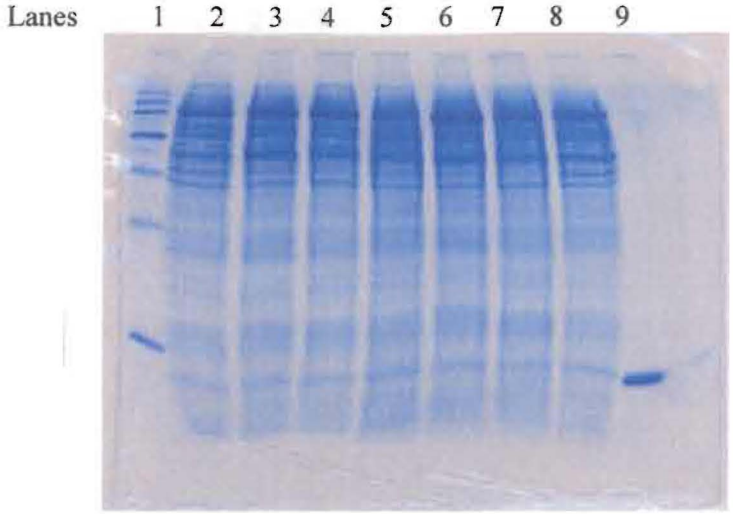


Figure 3-14: SDS-PAGE showing expressed protein bands at ~14 kDa in BMMY media by three colonies DM3-6a, 6b, and 6c at time points of 48, 72, and 96 hr of induction. Lanes 1 show MW marker, Lanes # 2 show cell lysate from 48 hr, 3-5 show cell lysate from 72 hr, Lanes # 6-8 from 96 hr, Lanes #9 show native lysozyme.

The amount of mutant protein in the medium increased steadily with induction time in BMMY media. Based on the results, the optimum methanol induction time for cultures was 72 hr.

Small scale expression in BMM was carried out as described on page 33. The cells were collected every 24th hr up to 96 hrs and cell lysates prepared for SDS-PAGE. The SDS-PAGE gels with cell lysates from three colonies of X-33 are shown in figures 3-15 and 3-16. Expression of a protein of ~14 kDa was observed in lysate collected at 72 and 96 hr samples but the bands were not as intense as the bands observed in BMMY media.

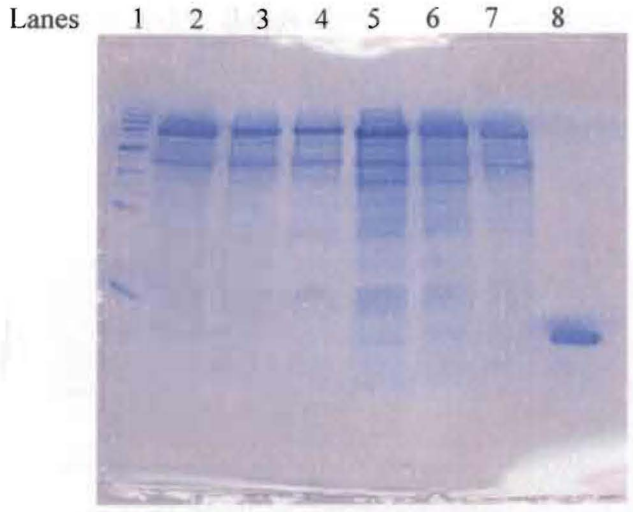


Figure 3-15: SDS-PAGE with cell lysate from BMM media. Lane 1 show marker, Lanes # 2-4 show cell lysate from DM3-6a, 6b, and 6c at 24 hr, Lanes # 5-7 show cell lysate at 48 hr and Lane 8 show native lysozyme.

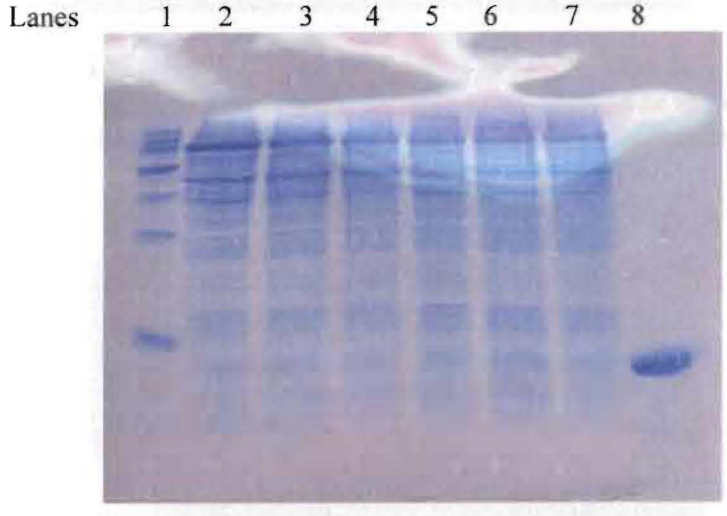


Figure 3-16: SDS-PAGE showing protein at ~14 kDa in BMM media. Lane 1 show marker, Lanes # 2-4 show cell lysate from DM3-6a, 6b, 6c at 72 hr, Lanes # 5-7 show cell lysate from DM3-6a, 6b, 6c at 96 hr and Lane 8 show native lysozyme.

The cell lysates from cultures grown in MMH medium were prepared by the same procedure as that described for BMMY medium and investigated by SDS-PAGE. Shown below are SDS-PAGE gels of cell lysate collected every 24th hr over a period of 96 hr

from three colonies of X-33. Bands corresponding to native lysozyme were not observed upon staining the gels (Figure 3-17 and 3-18).

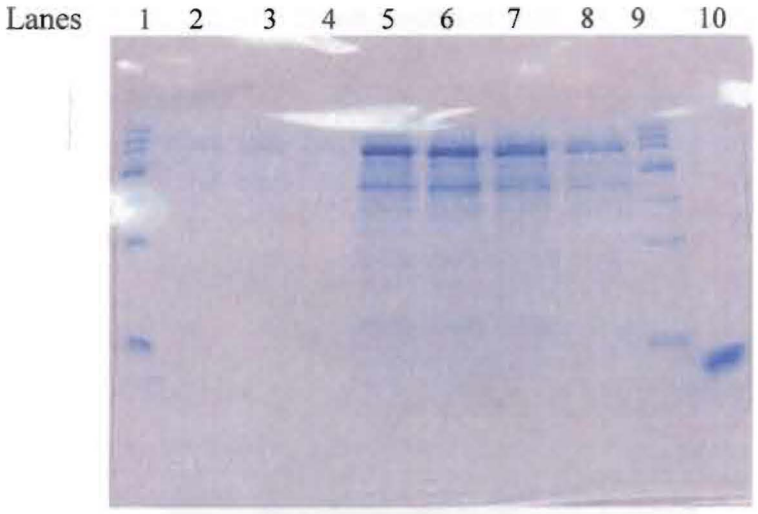


Figure 3-17: SDS-PAGE shows no bands at ~14 kDa in MMH. Lane 1and 9 show the molecular weight marker, Lanes # 2-4 show cell lysate from DM3-6a, 6b, and 6c at 24 hr, Lanes # 5-7 show cell lysate from DM3-6a, 6b,and 6c at 48 hr and Lane 10 show native lysozyme.

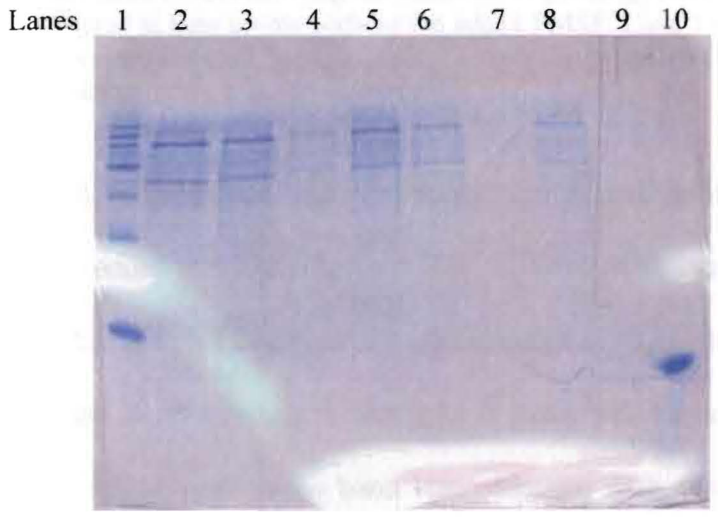


Figure 3-18: SDS-PAGE shows no protein at ~14 kDa in MMH. Lane 1 show marker, Lanes # 2-4 show cell lysate from DM3-6a, 6b, and 6c at 72 hr, Lanes # 5-8 show cell lysate from DM3-6a, 6b, and 6c at 96 hr and Lane 10 show native lysozyme.

Expression in synthetic media

18% and 12% gels were run with lysate collected at time points from the 4 flasks. Gels with less intense bands were obtained but the results were inconsistent. Figure 3-19, is a 12% SDS-PAGE gel with lysate collected from 24, 48, 72, 96 hr cell pellets without the added PMSF, showing bands corresponding to lysozyme.

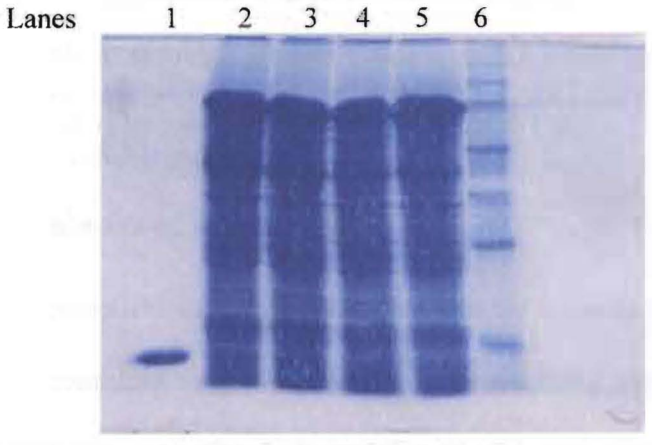


Figure 3-19: A 12% SDS-PAGE showing the bands corresponding to the native lysozyme. The lysate was collected at time points without the added PMSF. Lane 1 shows native lysozyme, lane # 2-5 shows lysate collected from 24, 48, 72, and 96 hr cell pellets. Lane 6 shows the molecular weight marker.

An 18% gel was run with lysate collected from the cell pellet at time points from flask 2 with PMSF added at 48 hr (Figure 3-20). A SDS-PAGE sample prepared with 40 µl of lysate (all other samples contained 20 µl) collected at 96 hr was also run along with control and samples at time points in this gel. A band was observed in the lane with sample containing 40 µl whereas no band was observed with control lysate. All other samples gave very faint bands.

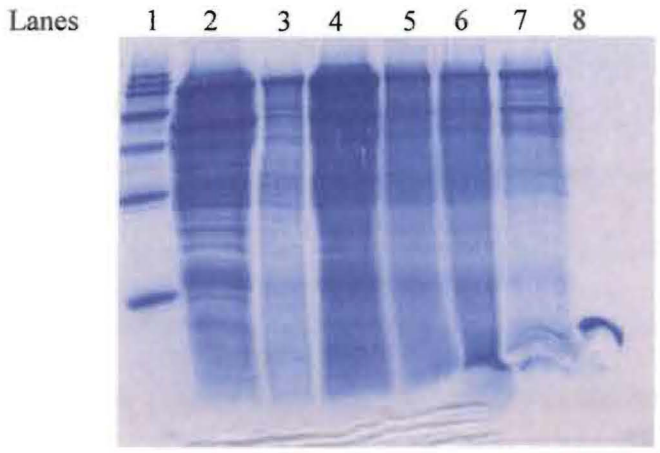


Figure 3-20: An 18% SDS-PAGE gel with lysate collected at time points from flask 2 with PMSF added at 48 hr. Lane 1 shows molecular weight marker. Lane 2 shows sample prepared with 40 µl of lysate collected at 96 hr. lane 3 shows control, lane # 4-7 shows lysate at time points of 96, 72, 48, and 24 hr. lane 8 shows native lysozyme.

Section IV: Scale-up of expression

The cell paste obtained in the first large scale experiment was lysed and the time required for the complete lysis and protein release from the cells was determined. A 100 µl cell lysate was removed after 3, 4, and 5, min of beating the cells. The absorbance at 280 nm of a 100 fold was measured and the total protein concentration was estimated assuming a 1 mg/ml solution has an absorbance of 1. Based on this estimate the total protein was released from the cells after 3 min of lysis. Table 3-2 estimates the total protein concentration in the cell lysate.

Fraction /min	Concentration µg/µl
3	10.3
4	10.0
5	9.9

Table 3-2: Estimation of total protein concentration in the cell lysate during the 5 min lysis.

Enzyme assay

To determine the enzyme activity, a plot of absorbance versus time was constructed. The absorbance at 450 nm 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 by a 100 µl aliquot of a 10 µg/ml sample of native lysozyme is shown in the graph below (Figure 3-21). Lytic activity could not be detected with 100 µl of whole cell lysate collected at 72 hr grown in BMGY/BMMY (Figure 3-22).

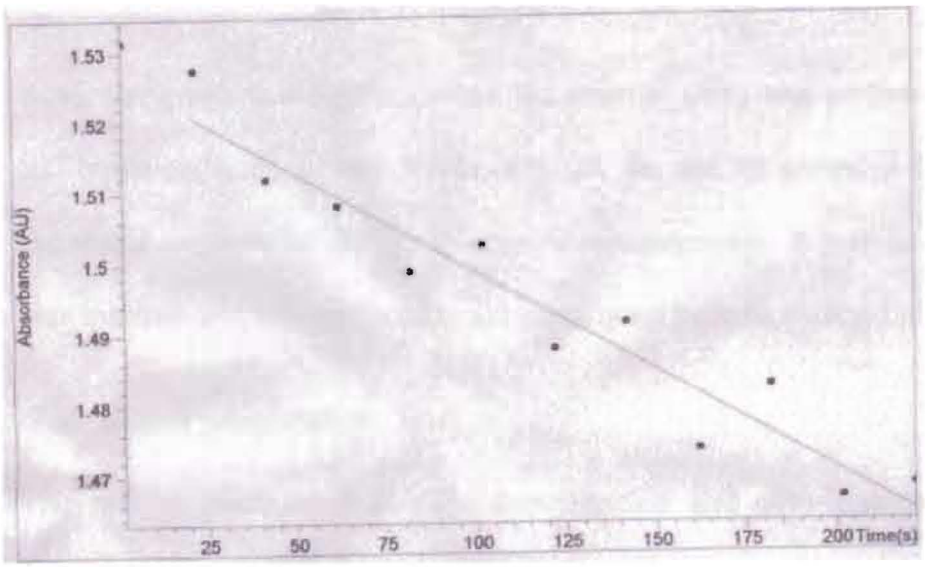


Figure 3-21: Graph showing the lytic activity of native lysozyme.

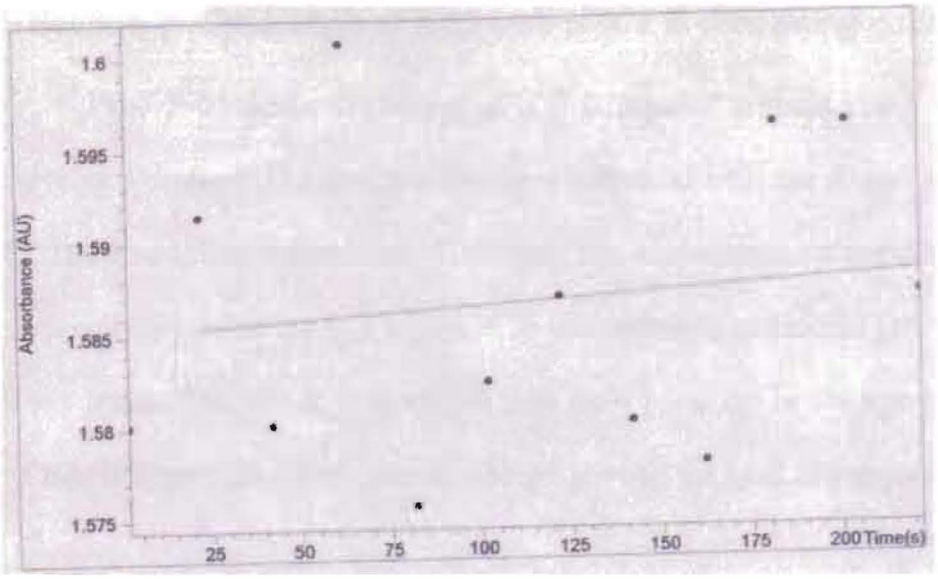


Figure 3-22: Graph showing the enzyme activity assay with whole cell lysate collected at 72 hr

A similar graph was obtained when the enzyme assay was performed with the whole cell lysate collected at time points of 0, 24, 48, and 96 grown in BMMY, SM media and eluted fractions by the gel filtration chromatography. It may be because the protein was inactive or it was produced in too low a quantity to be detected in the assay.

Ammonium sulfate precipitation

The protein precipitated at 30% concentration and 60% concentration was dialyzed using Tris, pH 6.8 buffer. The dialyzed protein was applied on to SDS-PAGE gel. The molecular weight marker was run in lane 1, 30% protein solution in lane 2, 60% protein solution in lane 3, supernatant solution in lane 4 and native lysozyme in lane 5. The gel was run at 150 V until the dye reached the bottom of the gel and stained with Gelcode® blue overnight. The band corresponding to the native lysozyme was not observed in SDS-PAGE gels (gel not included).

Section V: Generation of X-33 with pPICZ B containing no insert

The X-33 strain containing pPICZ B plasmid without insert was generated to serve as a control. The plasmid DNA was extracted from the *E. coli* and absorbance of the DNA sample was measured at 260 nm. The concentration was calculated assuming a 50 μg/ml sample has an A_{260} of one. The concentration of plasmid DNA was found to be 2.513 μg/μl. The pPICZ B linearized with *Bst*X I was run in 1% agarose gel at 96 V for 55 min (Figure 3-23). Ethidium stained gel showed the band of correct size.

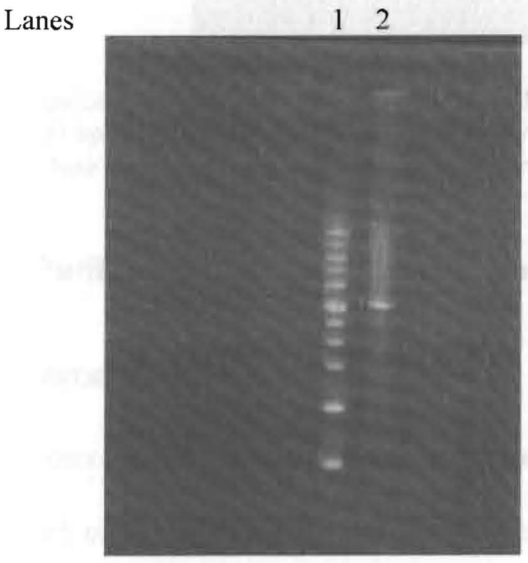


Figure 3-23: Agarose gel showing linearized pPICZ B. lane 1 shows 1.0 Kb DNA ladder and lane 2 shows linearized pPICZ B of ~4000 Kb.

The yeast, X-33 was transformed with linearized pPICZ B as described in Materials and Methods. The PCR products of the pPICZ B from the colonies 1A, 1B, 1C, 1D, 1E were run on a 2% agarose gel for 55 min at 96 V. The colony, 1D, was found be successfully transformed. Figure 3-24 is a picture of a gel showing positive transformant in lane 5 representing a band of ~300 bp (corresponding to *AOX1* gene)

DNA. This colony was grown in BMGY/BMMY, the lysate collected and was used in the analysis by SDS-PAGE and ELISA.

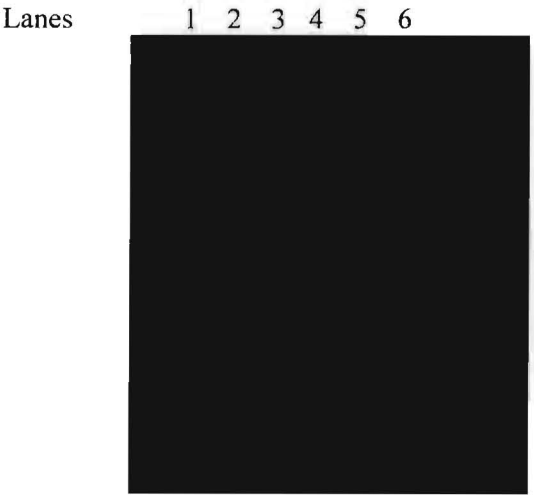


Figure 3-24: Agarose gel showing the PCR product of the pPICZ B from transformed yeast. Lane 1 shows 100 bp DNA ladder, lane 2 is from colony 1A, lane 3 is from colony 1B, lane 4 is from colony 1C, lane 5 is from colony 1D and lane 6 is from 1E.

Section VI: Purification and characterization of mutant lysozyme

Gel filtration chromatography

As a control, 1 ml of native lysozyme was loaded on to a 1.5 cm x 10.0 cm column. A graph of absorbance of the elutant versus tube number was plotted (Figure 3-25). In an attempt to purify the mutant lysozyme, 700 µl of the lysate sample was applied to the column and eluted with Tris-NaCl buffer. Figure 3-26 shows the graph of absorbance at 280 nm versus tube number. Enzyme assays of the elutant in tube numbers 8, 24, 42, 46, 64, 76 did not show lytic activity. The elutant form these tubes were also analyzed by SDS-PAGE. No band corresponding to the molecular weight of lysozyme appeared. Fractions of the eluted protein were pooled and concentrated. Enzyme assays performed on these fractions also did not show any lytic activity.

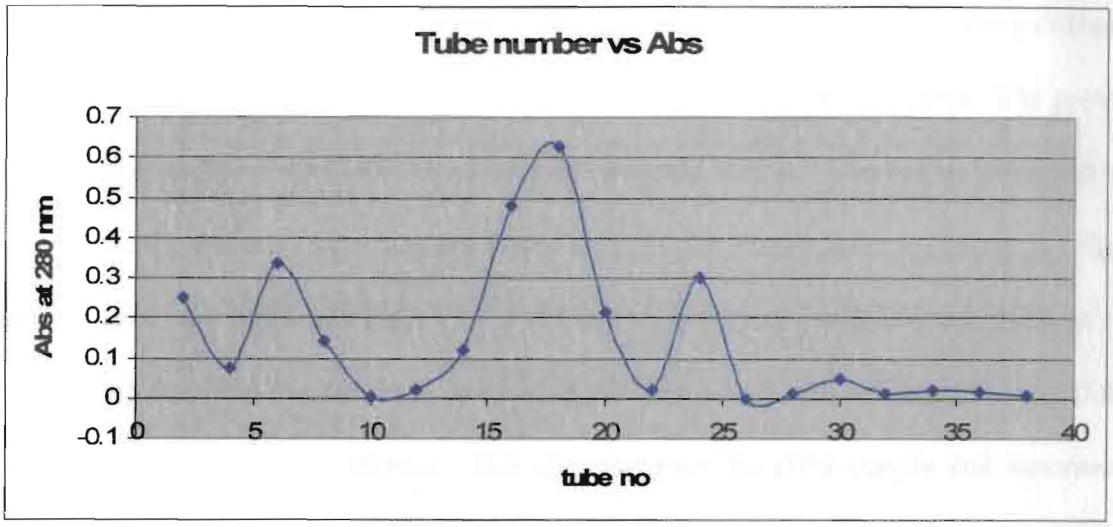


Figure 3-25: Graph of absorbance versus tube number for native lysozyme. The native lysozyme was eluted with Tris-NaCl buffer at a flow rate of 5 ml/hr. every 2.5 ml of effluent was collected in a test tube.

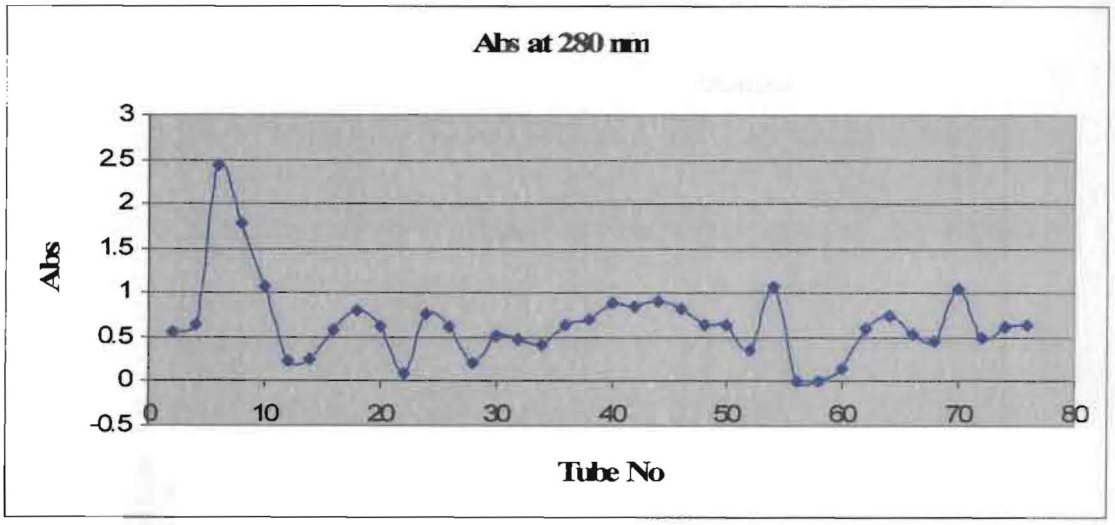


Figure 3-26: Graph of absorbance versus tube number for lysate sample. The proteins were eluted with Tris-NaCl buffer at a flow rate of 5 ml/hr. every 2.5 ml of effluent was collected in a test tube. The fraction falling under the peaks were pooled, concentrated and analyzed by enzyme assay and SDS-PAGE

Purification by HPLC

In an attempt to recover the mutant lysozyme by cation-exchange chromatography, 900 µl of lysate was loaded onto a PolyCAT A™ column. The proteins were eluted as described in the Materials and Methods section. The native lysozyme was also applied to the column and eluted with the HPLC buffers to serve as a control. Figure 3-27 shows the chromatogram for the native lysozyme and Figure 3-28 is the chromatogram for 30% sample eluted with potassium phosphate buffer containing 0.4 M NaCl at flow rate of 1.00 ml/min. The chromatogram for 60% sample and supernatant sample were similar to that of the 30% sample. The controls of native lysozyme showed peaks at 28-32 min of elution time whereas no peaks corresponding to lysozyme were observed in chromatograms for samples. All the protein was eluted in the void volume during the 1-6 min run.

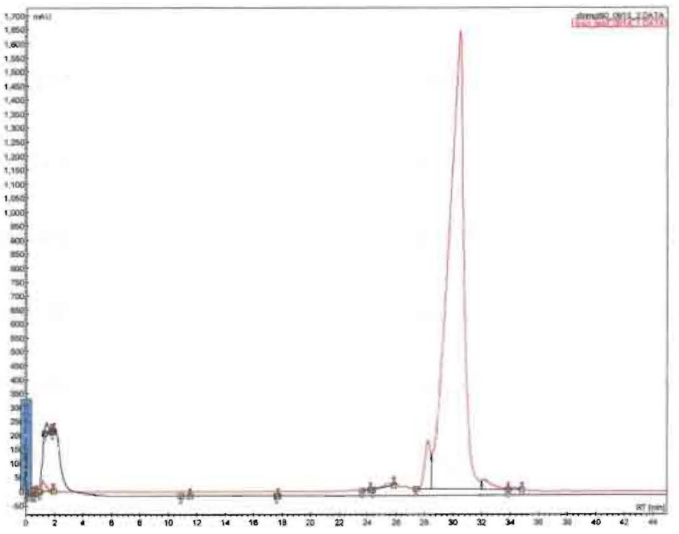


Figure 3-27: Chromatogram for the native lysozyme eluted with the phosphate buffer pH 6.0 containing 0.4 M NaCl at flow rate of 1.00 ml/min. native lysozyme was eluted during 28 to 32 min.

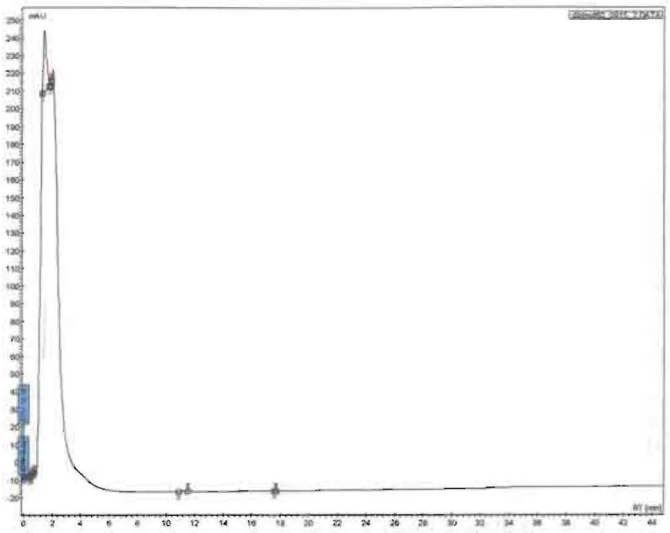


Figure 3-28: Chromatogram for 30% ammonium sulfate precipitated sample eluted with phosphate buffer pH 6.0 containing 0.4 M NaCl. All proteins were eluted in the void volume.

Similar chromatograms were obtained for dialyzed native lysozyme and for 30%, 60% and supernatant lysate eluted with 2-morpholinoethane sulphonic acid buffer, pH 5.0 containing 0.4 M NaCl.

Section VII: Quantitation of protein by Bradford Assay and ELISA

Bradford Assay for the lysate from the large scale expression

The lysate collected in the first large scale expression was assayed to determine the total protein content. The absorbance for the standard and test samples were obtained as described in the Materials and Methods section. A standard curve was obtained by plotting the absorbance for each BSA standard versus its concentration in μg/ml (Figure 3-29). Using the equation from the graph, the protein present in the lysate was calculated.

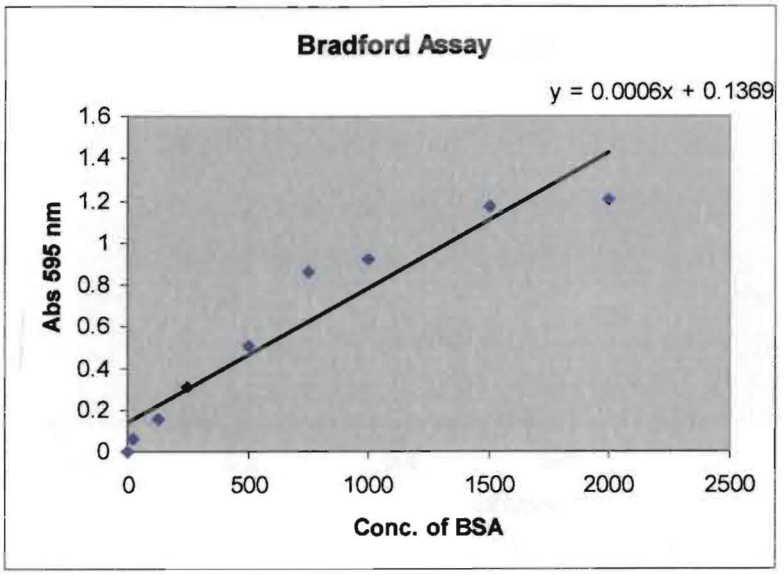


Figure 3-29: Standard curve of absorbance versus concentration of BSA.

The average of the absorbances for lysate was 0.2266. Using the equation from the graph, y = 0.0006x + 0.1369, the amount of protein was found to be 149.5 µg/ml. since the lysate was 100 fold diluted, 149.5 µg/ml X 100 = 14950 µg/ml or 14.95 mg/ml.

Bradford assay for the control and mutant lysate

The total protein concentration of lysate obtained in the second large scale expression experiment of control and mutant yeast was determined with Bradford assay.

The absorbances at 595 nm for various standard and test samples were measured. The blank value was subtracted from individual standard and test sample values and a standard curve was plotted with absorbance versus concentration of BSA in mg/ml (Figure 3-30).

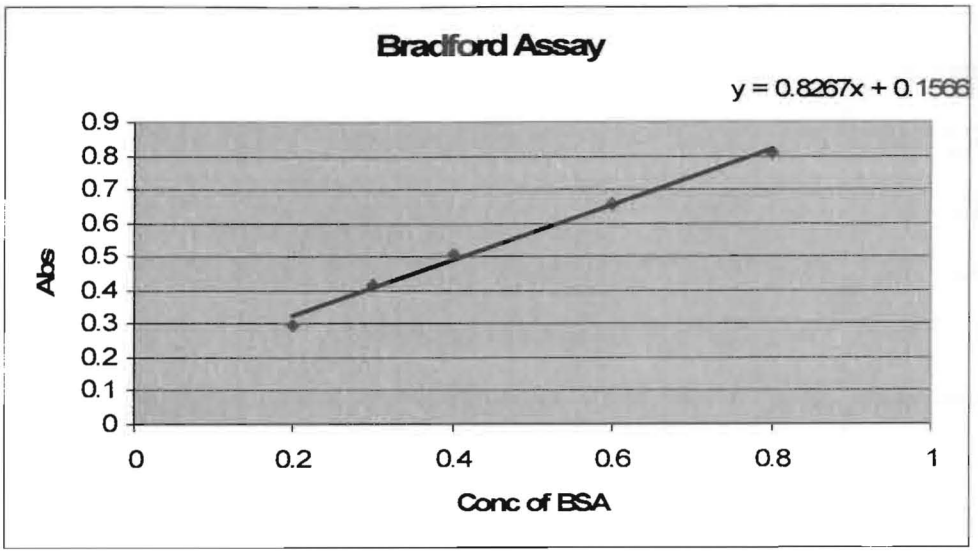


Figure 3-30: Standard curve plotted with absorbance versus concentration in mg/ml

Determining the unknown concentration

Using the equation from Figure 3-35, $y = 0.8267x + 0.1566$, the amount of protein in lysate was calculated. For mutant lysate, the average of absorbance at 595 nm was 0.7743. The amount of protein was found to be 7.47 mg/ml. For the control lysate the average of absorbance at 595 nm was 0.96855. The amount of protein was 9.82 mg/ml.

ELISA

ELISA was performed to detect and estimate the amount of the mutant protein. Table 3-3 shows the absorbances at 450 nm for the native lysozyme, lysate from the control yeast and lysate from the mutant yeast samples. The average of the blank values was found to be 0.1145. The blank value was then subtracted from each absorbance value. The average values for two sets of data obtained for the native lysozyme, control lysate and mutant lysate samples was calculated (Table 3-4). A graph was plotted with average of the absorbance versus the reciprocal of dilution (Figure 3-31).

		1	2	3	4	5	6	7	8	9	10	11	12
A	Lysozyme 10µg/ml	1.091	1.322	0.913	0.382	0.595	0.376	0.171	0.100	0.114	0.101	0.101	0.111
B		1.515	0.827	1.038	0.560	0.755	0.472	0.141	0.094	0.091	0.117	0.101	0.144
C	Control 5 mg/ml	0.500	0.427	0.444	0.441	0.445	0.560	0.473	0.477	0.492	0.351	0.494	0.480
D		0.521	0.393	0.449	0.450	0.453	0.536	0.454	0.362	0.447	0.447	0.463	0.434
E	Lysate 5 mg/ml	0.521	0.522	0.528	0.378	0.393	0.445	0.468	0.504	0.486	0.446	0.411	0.464
F		0.503	0.544	0.544	0.442	0.535	0.522	0.565	0.466	0.546	0.471	0.428	0.440
G	Controls	0.126	0.118	0.114	0.100	0.110	0.101	0.136	0.125	1.875	1.767	1.988	2.159

Table 3-3: Absorbances at 450 nm read by the microplate autoreader of native lysozyme, control, lysate, blank, positive control and negative control samples.

Reciprocal of dilution	Control lysate	Mutant lysate	Native lysozyme
1	0.396	0.3975	1.1885
2	0.2955	0.4185	
4	0.332	0.4215	0.96
8	0.331	0.2296	
16	0.3345	0.3495	0.861
32	0.4335	0.369	
64	0.349	0.402	
128	0.305	0.3705	0.3565
256	0.355	0.4015	
512	0.2845	0.344	0.5605
1024	0.364	0.305	
2048	0.3425	0.3375	0.3095

Table 3-4: Table showing the reciprocal of dilution and corresponding absorbance values of the native lysozyme, control lysate and mutant lysate samples.

5-fold dilutions were prepared for native lysozyme samples whereas 2-fold dilutions were prepared for control and mutant lysates (test samples). To compare the test samples with that of native lysozyme only a few data points falling in the range of

absorbances for test samples were used to plot the graph. The absorbance decreased with a decrease in the concentration for the native lysozyme sample whereas the absorbances were almost constant for the test samples for various concentrations. The ELISA indicates that no protein was detected in the test samples.

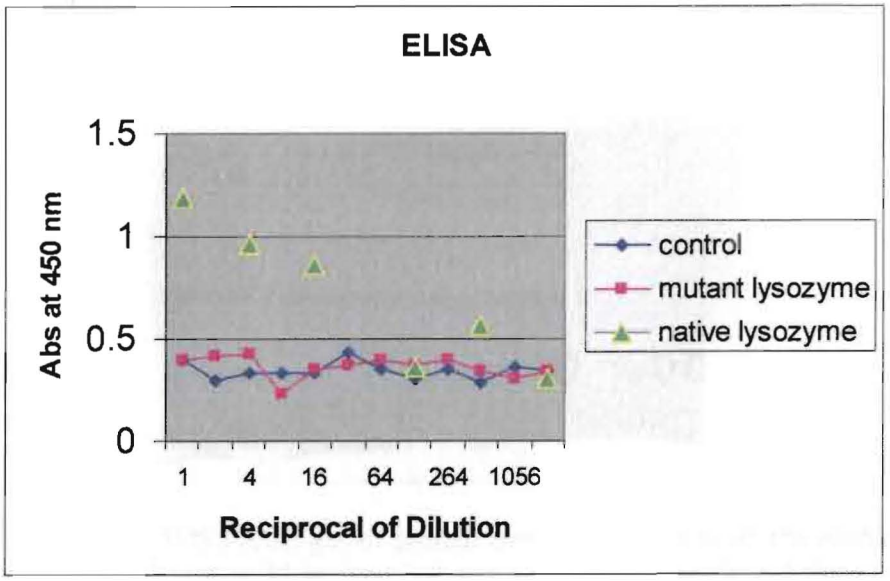


Figure 3-31: Graph plotted with absorbance at 450 nm versus reciprocal of dilution for the native lysozyme, control and lysate samples.

Section VIII: Expression in BMGY/BMMY with changed parameters

The cell lysates from the cells grown in the 1% and 2% methanol BMGY/BMMY cultures were analyzed for the presence of protein. The 15% SDS-PAGE gels run with the lysate collected at 24 hr time points in the 1% and 2% methanol cultures gave intense bands. No difference in the intensity of bands was observed among the 1% methanol induced cells and 2% methanol induced cells. Moreover samples with freshly prepared lysate gave more intense bands than the freeze thawed lysate.

The cell lysate collected at time points in the BMMY media with 1% methanol was run on 15% SDS-PAGE gel showed protein bands corresponding to lysozyme (Figure 3-32). An intense band was observed in the 72 and 96 hr sample whereas there was no such band in the control sample.

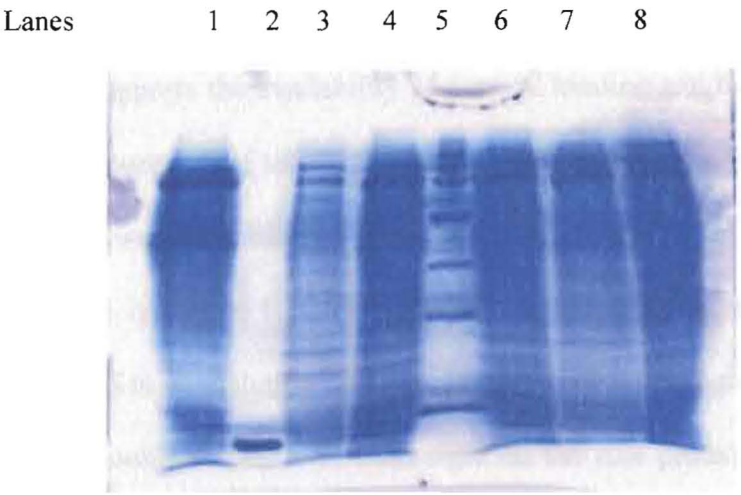


Figure 3-32: A 15% SDS-PAGE gel of protein over a time course of 1% methanol induction. Lane 1 and 6 shows lysate at 72 hr, lane 2 shows native lysozyme, lane 3 shows control lysate, lane 4 shows lysate at 96 hr, lane 5 shows the molecular weight marker, lane 7 shows lysate at 48 hr, and lane 8 shows lysate at 24 hr.

A 15% SDS-PAGE gel was run with a 30 µl of SDS-PAGE samples from 72 hr cell lysate in lanes 1, 2, 3, 5, 6, 7, 8 and native lysozyme in lane 4 (gel not included). The gel stained with Coomassie blue showed bands corresponding to lysozyme of ~14 kDa. Interestingly when the gels were treated with gel washing and destaining solutions (Coomassie stain protocol) most of the proteins were washed off the gels. The gel which retained few bands of protein of our interest were cut and sent to Ohio state university proteomics center for analysis of protein identity.

CHAPTER IV: DISCUSSION

Site-specific metal catalyzed oxidation is perceived as a "caged" reaction in which metal ions bind in a protective pocket on a protein's surface. On the other hand, evidence also suggests the availability of a metal binding residue alone is sufficient. To examine the importance of tertiary versus primary structure, a double mutant of hen egg white lysozyme was generated.

In native lysozyme His15 is in a protective pocket whereas in mutant lysozyme His77 is exposed to the solution. Comparison of the oxidation pattern of native lysozyme to that of the double mutant will shed light on the role protein structure plays in site-specific metal catalyzed oxidation.

As a model protein for these studies hen egg white lysozyme (HEWL) was chosen. HEWL is an extensively studied protein which has been used as a model in various fields of protein research and is structurally well characterized.

Two approaches were used in making the two mutations. One is the study of residue exchange calculations which show the 'safe' substitutions for amino acids that do not disturb the protein structure.[24] The second approach is the comparison of amino acid sequences of many closely related proteins. According to the residue exchange calculations, serine was suggested to be a `safe' residue substitution for histidine and Based on the comparison of similar proteins sequences, asparagine 77 was substituted with histidine.

Several attempts have been made to express various forms of lysozyme in *E. coli*. The gene product of recombinant canine milk lysozyme gene,[25] H-5 lysozyme when expressed in the *E. coli*, the gene product accumulated as inclusion bodies in an insoluble fraction.[26] Hen egg white lysozyme was expressed as a fusion protein in *E. coli*. The expression yielded soluble, enzymatically active and correctly folded recombinant lysozyme. However, the expression of active enzyme in *E. coli* consequently led to bacterial cell lysis due to hydrolysis of the peptidoglucan.[27, 28] Successful expression of mutant HEWL and N^{15} enriched HEWL has been accomplished using the yeast expression system *Pichia pastoris*.[29] Hence, *Pichia* is used to express the double mutant HEWL.

In this study, expression of double mutant lysozyme was attempted. The first part of the experiment involved generating the double mutant (H15S + N77H) lysozyme gene, and cloning the gene into the pCR®4-TOPO® vector. TOP10 *E. coli* was used for the cloning and the maintenance of plasmid. The gene was then verified through agarose gel electrophoresis and DNA sequencing. The mutant lysozyme gene was ligated into the *EcoR* I and *Xba* I sites of the pPICZ B vector. Finally, the cloned pPICZ B vector was used to transform the X-33 strain of *Pichia pastoris* for the expression of the gene.

HEWL is 129 amino acids long with a molecular weight of 14.3 kDa. A protein band of ~14 kDa was expected in the SDS-PAGE gels run with the samples of cell lysate collected at time points of 0, 24, 48, 72, and 96 hr grown in various media. An intense band of correct size was observed with small scale expression in complex buffered media, BMMY (100-130 ml). A ~14 kDa protein was also observed when yeast was grown in minimal media like BMM and Synthetic media but the bands in the SDS-PAGE

gels were not as intense as with the BMMY media. The ~14kDa protein is believed to be the double mutant lysozyme protein because of the absence of ~14kDa bands in the control lysate and 0 hr samples. Moreover the amount of protein produced was found to increase with time over the period of 96 hr.

Large scale expression in the BMMY media did not appear to produce the mutant lysozyme. Examination of the lysate by HPLC, gel filtration chromatography, and by enzyme assay indicated no HEWL was present. In cation exchange chromatography, no lysozyme peaks were visible in the sample (lysate) chromatograms. The eluted fractions of the gel filtration chromatography analyzed by SDS-PAGE did not show the presence of 14kDa band. Enzyme assays of the lysate also did not show any lytic activity. Therefore, the more sensitive method of ELISA was performed. The results of ELISA did not show any difference in the control and test lysate. These results would indicate either that the mutant lysozyme is not expressed or the amount of protein is too low to be detected by these methods.

The cells were cultured according to the methods described in the manual, Easy select™ Pichia expression kit (Invitrogen). The low level of expression in various media led us to change the parameters for expression. The cells were grown in BMMY media with 1% and 2% methanol at 28°C and 300 rpm. The 15% gels showed intense bands of ~14kDa and no difference in the intensity of bands was observed among the 1% and 2% methanol samples.

A gel run with the lysate collected from the 72 hr samples showed intense bands of 14kDa. The protein bands were trypsin digested and analyzed by mass spectrometry. The lysozyme sequence result obtained was as follows:

1 KVFGRCELAA AFKRHGLDNY RGYSLGNWVC AAKFESNFNT QATNRNTDGS
51 TDYGILQINS RWWC*NDGRTP GSRNLCNIPC SALLSSDITA SVNCAKKIVS
101 DGNGMNAWVA WRNRCKGTDV QAWIRGCRL

The peptide sequence in blue was sequenced but not 100% confident about its presence. The sequences in red were verified. The peptide sequences in black were not detected by MS/MS. The peptides sequenced were sufficient to verify the protein as lysozyme but the data was inconclusive about the mutations.

Different ways to increase the protein expression

Electrophoresis and various chromatographic methods indicated that intracellular expression of lysozyme occurred but at inadequate levels to continue this project. Review of the literature shows different options that exist to improve the expression of the protein. The number of copies of recombinant gene integrated into the yeast genome, its site of integration, and expressing the protein extracellularly are some of them.

In this research, the protein is expressed intracellularly because secretion requires the presence of a signal peptide on the expressed protein to target it to the secretory pathway. Previous research showed that the signal peptide is not always cleaved at the correct site. In addition, other work indicated that the N-terminal methionine residue was not cleaved as it normally should be.[29] The methionine residue is highly susceptible to oxidation, and addition of an extra Met was not desirable. To avoid these problems, the study explored conditions for intracellular expression in the hopes of obtaining properly modified lysozyme. The major advantage of expressing the recombinant protein as a secreted protein is that *Pichia pastoris* secretes very low levels of native proteins which

mean the majority of the total protein in the medium consists of the expressed protein. Thus also serves as a first step in the purification.

The phenotype of the yeast, X-33 was Mut^+ in which the gene is integrated upstream of the 5'*AOX1* region. Integration of the gene in 3'*AOX1* creates Mut^S transformants. Both recombinants are useful as one phenotype may favor better expression of the protein than the other and there is no way to predict before which phenotype will better express the protein of interest. The alcohol oxidase in the cell, induced by methanol may result in $\geq$ 30 % of the total soluble protein. Induction may also produce the same level as the protein of interest. Mut^S can be a preferred choice for intracellular expression because of the lower levels of alcohol oxidase activity (produced by *AOX2* locus) which make the expressed protein easier to purify. In a study by Brierly *et al.*, the secreted amount of bovine lysozyme per volume could be increased 6.5-fold by using a Mut^s strain instead of a Mut^+ strain.[30]

Some earlier studies have demonstrated that the gene dosage, i.e. the number of copies of the gene integrated into the yeast genome is also important for the level of expression of the protein.[31] The multicopy integrants produce a large amount of the recombinant protein. Recombinants derivatives of the pPICZ B plasmid, which had been linearized with restriction enzyme, usually are integrated at the AOX locus as a single copy, but multiple-copy integration can occur at a frequency of 1 to 10%.[32] Resistance to higher levels of Zeocin™ (500 μg/ml to 1000 μg/ml) correlates with higher copies of gene integration. In this research, the positive transformants were selected on the plates containing 100 μg/ml of Zeocin™, indicating that a single copy was integrated.

One of the reasons for the low level of protein in the large scale experiment might be due to relatively low level of bio-mass obtained in the 2L culture (10 g/L). *Pichia pastoris* is yeast and is particularly well suited to fermentation therefore, using a fermentor might increase the levels of protein expression.

Conclusions:

Although the cloning was successful, and the expression system is capable of producing the protein, the expression level of the protein was not adequate to carry out metal catalyzed oxidation studies. The expression of the gene in various media was carried out according to the standard protocol. Buffered complex media were found to be suitable for the expression of the protein. The cells were grown in shake flasks for 4 days with daily addition of a 0.5% (v/v) methanol to maintain the induction. Induction appeared to be enhanced by 1% methanol and even 2% methanol was not harmful to the yeast.

The mass spectrometry data verified the 14kDa band on SDS-PAGE as lysozyme but the data was inconclusive regarding the two sites of mutation.

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32. Easy select ^{TM} Pichia expression kit manual.

Appendix A: DNA sequence (390) and corresponding amino acid (129) sequence of hen egg white lysozyme.

AAA GTC TTT GGA CGA TGT GAG CTA GCA GCG GCT ATG AAG CGT
Lys Val Phe Gly Arg Cys Glu Leu Ala Ala Ala Met Lys Arg

CAC GGA CTT GAT AAC TAT CGG GGA TAC AGC CTG GGA AAC TGG
His Gly Leu Asp Asn Tyr Arg Gly Tyr Ser Leu Gly Asn Trp

GTG TGT GCT GCA AAA TTC GAG AGT AAC TTC AAC ACC CAG GCT
Val Cys Ala Ala Lys Phe Glu Ser Asn Phe Asn Thr Gln Ala

ACA AAC CGT AAC ACC GAT GGG AGT ACC GAC TAC GGA ATC CTA
Thr Asn Arg Asn Thr Asp Gly Ser Thr Asp Tyr Gly Ile Leu

CAG ATC AAC AGC CGC TGG TGG TGC AAC GAT GGC AGG ACT
Gln Ile Asn Ser Arg Trp Trp Cys Asn Asp Gly Arg Thr

CCA GGC TCC AGG AAC CTG TGC AAC ATC CCG TGC TCA GCC CTG
Pro Gly Ser Arg Asn Leu Cys Asn Ile Pro Cys Ser Ala Leu

CTG AGC TCA GAC ATA ACA GCG AGC GTG AAC TGT GCG AAG AAG
Leu Ser Ser Asp Ile Thr Ala Ser Val Asn Cys Ala Lys Lys

ATC GTC AGC GAT GGA AAC GGC ATG AAC GCG TGG GTC GCC TGG
Ile Val Ser Asp Gly Asn Gly Met Asn Ala Trp Val Ala Trp

CGC AAC CGC TGC AAG GGT ACC GAC GTC CAG GCG TGG ATC AGA
Arg Asn Arg Cys Lys Gly Thr Asp Val Gln Ala Trp Ile Arg

GGC TGC CGG CTG TAA
Gly Cys Arg Leu stop

The lysozyme sequence obtained from Lisa Crawford (University of Toledo) had changes in the sequence to incorporate the *Eco*R I at the beginning and *Xba* I at the end of the sequence.

1 AAA GTC TTT GGA
Lys Val Phe Gly

Was changed to

1 GAA TTC TTT GGA
Glu Phe Phe Gly

433 TGC CGG CTG TAA GTC GAC
Cys Arg Leu Stop Val Asp

was changed to

433 TGC CGG CTC TAG AGC GAC TTG GTT
Cys Arg Leu Stop Val Asp