EXPRESSION AND *IN VITRO* REFOLDING OF BUFFALO RECOMBINANT LYSOZYME FROM INCLUSION BODIES

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

MASTER OF SCIENCE

IN

DAIRYING

(ANIMAL BIOTECHNOLOGY)

BY

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Approved By

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This is to certify that the thesis entitled **"EXPRESSION AND *IN VITRO* REFOLDING OF BUFFALO RECOMBINANT LYSOZYME FROM INCLUSION BODIES"** submitted by **Mr. GATTU RUDRAPPA** towards the partial fulfilment of the requirement for the award of the degree of **MASTER OF SCIENCE IN DAIRYING (ANIMAL BIOTECHNOLOGY)** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated:14, June, 2010

(JAI K. KAUSHIK)
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Dedicated to

My Parents
&
My Nation

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ABSTRACT

Milk lysozyme is a 130 amino acid residues enzyme, which catalyzes hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. Lysozyme is an important hydrolyzing enzyme involved in the animal defense and digestive functions. In the present investigation, we have successfully cloned a new form (isoform) lysozyme in the pJET 1.2 cloning vector. The recombinant clones were selected and confirmed to contain the gene insert by PCR amplification. The nucleotide sequence analysis indicated an ORF of 447 nt for the insert cloned from the somatic cells of buffalo milk. The predicted polypeptide chain contained 148 aa, in which the first 18 were predicted to encode signal peptide and the remaining 130 residues constituting the mature lysozyme. Sequence alignment of buffalo milk lysozyme with lysozyme from other species indicated that the buffalo milk lysozyme shows 94 % - 95 % homology with the cattle at DNA level, 93 % similarity at protein level. On the induction with IPTG, buffalo milk lysozyme was expressed in *E. coli* in the form of inclusion bodies by raising the culture temperature to 42^{o}C to improve the yield of protein production, since lysozyme expressed in active form was highly toxic to *E. coli* cells. So, the expression of Lysozyme in *E. coli* was also improved by adding NAG, a lysozyme inhibitor. Purification of inclusion bodies was improved by washing them several times with detergents and 2 M urea. Refolding of the HEW lysozyme was achieved by dilution method, however similar method failed to refold buffalo lysozyme. Refolding of buffalo recombinant lysozyme was therefore attempted by screening large number of refolding conditions using a screening kit containing various refolding buffers at pH 7-8.5. Few of the combinations at pH 8.5 resulted in the refolding of buffalo lysozyme. Highest refolding of buffalo lysozyme was achieved in a mixture containing PEG4K and GSSG/GSH redox system.

सारांश

दूध लाइसोजाइम एक सौ तीस एमिनो ऐसिड रेजिडियूज का एजाइम है जो वाइटोडैक्सट्रिनस में एन-ऐस्टिाइल-डी-गलूकोसामीन अवशेषों के बीच ओर पेपडीटोडलाइकन में एन-ऐस्टिाइल मयूरामिक ऐस्डि और एन-ऐस्टिाइल-डी-गलूकासामीन अवशेषों के बीच बीटा 1,4 सम्बन्धें को ताड़ने की प्रक्रिया की कैटालाइज करता है। लइसोजाइम एक मत्वपूर्ण हाड्रोलाइजिंग एजांइम है जो पशु रक्षा और पाचक कार्यों में शमिल है। वर्तमान जांच में हमने सफलतापूर्वक पीजैट 1.2 क्लोनिंग किट की मदद से एक नया लाइसोजाइम का क्लोन किया है। पुनः संयोजक क्लोन चुने गए और पी.सी.आर प्रवर्धन द्वारा जीन सम्मलित हाने की पुष्टी की गई। न्यूक्लोटाईट अनुक्रम विश्लेषण से 447 न्यूक्लोटाईड ओ.आर एक होने का संकेत मिला। 148 अमीनों एसिड वाले इस पोलीपेप्टाईड में पहले 18 संकेत पेप्टाईड को दर्शातें है और शेष 130 परिपक्व लाइसोजाइम को अन्य प्रजातियों के लाइसोजाइम से संरेखन किया गया। जिसमें भैंस का लाइसोजइम डी.एन.ए स्तर पर गाय के "से 94 प्रतिशत-95 प्रतिशत की अनुरूपता दिखाता है जबकि प्रोटीन स्तर पर 93 प्रतिशत अनुरूपता दिखाता है। आई.पी.टी.जी के साथ शमिल करने पर ईक्को लाई में भैंस दूध लाइसोजइम को इन्कलूसन बोडीस के रूप में एक्सप्रेस कराया गया। प्रोटीन उत्पाद को बढ़ाने के लिए 42 डिग्री सेंल्सियस तक पहुंचाया गया क्योंकि परिपक्व लाईसोजाइम इको लाई के लिए हानिकारक थी। उत्पाद को बढ़ाने के लिए एन.ए.जी का उपयोग किया गया। इन्कलूजन बोडीज को कई बार डीअर्जेन्ट और 2 मोलर यूरिया से धोया गया। डाईल्यूशन से एच.ई डब्ल्यू लाइसोजाईम को रोफोल्ड करने की कोशिश विफल रहीं। अन्य तरीकों से इस कार्य काक करने की कोशिश की गई जिसमें सबसे उच्चतम रीफाल्डिग एक " पी.ई.जी, चार के" और जी.एस.एस.जी/जी.एस.एस रीडोक्स प्रणाली युक्त मिश्रण में प्राप्त की गई।

LIST OF ABBREVIATIONS

bp	Base Pairs
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cDNA	Complementary Deoxy-ribo Nucleic acid
CTP	C-Terminal Peptide
DNA	Deoxy-Ribo Nucleic Acid
dNTPs	Deoxy Nucleotide Tri Phosphates
EDTA	Ethylene Diamine Tetra Acetate
GSSG	Oxidized Glutathione
GSH	Reduced Glutathione
GuHCl	Guanidium Hydro Chloride
IPTG	Iso Propyle Thio β-D-Galactopyranoside
Kb	Kilo Bases
KDa	Kilo Daltons
LB	Luria-Bertani
LPS	Lipoplolysaccharides
MCS	Multiple Cloning Site
Mgcl2	Magnesium Chloride
mM	Millimolar
μM	Micromolar
mRNA	Messeger Ribo Nucleic Acid
MW	Molecular Weight
nM	Nanomolar
NaCl	Sodium Chloride
nM	Nanomolar
NTP	N-terminal Peptide
OD	Optical Density

PBS	Phosphate Buffered saline
PCR	Polymerization Chain Reaction
PEG	Poly Ethylene Glycol
RT-PCR	Reverse Transcritase - PCR
RNA	Ribo Nucleic Acid
r-Plasmid	Recombinant Plasmid
rpm	Rotations Per Minute
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis
TCEP	Tris(2-carboxyethyl)phosphine HCl
TEMED	Tetra Ethyl Methylene Diamine

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CHAPTER – *1*

Introduction

1. INTRODUCTION

More and more genes of interest could be expressed in heterologous expression systems to obtain high concentrations of desired proteins. However, such proteins are often produced as inactive inclusion bodies in *Escherichia coli*. These inclusion bodies do not have biological activity and therefore there is a need to solubilise the aggregates and refolds to their biologically active native structure. Protein folding is a complex process depending not only on the primary structure but also on the environmental conditions and might need assistance of proteins known as chaperones. In general, proteins themselves organize into specific three-dimensional structures through a myriad of conformational changes. Each conformational change itself is a solvent influenced event. The simplicity is owed to the global organisation of landscape of the energies of protein conformations into a funnel. This organisation is not characteristic of all polymers with any sequence of amino acids, but is a result of evolution.

A folding mechanism is an involvement of complex network of reactions. Protein folding from inclusion bodies involves a series of events, i.e. expression and isolation of inclusion bodies, dissolving them in strong denaturants and recovering the biological activity by controlled removal of denaturing agents. It is known that the refolding yield decreases with increase in the denatured protein concentration, this is because of kinetic competition between protein aggregation and protein refolding (Protein aggregates $\leftarrow$ unfolded protein $\leftrightarrow$ folded state). However, refolding at low protein concentration often leads to the requirement of large refolding reactor and quantities of buffer, and increasing difficulty in protein recovery. The aggregation is due to the exposure of inner hydrophobic core, which is a major reason for low refolding yield of recombinant proteins. The exact mechanism for aggregation is still unknown. But analysis of the kinetics of aggregation shows that the aggregation process exhibits an apparent reaction order $\geq$ 2 where as the correct folding step is generally first-order reaction, which suggests the competition between refolding at low protein concentrations. The impurities present in inclusion bodies may affect aggregation and thus, will influence the final refolding yield. Contaminants include nucleic acids, proteins, and phospholipids. There have been reports where removal of impurities improved refolding yields, or had no effect on refolding. The purifying inclusion bodies may be advantageous to reduce the impurities load on subsequent protein purification.

The most significant purification occurred during the removal of cell debris. The process advantages of expressing the cloned gene product as inclusion bodies can be exploited only if refolding yields are significant. The loss of activity at high protein concentrations is strongly linked to aggregate formation, caused by intermolecular interactions.

The high level expression of lysozyme which causes the formation of inactive inclusion bodies and the lack of knowledge to recover the soluble, active recombinant buffalo lysozyme from inclusion bodies, the present study was proposed with the following objectives:

1) Expression of buffalo lysozyme as inclusion bodies in *E. coli*.

2) Purification of recombinant lysozyme inclusion bodies and *in vitro* refolding to biologically active enzyme.

CHAPTER – *2*

Review of literature

2. REVIEW OF LITERATURE

C-type lysozyme is 129-130 amino acid residues enzyme, which catalyses the hydrolysis of 1, 4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins *(Arnheim et al., 1972).* This catalytic activity is non-specifically targeted to the bacterial cell membranes and related with general non-specific organism defence. Lysozyme is present in the mucosal secretion such as saliva and tears. In high concentration, about 3% from all proteins, lysozyme is present in chicken egg-white. This enzyme is only effective against Gram positive bacterial cells. Gram negative bacteria and yeast are completely resistant to lysis by it. Molecular weight of lysozyme is approximately 14-16 kDa. Alternative name for lysozyme are 1,4-N-acetylmuramidase, N,O - di acetyl muramidase, PR1-lysozyme, Globulin G1, Globulin G, lysozyme g, Mucopeptide N-acetylmuramoylhydrolase, Mucopeptide glucohydrolase and Muramidase. This catalytic activity is non-specifically targeted to the bacterial cell membranes and related with general non-specific organism defence.

Lysozyme from chicken egg was first described by Laschtschenko in 1909 *(Laschtschenko et al., 1909).* It was also reported in saliva by Bloomfield in 1919 *(Imoto et al., 1972).* Lysozyme was not officially named and understood to be present in many biological tissues and secretions until 1922 *(Fleming et al., 1922).* During these experiments Alexander Fleming discovered *Micrococcus lysodeikticus*, a bacteria especially susceptible to lysozyme, which is still used for lysozyme activity assays *(Imoto et al., 1972).*

In 1965, *Blake et al.* solved the structure of lysozyme, making it the second protein and first enzyme structure to be solved by X-ray diffraction methods *(Blake et al., 1965*). A year later, thye mechanism was explained (*Blake et al*., 1967). Throughout the 1960s and into the 1970s, interest in the enzyme increased as a "natural" antibiotic and aid in the diagnosis of disease *(Glynn 1968, Pruzanski and Saito et al., 1969).* Elevated lysozyme levels were found to be present in the urine and serum of leukemia patients *(Osserman and Lawlor et al., 1966Briere et al. 1974),* and in the cerebrospinal fluid of patients with a central nervous system tumor *(Newman et al., 1974).*

Lysozyme research in the 1980s included investigating enzyme intermediates *(Acharya et al., 1982, Desmadril and Yon et al., 1984),* analyzing the protein structure *(Delepierre et al., 1982),* and performing binding studies *(Perraudin and Preels, 1982, and Smitth-Gill et al., 1984).* In the 1990s, transcription control, silencers, and additional binding sites were investigated *(Bonifer, 1997, Baniahmad., 1987, and Madhusudan and Vijayan et al., 1992).*

Recent research has focused on obtaining more information about gene regulation of lysozyme both in the hen and other animals *(Shimizu et al., 2005),* gaining a better understanding of the secondary structure *(Schwinte et al., 2002)* and refining its use in biochemical applications *(Reischl, 2004 and Zhu et al., 2006).*

Escherichia coli has the dominant position as the first choice of host because of speed and simplicity. But expression of recombinant proteins in *E.coli* at high level leads to the formation of insoluble, inactive inclusion bodies. Protein products as inclusion body form can be some time advantageous over the soluble form, including high product concentration, less proteolytic degradation and reduced toxicity to the host cells. But these inclusion bodies do not have biological activity and there is a need to solubilise the aggregates and refolds to its native structure which is active. During protein refolding, many of the proteins tend to aggregate, causing a significant reduction in the total yield of active protein.

2.1. Lysozyme Evolution

Lysozyme of the chicken type (lysozyme *c)* and α-lactalbumin have undoubtedly evolved from a common ancestral protein because of the similarity of their amino acid sequences *(Brew et al., 1970),* and the intron-exon constitution of their genes *(Qasba et al., 1984),* although their functions are different. Lysozyme is a lytic enzyme which degrades peptidoglycan, a constituent of bacterial cell wall, while α-lactalbumin acts as a specificity modifier to convert galactosyltransferase to lactose synthase. However, it is still equivocal when α-lactalbumin diverged from lysozyme c and how α-lactalbumin obtained its function during evolution. At first, gene duplication leading to creation of the gene for α-lactalbumin was postulated to have taken place at the outset of mammalian evolution, after divergence of the avian and mammalian lineages. As a logical consequence, rapid evolution of α-lactalbumin was deduced because the

avian and the mammalian lysozymes are more similar in sequence than either is to α-lactalbumin. Secondly, an ancient duplication predating the last common ancestor of birds and mammals was postulated without the assumption of rapid evolution *(White et al., 1977*). Several articles have been published concerning the properties of α-lactalbumin and lysozyme. α-lactalbumin has been found to be a calcium-binding protein *(Hiraoka et al., 1980).* Although most lysozymes do not bind a calcium ion, some lysozymes have been suggested to bind this ion *(Stuart et al., 1986),* and, indeed, pigeon and equine lysozymes bind a calcium ion with quite high affinity *(Nitta et al., 1988).* One of the lysozymes of echidna *(Tachyglossus uculeatus*) has been reported to have both lysozyme and α-lactalbumin activities *(Hopper et al., 1974),* and it has been reported that preliminary results indicated the binding of a calcium ion to echidna lysozyme *(Godovac-Zimrnermann et al., 1987*). Lepidopteran lysozyme is of the *c* type in sequence *(Jolles et al., 1979),* and lysozyme from one of these, the giant silk moth *(Hyalophora cecropia),* has been sequenced from its cDNA, as well as from purified protein *(Engstrom et al., 1985).* The physicochemical properties of canine milk lysozyme (Calcium binding lysozyme) are very different from those of non calcium binding lysozyme (*Grobler et al., 1994; Kikuchi et al., 1998*). Though the amino acid sequences of hen and goose egg-white and bacteriophage T4 lysozyme have no detectable similarity, structural correspondence and functional similarities strongly suggests that all three lysozymes evolved from a common precursor, with goose egg-white being an evolutionary link between hen egg white lysozyme and Bacteriophage T4 lysozyme.

Lysozyme has been recruited as a digestive enzyme in ruminants, leaf eating monkeys and a bird, the hoatzin (*Dobson et al., 1984; Stewart et al., 1987; Kornegay et al., 1994*). In these species the lysozyme amino acid sequences have undergone some specific amino acid replacements that occurred convergently on all lineages (*Stewart et al., 1987; Jolles et al., 1989; Swanson et al., 1991; Kornegay et al., 1994*), these amino acid changes were required for adaptation to the stomach environment.

The genomes of ruminants (such as cow, sheep and deer) have approximately ten lysozyme like genes. Genomic blots indicate that the amplification for lysozyme gene family occurred 40-50 million years ago (*Irwin & Wilson, 1989; Irwin et al., 1989*) which is contradictory to the cDNA sequence analysis, which indicates that the stomach genes began

diverging from one another after the splitting of the deer and cow lineages, 25 million years ago (*Irwin & Wilson et al., 1989*). The comparison of the sequences of the sheep and cow stomach lysozyme genes shows a mosaic pattern of evolution with the introns and the flanking regions and the 3' untranslated region of the mRNAs have evolved in a divergent manner, while the coding exons have evolved by concerted evolution. The gene duplication events have occurred after the divergence of pig and ruminants about 55 million years ago (*Irwin et al., 1989, 1993*), the pig contains 3 allelic forms of lysozyme in the stomach but none shows adaptation to function in the stomach environment. Thus the pig represents an intermediate in the evolution of stomach lysozyme.

2.2. Source and Localization of Lysozyme

Lysozyme is widely distributed in animals, fish, insects, plants, fungi, bacteria, protozoa and bacteriophages (*Muller et al., 2005*). Lysozyme from these different sources have high structural homology, performing the same catalytic function, but has weak sequence homologies, differ in their specific activity, pH optimum, heat stability, electrophoretic mobility, isoelectric point, sedimentation coefficient (*Chandan et al., 1965: Parry et al., 1969*) and also on immunological properties (*Eitenmiller et al., 1976*).

Lysozymes are found in most tissues, but are present in high amounts in phagocytic cells like macrophages, granulocytes, and the paneth cells of the small intestine and in proximal tubules of kidney (*Klockars & Retimo 1975*). Synthesis of lysozyme has been detected in myeloid cells also (*McClelland et al., 1975*). Lysozyme from milk of human, equine, bovine, baboon (Buss, 1971), camel (*Benkerroum et al., 2004*), buffalo (*Priyadarshini & Kansal, 2002*) and dog (*Watanabe et al., 2004*) has been isolated and characterized. Human and equine milk are exceptionally rich sources of lysozyme, containing 400 and ~800 mg/L, representing ~4% and ~3% of total milk protein, respectively. The milk lysozyme concentration has been shown to vary with the period and season of lactation in human (*Montagne et al., 1998*) as well as ruminants.

In foregut fermenting mammals; the ruminants and colobine monkeys, lysozyme c has evolved as a digestive enzyme (*Dobson et al., 1984; Prieur, 1986; Stewart & Wilson, 1987*). The

cow & sheep stomach contains four and deer has two non allelic forms of lysozyme (*Irwin & Wilson, 1989*). Multiple forms of lysozyme have also been observed in the stomachs of other ruminants (*Dobson et al., 1984*). The G type lysozyme has also been reported in ruminants (*Prager et al., 1996*). On the other hand, monogastric animals possess a single gene, the c type lysozyme. That is expressed in both stomach and non stomach tissues (*Mei & Irwin, 1996*).

The lysozyme of bacteriophage T7and Klebsiella phage K11 are bifunctional proteins with amidase activity and inhibitory effect on T7 RNA polymerase and K11 phage RNA polymerase (*Cheng et al., 1994; Junn et al., 2005*), respectively. The Lyt C lysozyme of *Streptococcus pneumoniae* forms part of its autolytic system. It is composed of a C-terminal catalytic module, belonging to the GH25 family of glycosyl hydrolases, and an N-terminal choline-binding module, made of eleven homologous repeats, that specifically recognizes the choline residues that are present in pneumococcal teichoic and lipoteichoic acids (*Monterroso et al., 2005*).

Lysozymes are also found in many bacteria that are surrounded by a murein containing cell wall. Their physiological role in bacteria is still a debate, on the one hand they can autolyse the cell while on the other hand they may have an essential role during the enlargement and division of the cell wall by the controlled splitting of bonds in the murein sacculus.

A sperm lysozyme-like protein (SLLP1), a unique intra-acrosomal, non-bacteriolytic, c lysozyme-like protein has been found to be involved in sperm-egg binding and fertilization in human (*Mandal et al., 2003*) and mouse spermatozoa (*Herrero et al., 2005*).

2.3. Structure of lysozyme

All three types of lysozymes, the hen egg white (HEWL), goose egg white (GEWL) and T4 lysozyme are composed of two domains, first being predominantly alpha-helical and a smaller domain being mainly beta-sheet in nature. The lysozyme molecule is relatively rigid with the residues of the active site Glu35 and Asp52 adopting almost identical conformations in all structures determined so far. The NMR results also confirmed the presence of a similar conformation of HEWL in solution. The general acid/base residues in each lysozyme (Glu35 in

HEWL, Glu73 in GEWL and Glu11 in T4L) are contributed by the larger alpha-helical domain. The beta-sheet domains of HEWL and T4L contribute an aspartate to their respective active sites, which is likely involved in electrostatic stabilization of the oxycarbonium ion intermediate of the site D sugar on the hydrolytic pathway of oligosaccharides. There is no analogous aspartate carboxylate group in GEWL, although minor conformational changes could position one or other of Asp86 or Asp97 for such a stabilization role. The T4 lysozyme has an additional C-terminal domain whose function may be to bind the cross linking peptide of the *E. coli* peptidoglycan. The binding of substrate analogues, transition state mimics and oligosaccharide products of HEWL, GEWL and T4L hydrolysis have contributed greatly to our understanding of sugar binding to proteins. The observed subtle conformational differences of the free versus bound forms of these enzymes are best described by a narrowing of the active site clefts in the presence of the inhibitors (*Strynadka & James, 1996*).

Three-dimensional structure of the hen egg white lysozyme was determined by X-ray analysis of the tetragonal crystals (*Philips, 1967*). The structural domains in HEWL (129 amino acid residues) are formed by the larger α-domain (residues 1-35, 85-129; four α–helices and a short 3_{10}-helix) and the smaller ß-domain (residues 36–84; a triple-stranded antiparallel ß-sheet, a long loop, and a 3_{10}-helix). The α-domain contains a core of hydrophobic side-chains that are packed closely together (the hydrophobic box); In contrast, the ß-domain has no similar hydrophobic core; instead it formed hydrogen bonds and a number of small hydrophobic clusters and a long exposed loop region.

The X-ray structure of the wild-type human lysozyme has been determined at 1.5 Å resolution (*Artymiuk et al., 1981*). The patterns of atomic displacements in the human lysozyme crystals are broadly similar to that of hen egg white. It is a protein of 130 residues with two independent domains, the larger α-domain (residues 1-42 and 81-130; four α-helices and one 3_{10} helix) and the smaller β-domain (residues 43-80; a three- stranded antiparallel β-sheet and an irregular loop). In the α-domain, α1, α3, and α4 are relatively exposed to the solvent; whereas α2 is more buried in the interior region with its ends partially exposed to the solvent. In the β-domain, β1 is located on the protein surface near the end of the active site cleft; whereas β2 and β3 are situated in the protein interior. There are a total of four disulfide bonds in this protein: two

(C6-C127 and C30-C115) in the α-domain, one (C64-C80) in the β-domain and one (C76-C94) connecting these two domains.

The crystal structures of pheasant and guinea fowl lysozymes have been determined at a resolution of 1.9 Å. Three amino acid positions beneath the active site are occupied by Thr40, Ile55, and Ser91 in hen, pheasant, and other avian lysozymes, and by Ser40, Val55, and Thr91 in guinea fowl and American quail lysozymes. These substitutions do not result in appreciable structural changes. The pheasant enzyme has an additional N-terminal glycine residue, probably resulting from an evolutionary shift in the site of cleavage of prelysozyme. This amino acid partially fills a cleft on the surface of the molecule close to the Cα atom of Gly41 and absent in lysozymes from others species (which have a large side-chain residue at position 41: Gln, His, Arg, or Lys). The overall structures are similar to those of other C-type lysozymes, with the largest deviations occurring in surface loops (*Lescar et al., 1994*).

The crystal structure of calcium binding pigeon lysozyme at a 2 Å resolution was solved by molecular replacement technique. The main chain folding was found to be more similar to hen egg white lysozyme than that of baboon α-lactalbumin, the major difference between hen egg white lysozyme being in the loop region (*Yao et al., 1992*).

The three dimensional structure of Bacteriophage T4 has been observed to be quite different from that of hen white lysozyme. About 60% of the molecule is in helical conformation and a region consisting of antiparallel β-structure. The catalytic site is formed by folding of polypeptide backbone into two distinct lobes linked in part a long helix. Between the two lobes there is a cleft that deepens into a hole or cavity 6-8 Å in diameter, extending from one side of the molecule to another. The opening is closed off by side chains which extend to within 3-5 Å of each other (*Matthews & Remington*, 1981).

The structure of *Streptomyces erythmeus* lysozyme determined by x- ray diffraction at 2.9 Å resolution consists of three domains with 18% of its structure in helical domain and it was found to be completely different from that of both mammalian and phage lysozymes (*Zhangt et al., 1994*).

The X-ray crystal structure at 1.8 Å resolution of Rainbow trout lysozyme crystal grown from a mixture containing about 80% type I and 20% type II lysozyme shows the three-

dimensional structure to be very similar to the previously solved structures of other C-type lysozymes. The single polypeptide of 129 amino acids is folded into two domains separated by a deep cleft which contains the active site. Secondary-structure elements, four α-helices and a three-stranded β-sheet, are located in the same sequential positions as in the hen, turkey and human enzymes. The β-sheet is found to be common for structures of both C-type and G-type lysozymes. But they show a higher activity than egg-white lysozyme and lysozymes from other fish species against a variety of pathogenic bacteria. The differences in antibiotic activity of the two forms of Rainbow trout lysozyme may be due to small differences in the hydophobicity of a small surface region (*Karlsen et al., 1995*).

2.4. Isoforms of Lysozyme

Lysozymes are mainly classified into C type (chicken), g type (goose), I type (invertebrate) and phage lysozymes depending on the source of discovery. C-type lysozyme is the most common lysozyme found in animals, insects and plants (Prager & jolles, 1996). There are 129-130 amino acids in C-type as compared to 185 amino acids in G-type, the active residues Glu35 & Asp52 in C-type is replaced by Glu73 & Asp86 in G-type (*Banks & Tranter*, 1985).

2.5. Proposed Functions of Lysozyme as an Anti-Bacterial Agent

An antibacterial property was observed in hen's egg white and identified to be due to an enzyme (*Laschtschenko, 1909*). Later in 1922 Alexander Fleming observed that an enzyme present in nasal mucus, tears, sputum, saliva and other body fluids caused similar lytic activity on many types of bacteria. He proposed the name lysozyme for the enzyme. Later lysozyme was detected in human blood serum, saliva, milk, and a wide variety of other fluids (*Jolles & Jolles 1967; Hankiewicz & Swierczk 1974*), and among milks of various species (*Bordet & Bordet, 1924; Chandan et al. 1965*).

Lysozyme functions by attacking peptidoglycans (found in the cell walls of bacteria, especially Gram-positive bacteria) and hydrolyzing the ß (1-4) glycosidic bond between residues of *N*-acetylmuramic acid and *N*-acetylglucosamine. In addition to glycosidase activity, lysozyme

also shows transglycosylation (*Chipman & Sharon, 1969*) and esterase activities (*Pirzkiewiez & Bruice, 1969*). In addition to T7 lysozyme's muraminidase activity, it shows an inhibitory effect on T7 RNA polymerase by forming a complex with it (*Moffatt, et al., 1987; Huang et al., 1999*). Lysozyme also serves as a non-specific innate opsonin by binding to the bacterial surface, reducing the negative charge and facilitating phagocytosis of the bacterium (*Kokoshis et al., 1978; Thakur et al., 1999*) and stimulates proliferation and antitumour functions of monocytes (*Lemarbre et al., 1981*).

Lysozymes are found in tissues, fluids and secretions and are an important part of the innate immune system, protecting against bacterial invasion (*Jolles & Jolles, 1984*). Bacteriolytic property of lysozyme plays an important role in passive protection of breast fed newborns (*Ibrahim, 1998; Newman, 1995*). Lysozyme has a synergistic effect along with lactoferrin (*Montagne et al., 1998*). Lysozyme also has an anti inflammatory and anti allergic functions resulting from histamine antagonism, an anti viral property due to its capability of binding acid radicals. Because of potent antimicrobial activity, lysozyme is used as a natural antibiotic (*Seyfert, 1999*) as well as in combination with a variety of pharmaceutical preparations for the treatment of viral and bacterial infections like HIV infection (*Sylvia Lee-Huang et al, 1999*), leucopenia, maintaining oral cavity health, mastitis (*Sun et al., 2004*) and coliform infections of the bladder.

In viruses (or bacteriophages), lysozyme is used as an agent to break into the host bacterial cell. Lysozyme from the tail of the virus (or bacteriophage) destroys the peptidoglycan bacterial cell wall and then virus can injects its DNA. After multiplication in bacteria, many lysozyme molecules are created to lyse the bacterial cell wall and release new viruses.

2.6. Proposed Mechanism of Action

The groove between the two structural domains in the surface of lysozyme is its active site. Six N –acetylglucosamine (GlcNAc) or N-acetylmuramic acid (MurNAc) rings of the polysaccharide substrate fits into six sub sites (designated A to F) in the active site, it is not a perfect fit. In order for the fourth sugar unit to fit into its position "D", it must be distorted to form a less stable conformation. This increases the strain on the bond between the fourth and

fifth sugar units. The bond between the fourth and fifth sugar (sub sites D and E) is then cleaved. At the active site, the side chain carboxyl group of Glu 35 is positioned correctly to serve as the proton donor, while the carboxyl of Asp 52 lies on the opposite side of the groove and stabilizes the oxocarbenium ion intermediate. The lysozyme catalyzed reaction is completed by stereo specific addition of a hydroxyl ion to the oxocarbenium ion with the original β configuration being retained in the product.

Replacement of Glu 35 by Gln destroys all catalytic activity and replacement of Asp 52 by Ala or Asn decreases activity to ~ 4-5% of the original (*Matsumura et al., 1996; Kuroki et al., 1986*). Less than 1% activity remained for the D52S mutant (*Hadfield et a.l, 1994*). Nevertheless Asp 52 is not absolutely essential for lysozyme activity. Goose lysozyme lacks this catalytic aspartate (*Weaver et a.l, 1995*). It has been suggested that carboxyl groups of glycine residues covalently attached to N-acetylmuramic acid rings in the natural substrates may participate in catalysis (*Matsumura et al., 1996*).

The residues selected for active site formation are, in order: Asp101; Trp62, Trp63, Asn59, Ala107; Val109 and Gln57. The next residues (Asp52 and Glu35) are particularly important in the catalytic mechanism.

2.7. Recombinant expression of lysozyme

Lysozyme has been expressed in most of the available expression systems like bacteria (*Karina et al., 2005*), yeasts (*Sun et al., 2004*), insect (*Bang et al., 2006*), and plants (*Kato et al., 1998; Huang et al., 1999*). Bacterial expression (*E. coli*) has some important drawbacks while expressing secreted or post transitionally modified eukaryotic proteins. Bacteria synthesize proteins in the cytosol where disulfide bonds and glycosylation do not occur. Therefore, the recombinant polypeptides that are not folded are stored as inclusion bodies. Lysozyme containing four native disulfide bonds is therefore expressed as inclusion bodies in bacterial systems. Apparently this situation has proved advantageous since the active lysozyme can be toxic to the host cell. The denatured lysozyme can be *in vitro* refolded (*Karina et al., 2005*). Use of genetically engineered host cells for improving the codon usage and cytosolic disulfide bond

formation has been shown to improve the lysozyme yield. Low levels of induction also give higher amount of active protein (pET system manual, 11th edition).

Shrimp lysozyme was expressed using *E. coli* BL21 Rosetta Gami-LacZ and vector pET5a as insoluble protein in inclusion bodies, its refolding gave active protein with a yield of ~ 10% (*De La Re Vega, et al., 2004*). Canine milk lysozyme was overexpressed using plasmid pSCREEN 1-b(+) in *E. coli* (BL21/DE3)/pLysS), the protein also formed inclusion bodies from which lysozyme was completely refolded in 100h using reduced/oxidized thioredoxin in the presence of $CaCl_2$ (*Koshiba et al., 1999*). The thermal stability of the recombinant canine milk lysozyme was significantly lower than the authentic canine milk lysozyme due to the presence of an extra N-terminus methionine residue (*Koshiba et al., 1999*). The extra methionine residue at the N-terminus in recombinant hen egg white lysozyme brings about a decreased refolding yield and solubility in comparison with the authentic protein (*Imoto and Mine et al., 1997*). Bacteriophage ΦEa1h lysozyme was expressed in *E. coli* strain M15 and vector pQE-30, and an active lysozyme was detected when cells (kanamycin resistant) were grown in the presence of kanamycin and not in its absence (*Kim et al., 2004*).

The lysozyme gene of the *Klebsiella* phage K11, a bifunctional protein with amidase activity and inhibitory effect on K11 phage RNA polymerase, was expressed under the control of tac promoter in *E. coli* XL1-blue cells. Induction at 37^{o} C produced majority of the lysozyme as insoluble protein in inclusion bodies, however induction at lower temperatures produced more of soluble protein. The insoluble protein was refolded in the presence of oxido-shuffling agent (GSH/GSSG), giving active bifunctional protein (*Junn et al., 2005*).

2.8. Protein Folding

A folding mechanism must involve a complex network of elementary reactions. However, simple empirical patterns of protein folding kinetics, such as linear free energy relationships, have been shown to exist. This simplicity is owed to the global organization of the landscape of the energies of protein conformations into a funnel. This organization is not characteristic of all polymers with any sequence of amino acids, but is a result of evolution. The discovery of simple kinetic patterns *(Onuchic et al., 2000)* and the existence of a theoretical framework based on the

global properties of the energy landscape *(Miranker, 1996, Bryngelson et al., 1987)* have, in recent years, allowed a very fruitful collaboration between theory and experiment in the study of folding. The locations of atoms in proteins can be determined, in favourable cases, to an accuracy of less than 3Å using X-ray crystallography. This specificity of structure arises from the heterogeneity of the protein chain. The differing energies associated with positioning different residues near or far from each other or from solvent enable some structures to be more stable than others.

In spite of recent progress in theoretical and computational approaches (*Alm et al., 2002*) to understanding protein folding and refolding, efforts to manipulate folding *in vitro* often are plagued by competing off-pathway aggregation processes. Protein aggregation is the subject of intense investigation in disciplines including human medicine, fundamental protein chemistry, and biotechnology. For example, aggregation can have severe consequences in human diseases (e.g., Alzheimer's disease and Parkinson's disease) (*Lansbury et al., 1999*) and in the manufacturing, shipping, storage, and delivery of protein therapeutics (*Carpenter et al 1997*).
In particular, exploitation of the unique medical benefits of recombinant protein therapeutics often is hindered by the formation of non-native protein aggregates from native protein molecules. If even a minor fraction (e.g., 1%) of a initially delivered protein is aggregated, adverse reactions, including anaphylactic shock, can be induced (*Moore, 1980, Ratner, 1990, Thornton et al., 1993*). Aggregation can occur during refolding, purification, concentration, vial filling, freeze-thawing, lyophilization, rehydration, and delivery to patients. Particularly dramatic manifestations of the competition between proper folding pathways and off-pathway formation of non-native aggregates occur during attempts to obtain native recombinant proteins from precipitates formed during processing or inclusion bodies, both of which are essentially completely aggregated protein with substantial non-native structure *(Bowden, 1991; Mitraki, 1989; Oberg, 1994; Przybycien et al., 1994*). Currently, refolding proteins from non-native aggregates and inclusion bodies requires proteins to be disaggregated and then refolded into their native conformation. Most commonly, aggregates are solubilized in a strong chaotrope, such as 8 M guanidine hydrochloride (GdmHCl) (*Rudolph et al., 1996*), which results in nearly complete unfolding of the protein molecules. Once soluble and unfolded, the proteins are first diluted with additional GdmHCl solution and then refolded by removing the chaotrope by dialysis or additional dilution. The refolding step, however, is difficult and depends strongly on renaturing

conditions (*Rudolph, 1996, Valax et al., 1993*). For example, redox conditions, pH, rates of dialysis, and protein concentration all must be empirically optimized for each protein (*Rudolph et al., 1996*). Furthermore, because the process of protein folding is first order in protein concentration, and the overall aggregation process is at least second order, aggregation is favored over refolding at higher protein concentrations. Hence, achieving acceptable yield (e.g., 10%) of refolded protein often requires protein to be refolded at very low concentrations *(Rudolph, 1990, Goldberg, 1991, Maachupalli et al., 1997).* As a result, once a therapeutic protein is refolded, it must be concentrated (typically 100- to 1,000-fold) to final dosage concentration. Losses of native protein also can occur during this concentration step. In addition, yield of properly folded protein upon renaturation is often low regardless of refolding conditions.

2.9. Inclusion bodies

When genes from one organism are expressed in another the resulting protein sometimes forms inclusion bodies. This is often true when large evolutionary distances are crossed: a cDNA isolated from Eukarya for example, and expressed as a recombinant gene in a prokaryote risks the formation of the inactive aggregates of protein known as inclusion bodies. While the cDNA may properly code for a translatable mRNA, the protein that results will emerge in a foreign microenvironment. This often has fatal effects, especially if the intent of cloning is to produce a biologically active protein. For example, eukaryotic systems for carbohydrate modification and membrane transport are not found in prokaryotes. The internal microenvironment of a prokaryotic cell (pH, osmolarity) may differ from that of the original source of the gene. Mechanisms for folding a protein may also be absent, and hydrophobic residues that normally would remain buried may be exposed and available for interaction with similar exposed sites on other ectopic proteins. Processing systems for the cleavage and removal of internal peptides would also be absent in bacteria. The initial attempts to clone insulin in a bacterium suffered all of these difficulties. In addition, the fine controls that may keep the concentration of a protein low will also be missing in a prokaryotic cell, and overexpression can result in filling a cell with ectopic protein that, even if it were properly folded, would precipitate by saturating its environment.

2.10. Molecular Chaperons

The beauty and the frustration of science are that they are constantly producing surprises. Almost three decades after Christian Anfinsen had won the Nobel Prize for demonstrating that protein folding is governed solely by the protein itself, other scientists discovered that some proteins have helped in the process. This help consists of proteins called chaperones (or chaperonins) that are associated with the target protein during part of its folding process. However, once folding is complete (or even before) the chaperone will leave its current protein molecule and go on to support the folding of another. Proper folding of some proteins appears to call for not just one chaperone, but several. Especially clear evidence for such multi-step chaperoning is provided by test-tube experiments on a protein known as rhodanese. Several lines of evidence suggest that chaperones primary function may be to prevent aggregation. A role for chaperones in preventing aggregation is also suggested by what happens to mammalian proteins produced in bacteria. Although bacteria have chaperones, they are not the same as those in mammals. It is thus easy to imagine that they may be relatively ineffective toward mammalian proteins, and that this results in the aggregation so often seen. Indeed, there has been one case in which bacteria engineered to overproduce their own chaperones successfully produced a mammalian protein that otherwise irretrievably aggregated. Unfortunately, this approach has failed in other cases. And no one has yet reported introduction of mammalian chaperones into bacteria to help produce soluble mammalian proteins.

CHAPTER – *3*

Materials and Methods

3. MATERIALS AND METHODS

3.1 DESIGNING OF PRIMER FOR PCR AMPLIFICATION OF BUFFALO MILK LYSOZYME

The Lysozyme gene sequences of different animal species were retrieved from NCBI nucleotide data base and were aligned using Clustal W (1.83) Multiple Alignment Program. By analyzing the conserved regions of the aligned sequences of bovine and human, primers were designed and got custom synthesized from Sigma Pvt. Ltd., Bangalore.

3.2 RNA ISOLATION

3.2.1 Collection and storage of tissues

Buffalo mammary tissue brought from Delhi slaughter house in the RNA later solution and kept in ice.

3.2.2 Preparation of reagents and glasswares for RNA isolation

Reagents

All reagents were prepared in 0.1 per cent DEPC treated sterilized milli Q water and autoclaved at 121°C for 15 min at 15 lbs pressure. All solutions were prepared using RNase free glass wares and DEPC treated water. The chemicals reserved for RNA work were handled with baked spatula. Wherever possible the solutions were treated with 0.1 per cent DEPC for 12 h at 37°C and autoclaved.

Glasswares and Plasticwares

Sterile disposable plasticwares were used for the preparation and storage of RNA. Beakers, tubes and other glasswares used for the RNA work were treated with DEPC treated water (0.1% in milli Q water) and allowed to stand for 2 h at 37°C and ringed several times with sterile water and then heated to 100°C for 2 - 3 h. Disposable gloves were worn during the preparation of materials and solutions used for the isolation and analysis of RNA.

3.2.3 TRIzol reagent method

TRIzol reagent was used to initially lyse the minced tissues and to solubilize nucleic acids and proteins. TRIzol is a ready to use reagent for the isolation of total RNA from cells and tissues. It also protects the RNA from the action of RNase. Reagents needed for isolation of RNA were TRIzol reagent (Invitrogen), chloroform, isopropyl alcohol, 75% ethanol (in DEPC water) and RNase free water.

Protocol

1. ***Tissue Homogenization*:**
    - An aliquot of 50-100 mg tissue sample was homogenized in a sterile glass homogenizer followed by addition of one ml of TRIzol reagent to make a paste.
2. ***Phase separation:***
    - Homogenized samples were incubated at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes. The mixture was transferred to an eppendorf tube.
    - Chloroform (0.2 ml) was added to the above sample and the eppendorf tube was shaken vigorously by hand for 15 sec followed by incubation at room temperature for 2-3 min.
    - The sample was then centrifuged at 12,000 g for 15 min at 4°C which led to the formation of a lower red color phenol-chloroform phase, and a gelly interphase, and a colorless upper aqueous phase.
    - RNA remains exclusively in the aqueous phase. The volume of the aqueous phase was kept about 60% of the volume of TRIzol reagent used for homogenization.
3. ***RNA precipitation*:**
    - The aqueous phase was transferred to a fresh eppendorf tube.
    - The RNA from the aqueous phase was then precipitated by mixing with 0.5 ml isopropyl alcohol.
    - The sample was incubated at room temperature for 10 min.
    - The sample was then subjected to centrifugation at 12,000 g for 10 min at 4°C. A gel like pellet of RNA precipitate was formed on the side of the tube.

4. ***RNA wash:***
    - After centrifugation, the supernatant was removed and RNA pellet was washed once with 1ml of 75% ethanol.
    - Sample was mixed with ethanol by vortexing and centrifuged at 7,500 g for 5 min at 4°C.
    - After centrifugation, ethanol was decanted and the pellet was recovered.
5. ***Redissolving RNA:***
    - At the end of the procedure, RNA pellet was briefly dried under vacuum for 10 min without centrifugation.
    - Dried RNA was dissolved in the RNase free water by passing the solution a few times through a pipette tip, and incubating for 10 min at 55-60°C.

3.2.4 DNase treatment for RNA sample

1. 20µLof RNA sample was taken.
2. 2µL of DNase buffer along with 1µl DNase enzyme was added.
3. The mixture was incubated at 37°C for 30 min.
4. After that 2µl DNase inactivation agent was added.
5. The mixture was incubated for 2 min at room temperature followed by centrifugation at 7200g for 2 min.
7. Carefully the upper aqueous layer which contains RNA sample was transferred in to fresh eppendorf tube. 8. 1µL of RNA was run on PAGE for checking integrity & removal of cellular DNA from sample.

3.2.5 Determination RNA concentration

2 µl of the isolated RNA was diluted 30 times making the volume up to 60 µl, after mixing the sample carefully OD was read at 260 nm using TE buffer as a

reference. The conversion factor for RNA was taken as 0.040 µg/µl per OD_{260} unit. Ratio of OD at 260 nm and 280 nm >1.8 indicates the workable quality of RNA preparation.

RNA (mg/ml) = observed OD x df x 0.040 mg/ml

3.2.6 Agarose gel electrophoresis of RNA

The 1.5% gel of high quality agarose for molecular biology was prepared by dissolving the agarose in 1X TAE buffer (pH 8.0) followed by heating in a microwave oven or by keeping in boiling water bath. Ethidium bromide stock solution was added directly to molten agarose solution at the rate of 0.5 µg/ml of gel before casting the gel. The surface was levelled before pouring the gel. After complete setting of the gel, the comb was removed carefully and the gel casting tray was placed in the electrophoresis tanks containing 1X TAE (pH 8.0) buffer. The RNA samples were mixed with 5 µl of tracking dye and were loaded slowly into the slots of submarine gels using micropipette. Electrophoresis was carried out at 8 V/cm for half an hour. After completion of electrophoresis, the gel was examined under UV transilluminator and photographed using an imaging system equipped with a digital camera (GE Health Sciences).

3.3 FIRST STRAND cDNA SYNTHESIS

The first strand using mRNA as a template was synthesized using AccuScript *PfuUltra* II RT-PCR kit (catalogue No: 600184, STRATAGENE).

The following reagents were mixed in the reaction mixture.

Table 1: Composition of reaction mixture for the synthesis of first strand

Reagents	Volume
RNase free water	3.9µl
10X Accusript RT reaction buffer	1.0µl
gene specific primer	1.0µl
dNTP mix (10Mm each dNTP)	1.0µl
RNA	2.0µl

2. The reaction mixture was incubated at 65°C for 5 min.
3. The reaction was cooled down to room temperature for 5 min., allowing the primers to anneal with RNA template.
4. 1.0 µl of 100 mM DTT was added.
5. After that 0.5µl of *Accuscript* high fidelity RTase was added to the reaction.
6. The tube was placed in the temperature controlled thermal block at 42°C and incubated for 90 min.
7. The first strand cDNA synthesised was stored at -20°C for long term storage.

3.4 SECOND STRAND cDNA SYNTHESIS

Various components for the synthesis of cDNA were prepared as given in Table 2.

Table 2: Composition of reaction mixture for synthesis of cDNA

Components	Volume / concentration
RNase free water	39 µl
10X PCR reaction buffer	5.0µl
dNTP mix (10Mm each dNTP)	1.0µl
Forward primer	1.0µl
Reverse primer	1.0µl
First strand cDNA	2.0µl
Pfu ultra II HS DNA polymerase	1.0µl
Total	**50 µl**

All the above components were added in a sterile thin walled PCR tube for each PCR amplification reaction and the PCR amplification was carried out in a thermal cycler by running the following programme:

Table 3: PCR cycle program for synthesis of Lysozyme cDNA

Cycles	Programme	Temperature	Durations
1	Initial denaturation	95°C	2 minutes
40	Denaturation	95°C	30 seconds
	Annealing	58°C	30 seconds
	Extension	68°C	1min20seconds
1	Final extension	68°C	5 minutes

3.4.1 Agarose gel electrophoresis of the PCR product

The 1.2% agarose gel of high quality agarose for molecular biology (SIGMA Chem Co., USA) was prepared by dissolving the agarose in 1X TAE buffer (pH 8.0) followed by heating in a microwave oven or by keeping in boiling water bath. Ethidium bromide stock solution was added directly to molten agarose solution at the rate of 0.5 μg/ml of gel before casting the gel. The surface was leveled before pouring the gel. After complete setting of the gel, the comb was removed carefully and the gel casting tray was placed in the electrophoresis tanks containing 1X TAE (pH 8.0) buffer. The DNA sample was mixed with 5 μl of tracking dye and loaded slowly into the slots of submarine gels using a micropipette. Electrophoresis was carried out at 8 V/cm for an hour. After completion of electrophoresis, the gel was examined under UV transilluminator and photographed using gel documentation system.

3.5 EXTRACTION OF PCR PRODUCTS FROM AGAROSE GEL ELECTROPHORESIS

PCR products were separated on 1.2% Agarose gel as per the protocol described previously. The separated products were observed under UV transilluminator and excised immediately. Products were purified from the gel as per manufacturer's protocol using the SIGMA Gen Elute Gel and PCR Clean-up System and quantified by using UV-spectrophotometer

3.6 CLONING OF PCR AMPLIFIED LYSOZYME GENE

3.6.1 Ligation Reaction

Table 4: Composition of the ligation mixture for cloning of PCR product

Reagents	**Volume**
Fresh PCR product	3.5 µl
2x ligation buffer	10 µl
pJET1.2 vector (50ng/ul)	1 µl
T4 DNA ligase (5U/ul)	1 µl
Sterile water	4.5 µl
Total volume	20 µl

Reaction was setup for the ligation of lysozyme cDNA with the pJET vector using the following compositions. The reaction mixture was incubated at 16^{o}C for O/N for the completion of the ligation reaction.

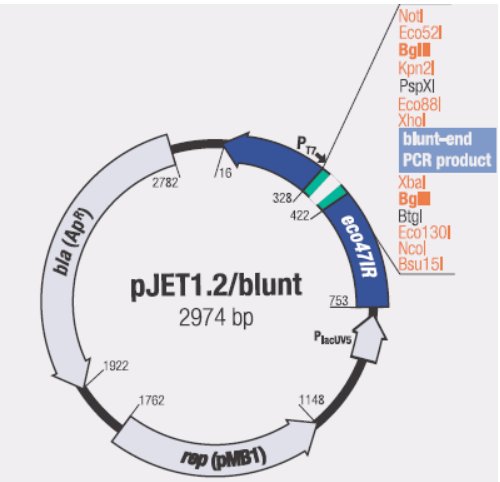


Figure 1: Schematic diagram of the pJET1.2 vector used for cloning of LYSOZYME cDNA.

3.6.2 BACTERIAL TRASFORMATION

3.6.2.1 Protocol for the transformation

- An aliquot of 5 µl of ligation mixture was added to 50 µl of competent *E. coli* (TOP10) cells and mixed gently by flicking. The mixture was incubated on ice for 30 min.
- The tubes were then transferred to water bath maintained at 42°C and held for exactly 90 seconds. The tubes were immediately transferred back on ice and allowed to chill for 1 to 2 min.
- To the above mix, 250µl of SOC media was added and mixed. The tubes were incubated at 37°C for 45 min in a rotary shaker to allow the bacteria to recover and develop the antibiotic resistance. After incubation, 150 µl of transformed mixture was spread evenly on LB agar plates containing ampicillin (100 µg/ml) and incubated at 37°C for 16-20 hrs.
- The transformants obtained on LB agar plates with ampicillin were inoculated into LB broth containing ampicillin (100 µg/ml) and incubated in shaking incubator at 37°C / 12-16 hrs after which the cells were pelleted and used for plasmid isolation by alkali lysis method.

3.7 PLASMID ISOLATION BY ALKALI LYSIS METHOD

Materials and Composition

Alkaline Lysis Solution I

50 mM glucose

25 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0

Alkaline Lysis Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1% (w/v) SDS

Prepare Solution II fresh and use at room temperature.

Alkaline Lysis Solution III

5 M potassium acetate, 60.0 ml

Glacial acetic acid, 11.5 ml

H_2O, 28.5 ml

Protocol:

1. Inoculated 2 ml of LB medium containing the appropriate antibiotic with a single colony of transformed bacteria. Incubated the culture overnight at 37°C with vigorous shaking.
2. Poured 1.5 ml of the culture into a microfuge tube. Centrifuged at maximum speed for 30 seconds at 4°C in a microfuge. Stored the unused portion of the original culture at 4°C.
3. Removed the medium by aspiration, leaving the bacterial pellet as dry as possible. Resuspended the bacterial pellet in 100 µl of ice-cold alkaline lysis solution I by vigorous vortexing.
4. Added 200 µl of freshly prepared alkaline lysis solution II to each bacterial suspension. Closed the tube tightly, and mixed the contents by inverting the tube rapidly five times. Stored the tube on ice.
5. Added 150 µl of ice-cold alkaline lysis solution III. Closed the tube and disperse alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3-5 minutes.

6. Centrifuged the bacterial lysate at maximum speed for 5 minutes at 4°C in a microfuge. Transferred the supernatant to a fresh tube.
7. Added an equal volume of phenol:chloroform mixture and vortexed to mix the organic and aqueous phases and then centrifuged the emulsion at maximum speed for 2 minutes at 4°C in a microfuge.
8. Transferred the aqueous upper layer to a fresh tube.
9. Precipitated nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mixed the solution by vortexing and then allowing the mixture to stand for 2 minutes at room temperature.
10. Collected the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.
11. Removed the supernatant by gentle aspiration as described in Step 3 above. Kept the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Used a disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
12. Added 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes at 4°C in a microfuge.
13. Removed all of the supernatant by gentle aspiration as described in Step 3. Care was taken care in this step, as the pellet sometimes did not adhere tightly to the tube.
14. Removed drops of ethanol that form on the sides of the tube. Stored the open tube at room temperature until the ethanol has evaporated and no fluid was visible in the tube (5-10 minutes) or by vacuum drying.
15. Dissolved the nucleic acids in 50 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). Vortexed the solution gently for a few seconds and stored the DNA solution at -20°C.

3.8 CONFIRMATION OF THE POSITIVE CLONES FROM TRANSFORMED COLONIES

3.8.1 By Agarose gel electrophoresis

The recombinant plasmids, isolated from the transformed colonies, were run on 0.8 % agarose gel and the size of the recombinant vector was determined using the DNA markers.

3.8.2 By PCR amplification

The recombinant plasmid was isolated from the transformed colonies and the positive clones were confirmed by carrying out PCR amplification using the same set of primers that were used for the synthesis of the cDNA. The size of the resultant PCR product was compared with the size of the insert used for cloning.

3.9 Plasmid isolation by using miniprep kit for sequencing

The recombinant plasmid was purified by using a plasmid miniprep kit (Sigma Chem Co., USA) by using following protocol:

1. Pelleted cells from 1-5 ml of overnight culture by centrifuging at high speed for 1 min. Discarded supernatant.
2. Resuspended cells in 200 µl resuspension solution. Pepetted up and down. 200 µl of lysis solution was added and inverted gently to mix.
3. 350 µl of neutralization solution was added and inverted and mixed 4-6 times. Pelleted the debris at high speed for 10min.
4. 500 µl of column preparation solution was added to binding column in a collection tube and spined at 12000 g / 1 min discarded flow-through.
5. Transferred cleared lysate into binding column and centrifuged for 1 min. at high speed and discarded flow-through.
6. 750µl of wash solution was added to column and centrifuged for 1 min at high speed and discarded flow-through. Spined 1 min. again to dry column.

7. Transferred the column to a new collection tube and added 100 µl elution solution or nuclease-free water and centrifuged at high speed for 1 min.

3.10 DNA sequencing and sequence analysis

After confirmation of the recombinant clone of LYSOZYME, the clones were sent for custom sequencing. The insert was sequenced from both the ends using T7 and the reverse primers present in the cloning vector. The ORF in the sequenced DNA was recognized and translated to predict the protein primary structure by employing molecular biology Web-tools available on the internet and aligned using CLUSTAL W (1.83) multiple sequence alignment tool.

3.11 Preservation of confirmed lysozyme clones

Positive clones were preserved by reculturing them in LB broth and by streak plating in LB agar containing Ampicillin (100µg/ml). Individual colonies were picked and grown in LB media (with ampicillin) to log phase, followed by transfer of the culture to a sterile glycerol solution in cryovials to a final concentration of 20%. The vials were cryopreserved at –80°C.

3.12 PRODUCTION OF RECOMBINANT LYSOZYME INCLUSION BODIES

E. coli strain BL-21 containing the plasmid vector PET-22b+ expresses buffalo mammary gland recombinant lysozyme as insoluble inclusion bodies. An overnight culture was grown in the LB medium supplemented with ampicillin antibiotic (100 microgram/ml) at 37°C and induced when cell density reached yp OD600nm 0.78 by the addition of isopropyl-thiogalactoside (IPTG). At this point, the growth temperature was increased to 42°C. After 3-4 hours the cells were collected by centrifugation and then broken by using Bead-Beater (cooled to 4°C between each pass). The cell homogenate was centrifuged for 15 min at 6000 × *g* at 4°C, the supernatant was discarded and the inclusion bodies were recovered.

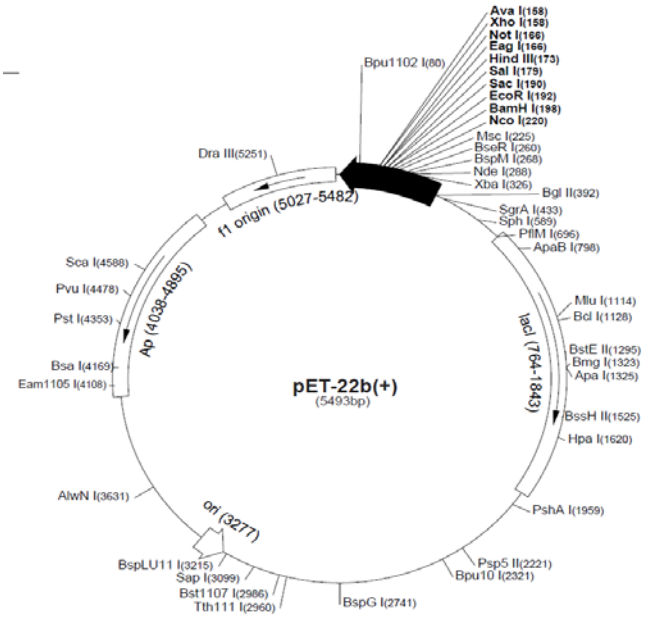


Figure 2: Schematic diagram of the pET22b+ vector used for expression of LYSOZYME

3.13 SDS-PAGE ANALYSIS

Composition of stock solutions for use in SDS-PAGE:

1. Acrylamide / bis Acrylamide (30% T, 2.67% C)

$$\text{Total monomer concentration (\% T)} = \frac{(\text{gm Acrylamide + gm bis-Acrylamide})}{(\text{Total volume})} \text{ X } 100$$

$$\text{Cross linking monomer concentration (\% C)} = \frac{\text{gm bis-Acrylamide}}{(\text{gm Acrylamide + gm bis-Acrylan}} \text{ X } 100$$

Acrylamide	29.2 g / 100 ml.
N' N' bis-methylene – Acrylamide	0.8 g / 100 ml

Filtered and stored at 4 ^{0}C in the dark (30 days maximum)

2. 1.5 M Tris-HCl , pH 8.8

18.15 g Tris base / 100 ml. pH is adjusted with 6 N HCl and final volume was made to 100 ml with deionized water

3. 0.5 M Tris-HCl, pH 6.8

6 g Tris base / 100 ml. pH is adjusted with 6 N HCl and final volume was made to 100 ml with deionized water

4. 10 % SDS

1. 4X sample buffer

1 M Tris –HCl, pH 6.8	2.5 ml
SDS	0.8 g
2-Mercapto ethanol	1 ml
Glycerol	3 ml
Bromophenol Blue	2 mg
Total volume	**10 ml**

2. 5X Running Buffer, pH 8.3

Tris base	15g / lit.
Glycine	72 g / lit.
SDS	5 g / lit.

3. 14 % Separating Gel Preparation – 0.375 M Tris, pH 8.8

Deionized water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10 % SDS	100 µl
Acrylamide / Bis (30% stock)	4.0 ml

10% Ammonium per sulfate	50 µl
(Freshly prepared)	
TEMED	5 µl
Total volume	**10 ml**

4. 4 % Stacking Gel Preparation – 0.125 M Tris, pH 6.8

Deionized water	3.05 ml
1.5 M Tris-HCl, pH 8.8	1.25 ml
10 % SDS	50 µl
Acrylamide / Bis (30% stock)	0.665 ml
10% Ammonium per sulfate	25 µl
TEMED	5 µl
Total volume	**5 ml**

Running conditions

At constant ampere of 16 mA till the dye reaches separating gel, then increased to 20 mA.

Coomassie Blue staining

Stain ½ hr with 0.1% Coomassie blue R-250 in fixative (40% Methanol and 10 % glacial Acetic acid).

Destaining

Destain with several changes of 40% methanol and 10% glacial acetic acid to remove background.

3.13 PURIFICATION OF RECOMBINANT LYSOZYME INCLUSION BODIES

Following disruption, the inclusion bodies were recovered by centrifugation and washed using urea and detergents. The improvement in inclusion body purity was obtained through several washings coupled with centrifugation. Centrifugation of the lysate

removed the cell debris and associated impurities. Washing the resulting pellet with PBS and EDTA, and centrifuging further can increase of the inclusion bodies. EDTA was used to remove the lipopolysaccharide (LPS) contaminants (*Hlodan et al., 1991*). This pellet was washed with 2M urea to selectively solubilise some of the impurities from the inclusion bodies (*Thatcher et al., 1996*). The final wash step was done by a detergent, Triton X-100, to further solubilise any membrane proteins bound to the inclusion body pellet. After the last wash, other proteins were still present in the inclusion body material in addition to the lysozyme.

After washing the inclusion bodies, they were denatured in high concentration of urea (8M). These denatured inclusion bodies are purified by HPLC by using Tris-HCl buffer (pH 8.7), containing 8M urea and 150 mM NaCl.

3.14 REFOLDING OF INCLUSION BODIES INTO BIOLOGICALLY ACTIVE FORM

3.14.1 By Dilution Method

Denatured and reduced lysozyme (1-1.5 mg ml $^{-1}$) was diluted 100 times into the refolding buffer containing 2 M urea, 3-6 mM reduced glutathione, 0.3-0.6 mM oxidised glutathione, 0.1 M Tris–HCl, pH 8.2 and 1 mM EDTA at 30°C for 24 hours.

3.14.2 By Protein Refolding Screening Kit

Components Used in Refolding Screening Kit are-

1) 96 well Protein Refolding Plate
2) 2 Aluminum Plate Sealers
3) 30 ml 10X IB-Prep Buffer (0.5 M Tris-HCl, 0.5 M NaCl, 5.0 mM EDTA, 50% (v/v) glycerol, pH 8.0)
4) 3 µl Benzonase Bioprocessing Reagent
5) 0.5 ml 1 M TCEP
6) 1.5 ml Triton X-100
7) 10 ml 30% *N*-Lauroylsarcosine
8) 50 ml 50X iFOLD Dialysis Buffer (500 mM Tris-HCl, 2.5 mM EDTA, pH 8.0)

Table 5. Components of that are pre filled in the protein refolding screening kit matrix

Buffer (50 mM Tris)	pH** 7.0, 7.5, 8.0, or 8.5
Ionic strength	NaCl, 100 mM or 250 mM
Detergent trap	Methyl-beta-D-Cyclodextrin, 12.5 mM
Redox agents	TCEP, 1 mM
	GSH/GSSG, 3.8/1.2 mM
Additive	Glycerol, 20% (v/v)
	PEG 6000, 0.1% (w/v)
	L-Arg, 500 mM
	GuHCl, 500 mM
	$CaCl_2/MgCL_2$, 1 mM each
	EDTA, 1 mM

3.14.2.1. Preparation of inclusion bodies

1. Prepared 30 ml 1X cell resuspension buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM TCEP, 0.5 mM EDTA, 5% glycerol, pH 8.0) by combining 3 ml 10X IB-Prep Buffer and 30 µl 1M TCEP with 27 ml ddH2O.
2. Prepared 20 ml 1X IB wash buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM TCEP, 0.5 mM EDTA, 5% glycerol, and 1% Triton X-100, pH 8.0) by combining 2 ml 10X IB-Prep Buffer, 20 µl 1 M TCEP, 0.2 ml Triton X-100, and 17.6 ml ddH2O.
3. Resuspended 4.53g cell paste in 10 ml 1X cell resuspension buffer.
4. Added 3 µl Solution per 1X cell resuspension buffer that contain 4.48 g cell paste. Stired gently at room temperature for 15 min.
5. Lysed cells by mini bead-beater. To prevent overheating, kept sample in an ice bath.
6. Added Triton X-100 to a final concentration of 1.0% (v/v). Stired slowly for 15 min at room temperature.
7. Centrifuged at 8000 x *g* for 15 min at 10°C. Discarded supernatant.
8. Washed IB, resuspended pellet in 10 ml 1X IB wash buffer.
9. Centrifuged at 8000 x *g* for 15 min at 10°C. Discarded supernatant.
10. Washed IB again by resuspending IB pellet in 10 ml 1X IB wash buffer.

11. Centrifuged at 8000 x *g* for 15 min at 10°C. Discarded supernatant.
12. Removed residual Triton X-100 from IB, resuspended pellet in 10 ml 1X cell resuspension buffer.
13. Centrifuged at 8000 x *g* for 15 min at 10°C. Discarded supernatant.
14. Removed 20 µl resuspended IB pellet and reserved for SDS-PAGE analysis.
15. Centrifuged at 8000 x *g* for 15 min at 10°C. Discarded supernatant.
16. Analyzed IB pellets by SDS-PAGE.

3.14.2.2. Denaturation of inclusion bodies

1. Prepared 10 ml 1X IB denaturation buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM TCEP, 0.5 mM EDTA, 5% glycerol, pH 8.0) by combining 1 ml 10X IB-Prep Buffer and 50 µl 1M TCEP with 9 ml ddH2O.
2. Prepared 400 ml 1X iFOLD dialysis buffer (10 mM Tris-HCl, 0.05 mM EDTA, 0.1 mM TCEP, and 0.06% (w/v) *N*-Lauroylsarcosine, pH 8.0) by adding 8 ml 50X Dialysis Buffer, 40 µl 1 M TCEP, and 800 µl 30% *N*- Lauroylsarcosine solution to 391.2 ml ddH2O.
3. Transfered ~0.48 g IB to glass beaker.
4. Added 10 ml 1X IB Denaturation Buffer per 0.48 g IB pellet.
5. Disrupted IB pellets by brief mini bead-beater.
6. Added 1.75 ml 30% *N*-Lauroylsarcosine to 0.48 g IB pellet.
7. Stired IB pellet at room temperature until solution becomes clear. Sample was heated gently (40–50°C for 5–10 min) to hasten IB solubilization.
8. Centrifuge solution at 25,000 x *g* for 15 min at 4°C. Discard pellet and continue with supernatant. Alternatively, solution may be passed through a 0.45 µm filter.
9. Dialyzed IB sample against 1X iFOLD dialysis buffer.
10. Estimated protein concentration of sample.
11. 5 ml of protein solution was used for the refolding experiment by refolding matrix.

3.14.2.3. Folding of target protein

1. Thawed refolding matrix **(Figure 3)** at room temperature for 90–120 min.
2. Mixed each well of the refolding plate with a multi-channel pipet, prior to adding the denatured protein.
3. Using a multi-channel pipet, added 50 µl protein solution (2 mg/ml) to each well.

Rapidly mixed each well by pipetting up and down at least 10 times.

4. Gently shaked refolding matrix overnight at room temperature.

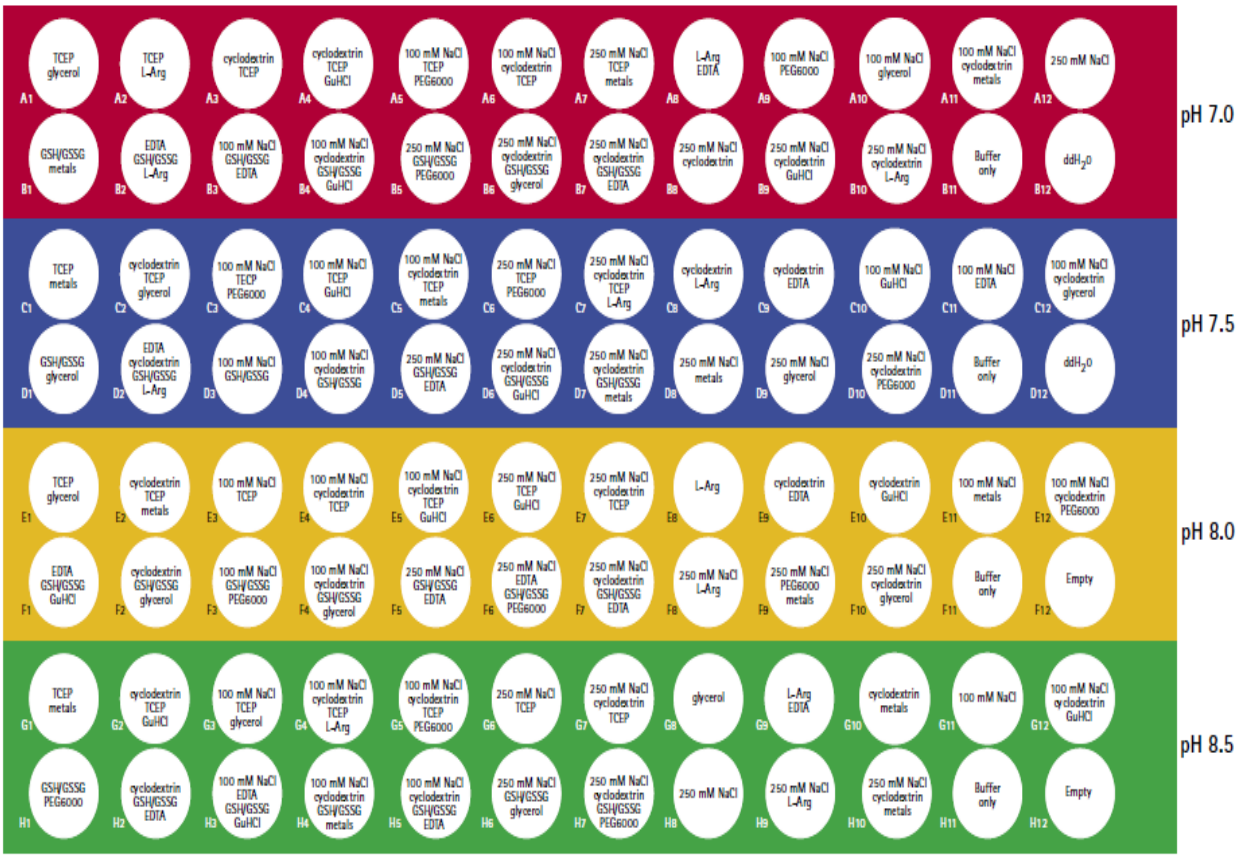


Figure 3: Different conditions that were used for refolding of buffalo recombinant lysozyme by protein refolding screening kit.

3.15. ANALYTICAL METHODS

Lysozyme concentration was determined spectrophotometrically at 280 nm using extinction coefficient 38940 for the denatured form. The protein composition of the inclusion bodies was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Lysozyme activity was measured at 25°C by following the decrease in absorbance (450 nm) of a *Micrococcus lysodeikticus* suspension.

CHAPTER – *4*

Results and Discussion

4. RESULTS AND DISCUSSION

4.1 Primer Designing for Milk Somatic Cell Lysozyme

The specific primers for lysozyme were constructed using the sequence information of lysozyme from several ruminant species like bovine, sheep, deer, goat etc. The forward and the reverse primers were designed from the non-conserved 5' and 3' untranslated regions for the amplification of the specific isoform of lysozyme expressing in milk/mammary gland of ruminants. Conserevd region was not used to avoid the amplification of various other forms of lysozyme expressing in these animals. A combination of primers could successfully amplify the respective lysozyme gene from the buffalo milk somatic cells.

Forward Primer

5` GGGATTTTGGCTTCTGTCAACATG `3

Reserve Primer

5` AGCTGAAGACGAAAACTCCACAG `3

4.2 Cloning and sequencing of lysozyme gene

4.2.1 cDNA synthesis

The total RNA purified from somatic cells of buffalo milk somatic cells showed good recovery and purity (**Figure 4A**) required for the synthesis of cDNA of lysozyme. First strand and consecutively cDNA corresponding to ~500 bp long sequence was successfully generated by RT-PCR using a pair of primers specific for milk lysozyme**.** Lysozyme amplicon contained approximately 490 bp including the sequence of the forward and reverse primer from the non-translated region. The presence of only one strong band on agarose gel corresponding to ~500 bp marker DNA indicated the amplification of the lysozyme gene (**Figure 4B**).

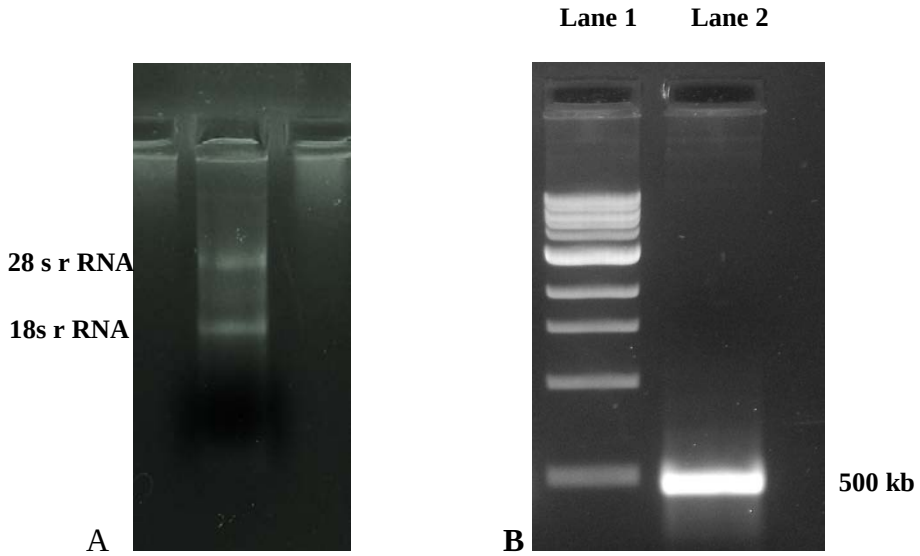


Figure 4: Panel A, Total RNA isolated from buffalo milk somatic cells. Panel B, PCR product of milk somatic cells lysozyme gene, the sharp single band corresponds to 500 bp (Lane 2).

4.2.2 Cloning, Transformation and Screening of Recombinant Clones

The ligation of the gel purified PCR product with the PCR cloning vector (pJET 1.2) followed by its transformation in Topo10 strain of *E. coli* resulted in the appearance of large number of colonies the following day of plating. Recombinant plasmid purified from the transformants showed larger plasmid size in comparison to the size of cloning vector without insert which suggested the success of ligation and transformation reaction. The confirmation of the presence of cloned insert in the plasmid isolated from the transformants was shown by PCR amplification of the insert using the corresponding pairs of specific primers. **Figure 5** shows the agarose gel electrophoresis of the PCR amplified product from clones containing lysozyme gene.

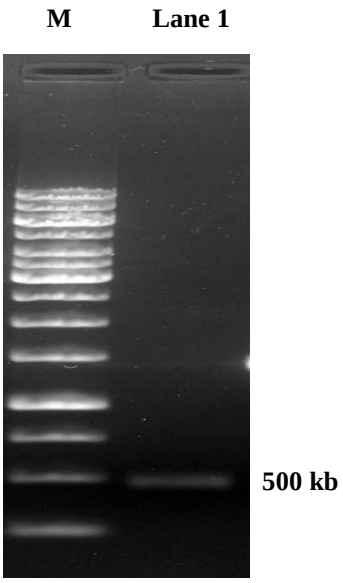


Figure 5: PCR amplification of lysozyme insert in recombinant clones. M- marker 10 kb DNA ladder, Lane 1-PCR product.

4.2.3 DNA Sequence Analysis of the Cloned Genes

Sequencing of the recombinant clones that were confirmed by the PCR amplification was carried out. Two recombinant clones of the lysozyme genes from the transformant were selected for parallel sequencing. The sequencing was carried out using T7 sequencing primer. The signals in the sequencing reactions for different bases were reliable up to 800-900 nt. read through. Given the small size of lysozyme gene (~500 nt.), the reliability of the sequencing reactions was satisfactory. The sequence of the nucleotides from different reactions set up for similar clones were compared and observed to be 100% identical, suggesting the reliability of the observed sequence. These results also suggested that the selected clones represents same gene. It is noteworthy that lysozyme gene has several duplicated copies expressing in mammary gland, stomach and non-stomach tissues of ruminants ((Irwin *et al*., 2004).), rodents and even of primates (Irwin *et al*., 2004).

5`ATGAAGGCTCTCCTTATTGTGGGGCTTCTCCTCCTTTCTGTTGCTGTCCAGGGCAAGAAA
TTTGAGAGGTGTGAGCTTGCCAGAACTCTGAAGAAACTTGGATTGGCTGGCTACAAGGGAGT
CAGCCTGGCAAACTGGATGTGTTTGGCCAGATGGGAAAGCAATTACAACACAAGTGCTATAA
ACTACAATCGTGGAAACAAAAGCACTGATTATGGGATATTTCAAATCAATAGCCGCTGGTGG
TGCAATGATGGCAAAACCCCAAGAGCAGTTAACGCCTGTGGTATACCCTGCAGCGCTTTGCT
GAAAGATGACATCACTCAAGCTGTAACATGTGCAAAGAGGGTTGTCAGCGATCCACGAGGCA
TTAGAGCATGGGTGGCATGGAGAAACAAGTGTCAAAACCGAGACCTCACGAGTTATGTTAAG
GGTTGCGGAGTG3`

Figure 6. Sequence of buffalo milk somatic cells lysozyme: Bases in blue colour indicate the sequence encoding signal peptide.

Figure 6 enlists the nucleotide sequence of lysozyme gene from the somatic cells of buffalo milk. The sequence was observed to contain an open reading frame corresponding to lysozyme gene. Prediction of the protein translated from these sequences resulted in polypeptide chains containing 148 amino acids (**Figure 7**) corresponding to 447 nt, the last codon being nonsense. Buffalo somatic cell clones contained an ORF spanning 447 nt (**Figure 6**). The lysozyme precursors from human placenta and non-stomach tissues of several species including closely related ruminants contain a single polypeptide chain with 148 amino acid (aa), while the chicken and stomach tissues of ruminants express lysozyme precursor with 147 aa.

Buffalo Milk Lysozyme(Translated Protein Sequence)

1⟶18

MKALLIVGLLLLSVAVQGKKFERCELARTLKKLGLAGYKGVSLANWMCLARWESNYNTSAIN
YNRGNKSTDYGIFQINSRWWCNDGKTPRAVNACGIPCSALLKDDITQAVTCAKRVVSDPRGI
RAWVAWRNKCQNRDLTSYVKGCGV **(148 aa)**

Figure 7. Predicted amino acid sequence of buffalo lysozyme. The first 18 amino acids shown in bold face indicates the signal peptide sequence.

Based on protein motif sequence analysis, the first 18 aa seems to code the signal peptide resulting in the matured polypeptide chain of 130 aa from the buffalo milk somatic cells. Irrespective of the species or tissue source, all known lysozymes contain lysine residue at N-terminal of the mature protein. Our results are consistent with the reported mature lysozyme from the corresponding tissues of most mammalian species including ruminants (*Dobson et al., 1984),* primates and rodents.

4.3 Sequence Analysis

4.3.1 DNA Sequence Analysis

Multiple sequence alignment of the lysozyme gene ORF from the buffalo milk was aligned with other species. By this alignment (**Table 6**) we predicted that, the buffalo milk (somatic cells) lysozyme shows higher homology with cattle milk lysozyme rather than with buffalo mammary gland lysozyme cloned by this group earlier (Kumar, 2005). **Table 6** shows that there is very high similarity existing between buffalo milk lysozyme (somatic cells), Bos taurus (95 %), and Bos taurus X Bos indicus (93 %) at DNA level. But only 90 % similarity existed between buffalo milk lysozyme and buffalo mammary gland lysozyme. These results indicate clearly that lysozyme genes expressing in mammary gland are divided into at least two groups and have evolved independently. High homology among these lysozyme indicates them to be descendent of the same gene, which might have undergone duplication and then evolved independently under the selection pressure of their environment without losing functionality in different species. It has been proposed that lysozyme genes in ruminants have undergone concerted evolution, which means that the multiple genes within a species have evolved in unison with similar rate (Irwin and Wilson, 1990). Based on sequence analysis of deer, sheep and cow lysozymes, it has been shown that the ancestral lysozyme gene duplicated some 40-50 million years ago, followed by divergence of deer (25 million years ago) and sheep (20 millions years ago) from the cow (Irwin and Wilson, 1990). In the beginning they evolved very fast followed by a refining or purifying step at a slow rate. The newly identified sequence of buffalo stomach and mammary gland tissue lysozymes are very close to the respective bovine lysozymes, indicating that divergence of buffalo and bovine is a much more modern event on the evolutionary path of ruminants.

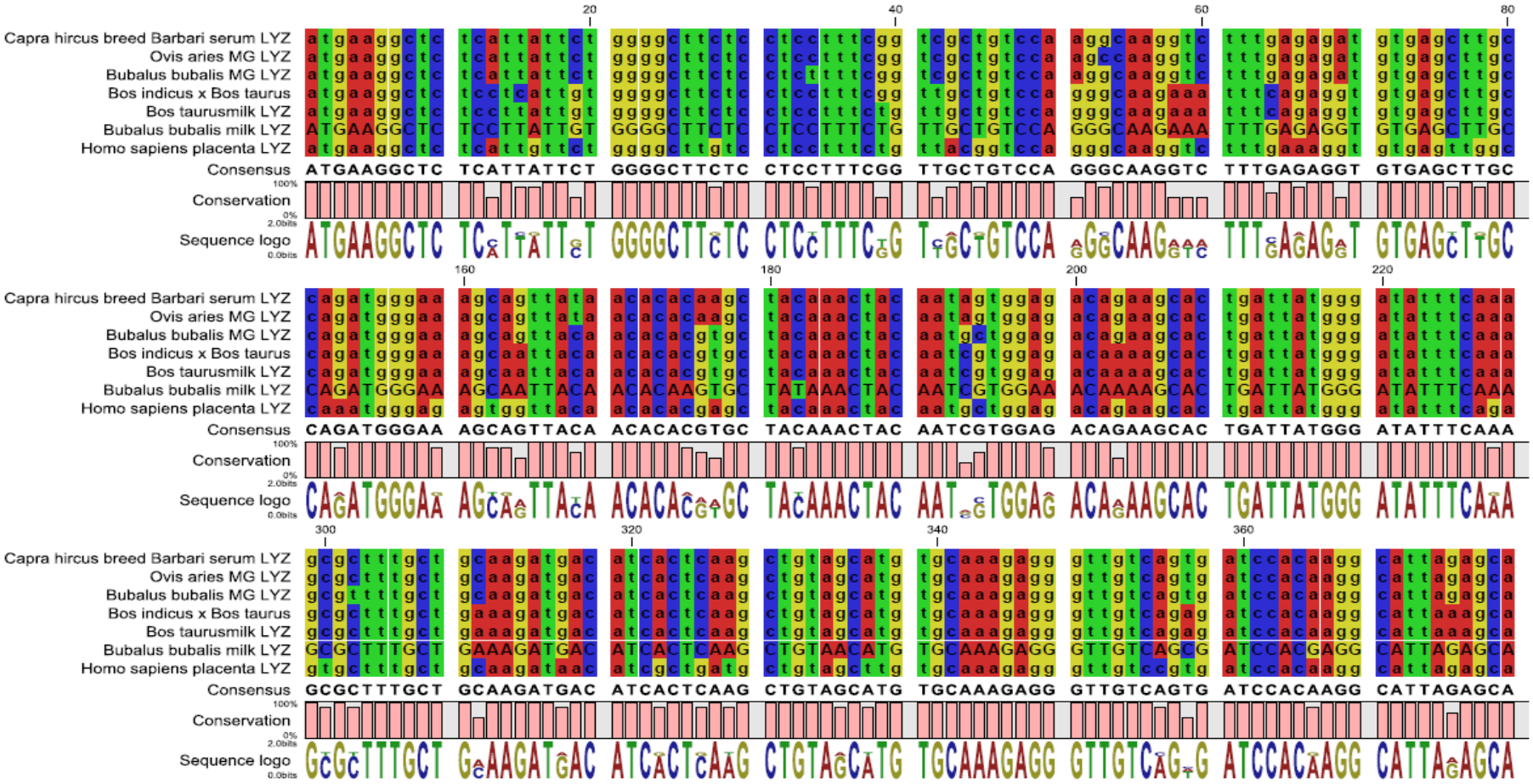


Figure 8: Nucleotide alignment of buffalo (somatic cells) recombinant lysozyme with other species

4.3.2 Protein Sequence Analysis

To find the changes at amino acid level in the translated gene product, the ORF was translated using the normal eukaryotic genetic code. The matured LYZ buffalo mik lysozyme contained 130 aa. The translated protein sequences from the corresponding genes were used to predict various properties of their polypeptide chains.

Table 6 shows the predicted pI, Molecular weight of lysozyme in different species predicted from their reported sequences. These results show that the pI of buffalo milk lysozyme (somatic cells) and cattle are almost similar i.e. ≥9.7, while the pI of buffalo mammary gland lysozyme was predicted ~ 8.9. Majority of ruminant species have been known to contain at least two sets of lysozyme genes, one of the kinds expressing in stomach or intestinal tissues while others in non-stomach tissues like kidney, macrophage, mammary gland, lachrymal glands and many other tissues involved in body defense and secretions. Bovine and other ruminants contain at least 10 lysozyme genes. In buffalo and cattle it seems, the milk lysozyme constitute a third group apart from the other two known to express in other tissues.

Table 6 also shows, at Protein level, 93 % similarity existing between buffalo and cattle, but between buffalo milk (somatic cells) lysozyme & buffalo mammary gland lysozyme both at DNA and protein level similarity was lower. By analyzing these data we obsereved that the sequence similarity existing both at DNA and protein level between similar isoforms from different species was higher as compared to the sequence similarity existing within the two different isoforms of lysozyme expressing in the mammary gland of a given species.

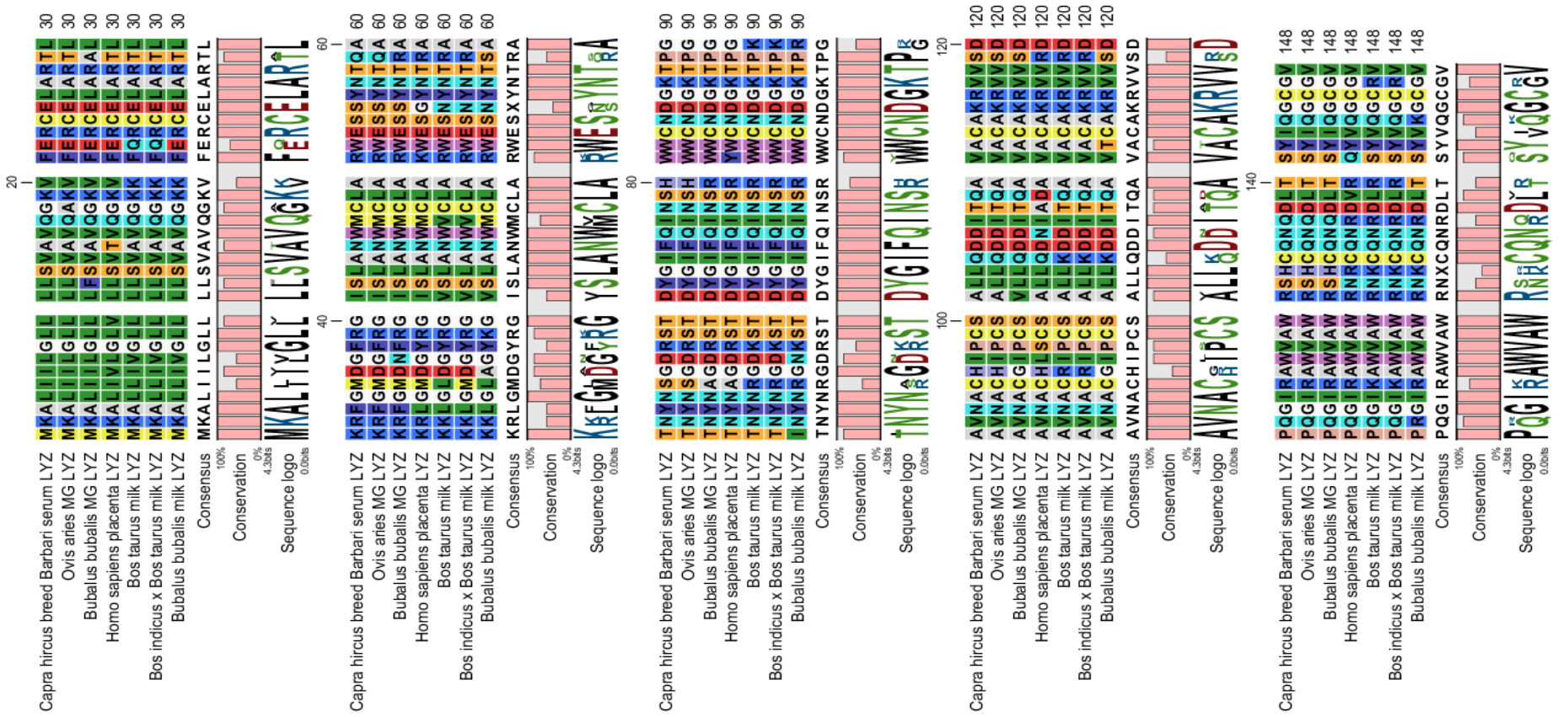


Figure 9: Amino acid alignment of buffalo (somatic cells) lysozyme with other species

Table 6: Comparison of buffalo (somatic cells) lysozyme with other species

	Bubalus bubalis (somatic cells)	**Bos taurus**	**Bos indicus X Bos taurus**	**Bubalus bubalis (mammary gland)**	**Capra hircus**	**Ovis aries**	**Homo speins (placenta)**
DNA (%)	100	95	94	90	89	89	85
Protein (%)	100	93	93	90	91	91	89
pI	9.69	9.92	9.92	8.87	8.46	8.46	9.38
M.W. (kDa)	14.65	16.68	16.80	16.48	16.44	16.45	16.53

4.4 Expression of Buffalo Recombinant Lysozyme as Inclusion Bodies in BL-21 host cells

The lysozyme gene was cloned at specific restriction sites, which are located at the multiple cloning sites (MCS) of respective vectors as described previously. They were inserted downstream to the T7 promoter in pET22b+ expression vector. T7 promoter is a strong and regulated promoter inducible by lactose or IPTG. On addition of the inducer, the T7 RNA polymerase would start transcribing the gene; the gene downstream to this would then start synthesizing the mRNA followed by protein synthesis. *E. coli* expression host like BL21 (DE3) was used for the expression of buffalo lysozyme gene previously cloned in the pET22b+ expression vector.

The lysozyme gene cloned in pET22b+ vector and transformed in to BL21 (DE3) expression host was induced with 0.2mM IPTG. The expressed recombinant protein was analyzed by SDS-PAGE as shown in Figure 8. The SDS-PAGE profile showed high level of expression of lysozyme in the presence of IPTG induced culture. The lysozyme gene product corresponded to 16-17 kDa, since lysozyme was expressed along with 6x His tag as a fusion protein. Therefore, the expressed protein showed **(Figure 10)** difference in the molecular weight level in comparison to hen egg white lysozyme used as a molecular weight marker protein.

Expression of lysozyme at 37 ^{o}C resulted in the majority of lysozyme in the inclusion body fraction, however at very lowe level, undetectable on SDS-PAGE, was also expressing as soluble and biologically active form. This soluble fraction when released from the cell over breakage during fermentation led to lysis of other cells and caused a very low yield of inclusion bodies. Therefore, the temperature of the culture during expression was raised to 42^{o}C to decrease soluble expression and promote formation of inclusion bodies. We observed a significant enhancement in the recovery of the inclusion bodies due to increased biomass during culturing of the recombinant clones of *E. coli*. Addition of lysozyme inhibitor also helped in production of greater biomass and better recovery of inclusion bodies.

The condition optimized for the good recovery of inclusion bodies was used for all future purpose for production of inclusion bodies in shake flasks in a culture volume of up to 100 ml.

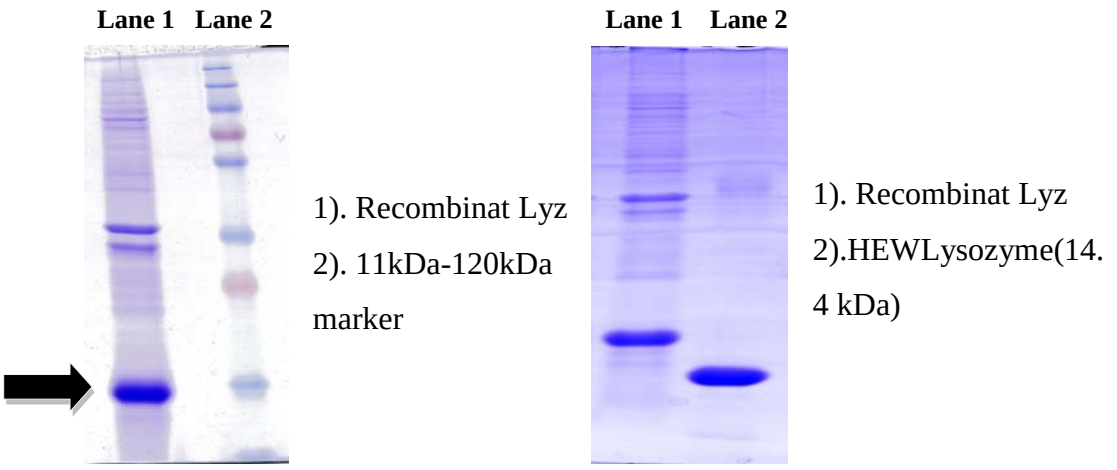
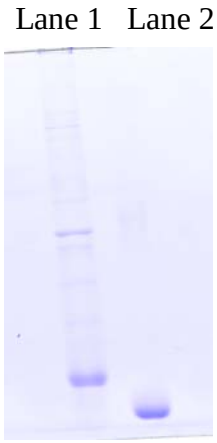


Figure 10: SDS-PAGE analysis of expression of buffalo recombinant lysozyme inclusion bodies

4.5 Isolation and Purification of Recombinant Lysozyme inclusion Bodies

Shake flask grown cells were broken by using a mini bead-beater with glass beads along with the resuspended pellet. Following disruption, the inclusion bodies were recovered by centrifugation. **Figure 11** shows the improvement in inclusion body purity obtained through

washing coupled with centrifugation. Centrifugation of the lysate removed the cell debris and associated impurities and increased the purity. Washing the resulting pellet with PBS and EDTA, and centrifuging further, increased the purity. EDTA was used to remove the lipopolysaccharide contaminants (*Hlodan et al., 1991*). This pellet was washed with 2 M urea to selectively solubilise adhering impurities from the inclusion bodies (*Thatcher et al., 1996*). The final wash step utilised a detergent, Triton X-100, to further solubilise any membrane proteins bound to the inclusion body pellet. After the last wash, other proteins were still present in the inclusion body material in addition to the lysozyme (lane 1). The expressed buffalo milk lysozyme was purified from cellular proteins initially by centrifugation method and analysed by SDS-PAGE. In this method the recombinant lysozyme was partially purified.



Lane 1-recombinant LYZ, Lane 2-HEWL used as marker.

Figure 11. Partially purified recombinant lysozyme by Centrifugation Method.

The inclusion bodies were solubilised in 8M urea and DTT (0.1 M). At higher concentrations urea promotes the denaturation of proteins and at lower concentrations (≤1.5 M) aggregation of proteins competes with refolding process. DTT involved in the reduction of disulfide bonds between the cysteine residues resulting in the linearization of polypeptides as well as separation of multimeric proteins due to breaking of interchange disulfide bonds. In the final purification stage, high resolution gel filtration chromatography was employed using a Superdex-200 column attached with the Akta Exlorer system (GE Health Sciences) with flow rate of 0.2 ml/min. Majorly two distinct peaks (peak A, B in **Figure 12**) were eluted at high concentration (8M) urea and 0.15 M NaCl**.** Then, the eluted fractions were anlysed by SDS-

PAGE. These results indicated that the recombinant lysozyme was fully purified from bacterial proteins (**Figure 14**).

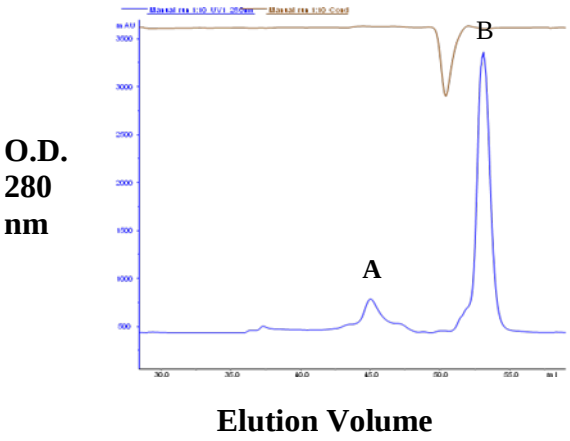


Figure 12: Elution profile of buffalo recombinant lysozyme expressed in *E. coli* as inclusion bodies. Peak A, indicates the purified buffalo recombinant lysozyme, and peak B indicates DTT separating out from lysozyme. On the top side the red line indicates the conductivity.

4.6 Denaturation and Separation of HEW Lysozyme from DTT

The HEW Lysozyme was denatured in the denaturation buffer (8M urea & DTT) to use it as a control in the refolding experiments. The HEWL was separated from DTT by gel filtration chromatography using Superdex-200 column attached with HPLC system. Here also two distinct peaks (peak A, B in **Figure 13**) were eluted at high concentration (8M) of urea and 0.15 M NaCl buffered with 50 mM Tris-HCl.

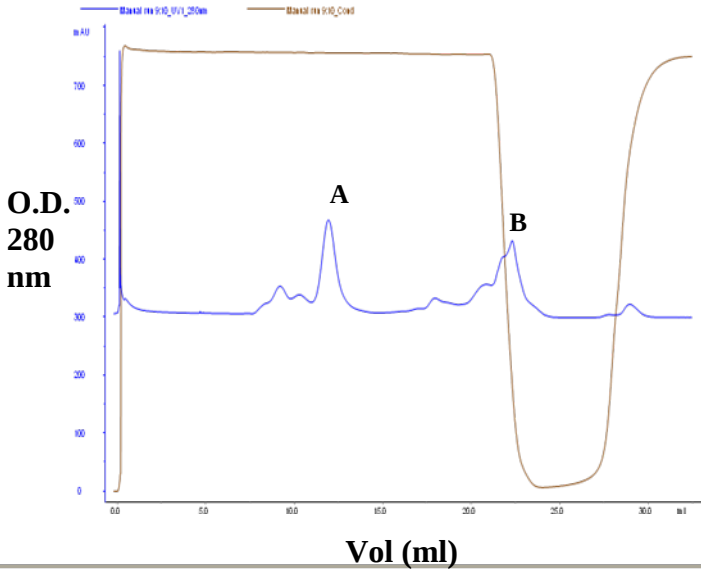


Figure 13: Elution profile of HEW Lysozyme. Peak A shows the HEWL, and peak B shows DTT which is well separated from HEWL. On the top side the red line indicates the conductivity.

Lane 1 Lane 2 Lane 3

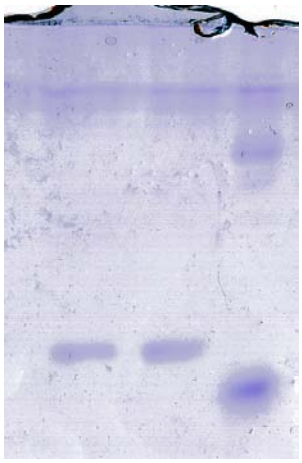


Figure 14: Purified recombinant lysozyme by Gel fitration. Lane 1 & 2 recombinant LYZ, Lane 3- HEW Lysozyme.

4.7 *In-Vitro* Refolding of Buffalo Recombinant Lysozyme and HEW Lysozyme

4.7.1 *In-Vitro* Refolding of Buffalo Recombinant Lysozyme and HEW Lysozyme by Dilution Method

Initially the refolding lysozyme was carried out by Dilution Method. In this method the denatured protein purified on Superdex-200 in the presence of 8M urea and free from DTT was diluted 100 times into the renaturation buffer. Before the addition of denatured protein into the renaturation buffer, the lysozyme in 8 M urea was concentrated such that the final urea concentration after mixing did not exceed 2.6 M, because higher urea concentration might cause lower yield od refolded protein due to denaturing effect of urea. Urea is not only a strong denaturing agent but also also an effective aggregation inhibitor at lower concentrations below 3M. It is found that urea at non-denaturing conditions improve the yield of properly folded protein.

The turbidometric assay for lysozyme activity after refolding was carried out to know the extent of refolding of buffalo. However, given the lower concentration of lysozyme after dilution, we used lysoplate method emplyoing *M. lysodeicticus* as a test culture to measure the refolded lysozyme. The activity assays showed that HEW Lysozyme could refold to native state as indicated by the clear zones of *M. lysodeicticus* lysis around the well. However buffalo lysozyme failed to show any activity under the conditions used in the experiment (**Figure 15**).

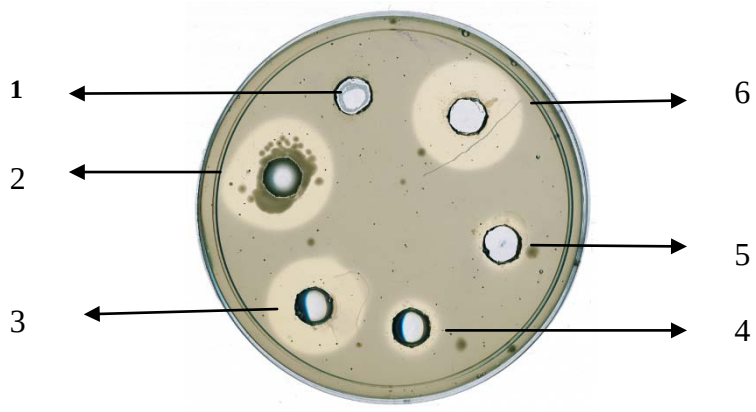


Figure 15: Activity assay of buffalo recombinant lysozyme and HEWL by Lyso plate method. 1. 200 µl Recombinant Lys 2. 200 µl HEW Lys (refolded) 3. 150 µl HEW Lys (refolded) 4. 100 µl HEW Lys (refolded) 5. 50 µl HEW Lys (refolded) 6. 200 µl Controle (native) HEWL

4.7.2 Refolding of Recombinant Protein by Protein Refolding Screening Kit

In this method the recombinant protein was tried to refold using various combinations of various additives know to promote protein refolding at various pH values. The matrix used for refolding experiments contained the additives and detergents along with the redox system at pH value of 7.0, 7.5, 8.0 & 8.5. Recombinant denatured lysozyme was mixed with content of the kits in an 1:10 ratio in a 96 well plate using 92 different conditions. After mixing the protein, the plate was incubated under shaking condition. After overnight incubation, the plate was used for measuring the activity assay in the indicative wells showing refolded protein. *M. lysodeicticus* solution prepared at 0.9 OD at 450 nm was added in the potential wells and change in absorbance at 450 nm was measure as a function of time. The activity of buffalo lysozyme measured by turbidometric method was observed under the following conditions with varying extents.

Table 7: Composition of buffers and additives promoting buffalo recombinant lysozyme refolding

S. No	Well No	pH	Solution Composition	Activity
1	H1	8.5	GSH/GSSG & PEG4K	+++
2	B1	7.0	GSH/GSSG & metals pH 7.0	++
3	C3	7.5	100 mM NaCl & TCEP	++
4	A5	7.0	100 mM NaCl, TCEP & PEG	+
5	C6	7.5	250 mM NaCl, TCEP & PEG	+
6	D3	7.5	100 mM NaCl & GSH/GSSG	+
7	F6	8.0	250 mM NaCl, EDTA, GSH/GSSG & PEG	+
8	F3	8.0	100 mM NaCl, GSH/GSSG & PEG	+
9	F5	8.0	250 mM NaCl, GSH, GSSG & EDTA	+
10	C1	7.5	TCEP & metals	+
11	B5	7.0	250 mM NaCl, GSH/GSSG	+
12	G1	8.5	TCEP & metals	+
13	H3	8.5	100 Mm NaCl, EDTA, GSH/GSSG & GuHCl	+

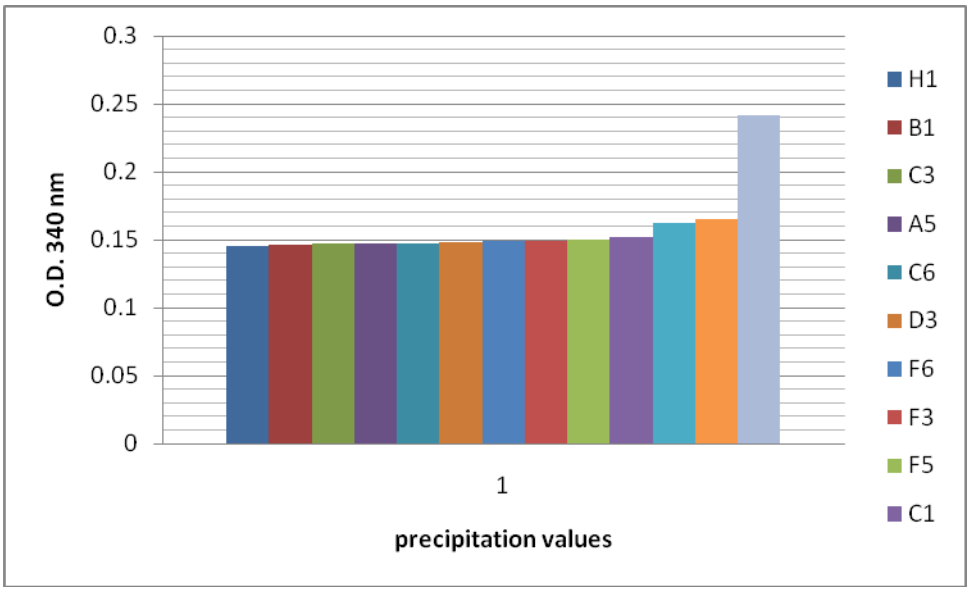


Figure 16: The diagram shows the precipitation values of refolded recombinant lysozyme in designated wells of the protein refolding screening kit. The composition of the wells has been shown in Table 2.

4.8 Activity Assay

100 µl of M. lysodeiticus was added to the each well and O.D. was measured at 450 nm as a function of time at an interval of 15 second for each of the well listed above. A fall in OD with time suggested the lysis of cells by lysozyme. Shorter time taken to reach a given OD value suggested a higher activity of the lysozyme. Among the 11 wells **(Figure 17)** where highest activity of lysozyme was observed in the H1well which contained PEG with Redox system (GSH/GSSG) **(Figure 18**).

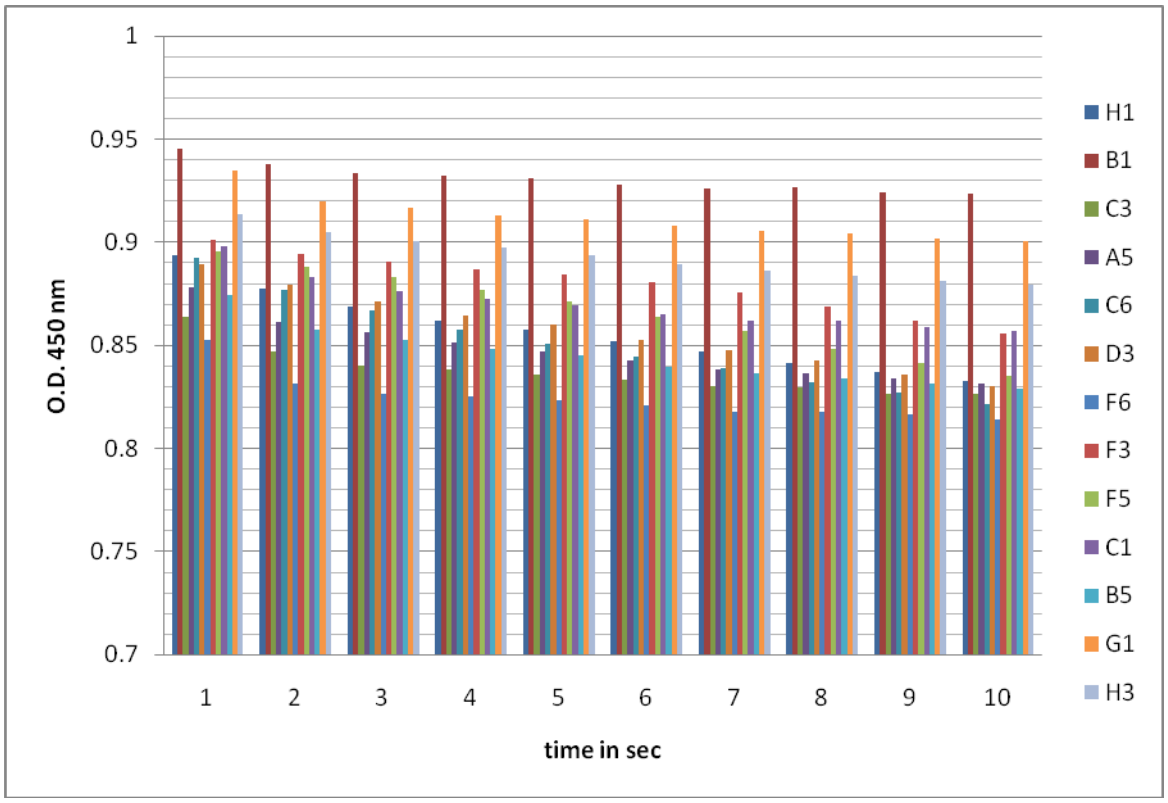


Figure 17: The diagram shows the activity of recombinant lysozyme in different chemical conditions.

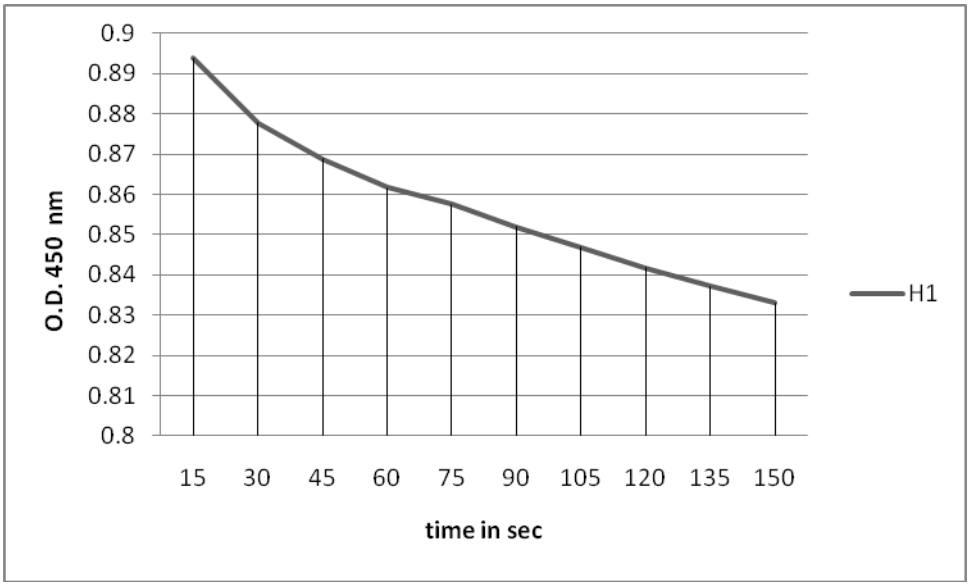


Figure 18: The diagram describes the best activity of recombinant lysozyme that was found in presence of PEG in line graph.

The **Figure 16** indicting the successful refolding reactions which were screened by measuring A340. Smaller A340 values correlate with less precipitation. However, soluble protein does not always correspond to correctly folded and active protein. Consequently, samples with lower A340 readings were analyzed with an activity assay specific to the lysozyme. Because the refolded proteins were do not undergo precipitation. So, lower the A340 value greater the activity.

Refolding of HEW Lysozyme and buffalo recombinant lysozyme were not followed the same method. Refolding of HEWL was carried out by urea (2M) and GSH/GSSG. Generally at 1.5 M-2.5 M concentration urea will promote the refolding of target protein. But the refolding of buffalo recombinant lysozyme was carried out by PEG along with GSH/GSSG redox system. PEG is known to stabilize native fold of the protein by causing preferential hydration of protein through steric hindrance (Bhat and Timasheff, 1994).

CHAPTER – *5*

Summary & Conclusion

5. SUMMARY AND CONCLUSION

The dissertation work "EXPRESSION & *IN-VITRO* REFOLDING OF BUFFALO RECOMBINANT LYSOZYME FROM INCLUSION BODIES" was carried out to clone a new form of lysozyme (isoform) and to optimize conditions for the expression of lysozyme in to inclusion bodies followed by refolding in to biologically active form. Lysozyme is an important hydrolyzing enzyme involved in the animal defense and digestive functions. It acts as antimicrobial enzyme by hydrolyzing the β-(1,4) glycosidic bond in peptidoglycan layer of gram positive bacteria. In ruminants, lysozyme is important not only as a defensive enzyme but also as a digestive enzyme. Realizing the potential of lysozyme application in therapeutics, diagnostics, functional foods, feed and fodder in dairy industry, we carried out the studies on buffalo lysozyme with the following objectives:

- Expression of Recombinant Buffalo Lysozyme as Inclusion Bodies in *E. coli.*
- Purification of Recombinant Lysozyme Inclusion Bodies and *in vitro* refolding to biologically active enzyme.

The summery of the work carried out is as follows:

1. Total RNA was isolated by TRIzol method using the RT-PCR. The cDNA was synthesized and amplified from the somatic cells of buffalo milk.

2. The PCR product was purified and subsequently cloned in a PCR cloning vector (pJET vector) and used for transformation of Top10 competent cells.

3. The recombinant clones were selected and confirmed to contain the gene insert by PCR amplification.

4. The sequencing of the cloned gene was carried out using the T7 primers (sequence available in the cloning vector).

5. The nucleotide sequence analysis indicated an ORF of 447 nt for the insert cloned from the somatic cells of buffalo milk.

6. The predicted polypeptide chain contained 148 aa, in which the first 18 were predicted to encode signal peptide and the remaining 130 residues constituting the mature lysozyme.

7. Sequence alignment of buffalo milk lysozyme with lysozyme from other species indicated that the buffalo milk lysozyme shows 94 % - 95 % homology with the cattle at DNA level, 93 % similarity at protein level.

8. Buffalo milk lysozyme was expressed in *E. coli* in the form of inclusion bodies by raising the culture temperature to 42^{o}C to improve the yield of protein production, since lysozyme expressed in active form was highly toxic to *E. coli* cells.

9. Expression of Lysozyme in *E. coli* was also improved by adding NAG, a lysozyme inhibitor.

10. Purification of inclusion bodies was improved by washing them several times with detergents and 2 M urea.

11. Refolding of the HEW Lysozyme was achieved by dilution method, however similar method failed to refold buffalo lysozyme.

12. Refolding of buffalo recombinant lysozyme was therefore attempted by screening large number of refolding conditions using a screening kit containing various refolding buffers at pH 7-8.5. Few of the combinations at pH 8.5 resulted in the refolding of buffalo lysozyme.

13. Highest refolding of buffalo lysozyme was achieved in a mixture containing PEG4K and GSSG/GSH redox system.

CHAPTER – *6*

Bibliography

6. BIBLIOGRAPHY

Acharya, KR., Stuart, DI., Walker, NCP., Lewis, M and Phillips, DC (1989). Refined structure of baboon alpha-lactalbumin at 1.7 A resolution. Comparison with C-type lysozyme. *J. Mol. Biol.* **208**: 99-127.

Acharya, A and Taniuchi, H (1982). Implication of the structure and stability of disulfide intermediates of lysozyme on the mechanism of renaturation. *Mol. Cell. Biochem.* **44**: 129-134.

Acharya, A and Taniuchi, H (1980). Influence of Solution Conditions on the Formation of an Intermediate from Hen Egg Lysozyme Containing Non-native Disulfide Bonds during Thiol-dependent Renaturation. *J. Biol. Chem.* **255**: 190-198.

Alm, E., Morozov, AV., Kortemme, T and Baker, D (2002). Simple physical models connect theory and experiment in protein folding kinetics. *J Mol Biol.* **322**:463-476.

Arnheim, N and Wilson, A (1972). Quantitative Immunological Comparison of Bird Lysozymes. *J. Biol. Chem.* **242**: 951-967.

Artymiuk, PJ and Blake, CCF (1981). Refinement of humanlysozyme at 1.5 resolution. Analysis of non-bonded and hydrogen-bond interactions. *J. Mol. Biol.* **152***:* 737-762.

Bang, IS., Yoe, SM and Kang, CS (2006). Expression and purification of *Artogeia rapae* lysozyme II cDNA in a baculovirus/insect cell system. *Entomological Research.* **36 (2)**: 116–121.

Baniahmad, A., Muller, M., Steiner, C and Renkawitz, R (1987). Activity of Two Different Silencer Elements of the Chicken Lysozyme Gene Can Be Compensated by Enhancer Elements. *E.M.B.O. J.* **6**: 297-310.

Bank, JG and Tranter, HS 1985: Lysozyme. Antimicrobial systems in milk. Proc. Symp. on Natural Antimicrobial Systems, England, pp. **3**:39-48.

Bell, K., McKenzie, HA., Muller, V., Rogers, C and Shaw DC (1981). Equine whey proteins. *Comp. Biochem. Physiol.* **68B**: 225–236.

Benkerroum, N., Mekkaoui, M., Bennani, N and Hildane, K (2004). Antimicrobial activity of camel's milk against pathogenic strains of Escherichia coli and Listeria monocytogenes. *Int. Journal of Dairy Technology*. **57**: 39–43.

Bhat, R and Timasheff, SN (1992). Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols. *Protein Science*. **1**: 1133-1137.

Blake, C., Koenig, D., Mair, G., North, A., Phillips, D and Sarma, V (1965). Structure of Hen Egg-White Lysozyme. A Three-Dimensional Fourier Dynthesis at 2 Angstrom Resolution. *Nature*. **206**: 757-767.

Blake, C., Johnson, L., Mair, G., North, A., Phillips, D and Sarma, V (1967). Crystallographic studies of the activity of hen egg-white lysozyme. *Proc. R. Soc. Lond. B Biol. Sci.* **167**: 378-384.

Bonifer, C., Jägle, U and Huber, M (1997). The Chicken Lysozyme Locus as a Paradigm for the Complex Developmental Regulation of Eukaryotic Gene Loci. *J. Biol. Chem.* **72**: 260-275.

Bordet, J and Bordet, M (1924). Le pouvoir bacteriolytique du colostrum et du lait. *Comptes Rendus.* **179**: 1109–1113.

Bowden, G., Angel, PM and Georgiou, G (1991). Structure and morphology of protein inclusion bodies in *E. coli. BioTechnology* **9**: 725–730.

Brew, K., Castellion, FJ., Vanaman, TC and Hill, RL (1970). The complete amino acid sequence of bovine alpha-lactalbumin. *J. Biol. Chem*. **245**: 4570-4582.

Briere, J., Barthelemy, J., Bachard, G and Fiet, J (1974). Usefulness of the determination of serum and urinary muramidase activity in chronic and sub-acute myeloproliferative syndromes and in leukemia with a low percentage of myeloblasts. II. Application to Various Pathological Cases. *Clin. Chim. Acta.* **50**: 265-271.

Bryngelson, JD and Wolynes, PG (1987). Spin-glasses and the statistical-mechanics of protein folding. *Proc Natl Acad Sci*. **84**:7524-7528.

Buss, DH (1971). Isolation and partial characterization of lysozyme from baboon milk. *Biochim. Biophys. Acta*. **236**: 587-592.

Carpenter, JF., Pikal, MJ., Chang, BS and Randolph, TW (1997). Rational design of stable lyophilized protein formulations: some practical advice. *Pharm. Res.* 14: 969–975.

Chandan RC., Parry RM Jr and Shahani KM (1965). Purification and some properties of bovine milk lysozyme. *Biochim Biophys Acta*. **110(2)**: 389-398.

Chipman, DM and Sharon, N (1969). Mechanism of lysozyme action. *Science.* **165**: 454-465.

Cooper, GM (1997). The Cell: a Molecular Approach. *American Society for Microbiology*. **32**: 543-551.

De-la-Re-Vega, E., García-Orozco, KD., Calderón-Arredondo, SA., Romo-Figueroa, MG., Islas-Osuna, MA., Yepiz-Plascencia, GM and Rogerio R. Sotelo-Mundo (2004). Recombinant expression of marine shrimp lysozyme in *Escherichia coli*. *Electronic Journal of Biotechnology*. **3***:* 221-234.

Delepierre, M., Dobson, C and Poulson, F (1982). Studies of beta-sheet structure in lysozyme by proton nuclear magnetic resonance. Assignments and analysis of spin-spin coupling constants. *Biochemistry*. **21**: 47-56.

Desmadril, M and Yon, J (1984). Evidence for intermediates during unfolding and refolding of a two-domain protein, phage T4 lysozyme: Equilibrium and einetic studies. *Biochemistry*. **23**: 11-17.

Dickerson, RE (1971). The structure of cytochromec and the rates of molecular Evolution. *J. Mol. Evol.* **1**: 26-45.

Dobson, D. E., Prager, EM and Wilson, AC (1984). Stomach lysozymes of ruminants. Distribution and catalytic properties. *J. Biol. Chem*. **259**: 11607-11616.

Duhiman, AS (1988). Purification of camel milk lysozyme and its lytic effect on *Escherichia coli* and *Micrococcus lysodeikticus*. *Comparative Biochemistry and Physiology.* **91B**: 793-796.

Ebner, KE., Denton, WL and Brodbeck, U (1966). The substitution of alpha-lactalbumin for the B protein of lactose synthetase. *Biochem. Biophys. Res. Commun.* **24**: 232–236.

Eitenmiller, RR., Friend, BA and Shahani, KM (1974). Relationship between composition and stability of bovine milk lysozyme. *J. Dairy Sci.* **59**: 834-839.

Engstrom, A., Xanthopoulos, KK., Boman, HG and Bennich, H (1985). Amino acid and cDNA sequences of lysozyme from *Hyalophora cecropia. EMBO J.* **4**:2119-2122.

Fleming, M (1922). On a remarkable bacteriolytic element found in tissues and secretions. *Proc. R. Soc. London Ser. B.* **93**: 306-317.

Garcı´ a-Orozco, KD., Lo´ pez-Zavala, AA., Puentes-Camacho, D., Caldero´ n-de-la-Barca, AM and Sotelo-Mundo, RR (2005). Recombinant bacterial expression of the lysozyme from the tobacco-hornworm Manduca sexta with activity at low temperatures. *Biotechnology Letters*. **27**: 1075–1080.

Gebler, J., Gilkes, NR., Claeyssens, M., Wilson, DB., Beguin, P., Wakarchuk, WW., Kilburn, DG., Miller, RC., Warren, RAJ and Withers, SG (1992) Stereoselective hydrolysis catalyzed by related *β*-1,4-Glucanases and *β*-1,4-xylanases. *J. Biol. Chem*. **267**: 12559–12561.

Glynn, A (1968). Lysozyme: Antigen, enzyme and antibacterial agent. *Sci. Basis Med. Ann. Rev.* **19**: 31-38.

Godovac-Zimrnermann, J., Shaw, D., Conti, A and McKenzie, H (1987). Identification and the primary structure of equine alpha-lactalbumin B and C (Equus caballus, Perissodactyla). *Biol. Chem. Hoppe-Seyler*. **368**:427-433.

Goldberg, M., Rudolph, R. and Jaenicke, R (1991). A kinetic study of the competition between renaturation and aggregation during the refolding of denatured-reduced egg white lysozyme. *Biochemistry* **30**: 2790–2797.

Grobler, JA., Ramakrishna, R., Pervaiz, S and Brew, K. (1994). Sequences of two highly divergent canine type lysozymes: Implications for the evolutionary origins of the lysozyme/α-lactalbumin superfamily. *Arch. Biochem. Biophys*. **313**: 360-366.

Grutter, M. G., Weaver, L.H and Matthews BW (1983). Goose lysozyme structure: an evolutionary link between hen and bacteriophage lysozymes? *Nature.* **303**: 828-831.

Hankiewicz, J and Swierczek, E (1974). Studies on serum and urinary lysozyme. *Pol Arch Med Wewn.* **51(6)**: 591-7.

Hankiewicz, J and Swierczek, E (1974). Lysozyme in human body fluids. *Clin Chim Acta.* **57(3)**: 205-9.

Herrero MB., Mandal A., Digilio LC., Coonrod SA., Maier B and Herr JC (2005). Mouse SLLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization. *Dev Biol.* **284(1)**: 126-42.

Hiraoka, Y., Segawa, T., Kuwajima, K., Sugai, **S** and Murai, N (1980). Kinetic correlations between the disulfide bond reduction and the induced conformational change of proteins. *Biochem. Biophys. Res. Commun.* **95**: 1098- 1104.

Hlodan, R., Craig, S and Pain, RH (1991). Protein folding and its implications for the production of recombinant proteins. *Biotechnol. Genet. Eng. Rev*. **9**: 47–87.

Hopper, KE and McKenzie, H**A** (1974). *Mol. Cell. Biochem.* **3**: 93-108.

Huang, JB., Villemain, J., Padilla, R and Sousa, R (1999). Mechanisms by which T7 lysozyme specifically regulates T7 RNA polymerase during different phases of transcription. *J Mol Biol.* **293**:457–475.

Ibrahim, HR Yamada, M., Matsushita, K., Kobayashi, K and Kato, A (1994). Enhanced bactericidal action of lysozyme to *Escherichia* coli by inserting a hydrophobic pentapeptide into its C terminus. *J Biol Chem.* **269**:5059–5063.

Ibrahim, HR., Thomas, U and Pellegsine, A (2001). A helix loop helex peptide at upper lip of the native site left of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *J. Biol. Chem.* **276**: 43767-43774.

Imoto, T., Doi, T., Hayashi, K and Funatsu, M. (1969). Characterization of Enzyme-Substrate Complex of Lysozyme. *J. Biochem. (Tokyo)* **65***:* 667-673.

Imoto, T., Johnson, L., North, A., Phillips, D and Rupley, J (1972). Vertebrate Lysozymes. *The Enzymes Vol. 7*, P. Boyer, Academic Press, NY, 665.

Irwin, D. M., A. Sidow, R. T. White, AC Wilson, Irwin DM., Sidow, A., White, RT and Wilson, AC (1989). Multiple genes for ruminant lysozymes in The Immune Response to Structurally Defined Proteins: The Lysozyme model, eds S. J. Smith-Gill & E. E. Sercarz. *Adenine Press, Schenectady. New York*. **8**: 73-85.

Irwin, DM and Wilson, AC (1990). Concerted Evolution Of Ruminant Stomach Lysozymes. *J. Biol. Chem*. **10**: 54944-4952.

Irwin, DM and Wilson, AC (1989). Multiple cDNA sequences and the evolution of bovine stomach lysozyme. *J. Biol. Chem.* **264**: 11387-11393.

Janet R Kornegay, James W. Schilling and Allan Wilson, C (1994). Molecular Adaptation of a Leaf-eating Bird: stomach lysozyme of the Hoazin. *Mo.Biol. Evol*. **11(6)**: 921-928.

Jolles J and Jolles P (1967). Monoclonal human tear and human milk lysozymes. *Biochemistry.* **6(2)**: 411-7.

Jolles P and Jolles J (1984). What's new in lysozyme research? Always a model system, today as yesterday. *Mol Cell Biochem*. **63(2)**: 165-89.

Jolles, J., Jollie, P., Bowman, BH., EM., Prager, CB., Stewart and Wilson, AC (1989). Episodic Evolution. In The Stomach Lysozymes of Ruminants. *J. Mol. Evol*. **28**: 528-535.

Jolles, J., Schoentgen, F., Croizier, G., Croizier, L and Jolles, P (1979). Insect lysozymes from three species of lepidoptera: Their structural relatedness to the c (chicken) type lysozyme. *J. Mol. Evol.* **14**: 267-271.

Junn, HJ., Youn, J., Suh, KH and .Lee, SS (2005). Cloning and expression of *Klebsiella* phage K11 lysozyme gene Protein Expression and Purification. *J Zhejiang Uiv Sci B.* **42(1)**: 78-84.

Karlsen, S., Eliassen, BE., Hansen, LK., Larsen, RL., Riise, BW., Smalås, AO., Hough, E and Grinde, B (1995). Refined crystal structure of lysozyme from the rainbow trout (Oncorhynchus mykiss). *Acta Crystallogr D Biol Crystallogr.* **51**:354-67.

Kato A, Nakamura S, Ibrahim H, Matsumi T, Tsumiyama C, Kato M (1998). Production of genetically modified lysozymes having extreme heat stability and antimicrobial activity against gram negative bacteria in yeast and in plant. *Nahrung*. **42(3-4)**: 128-30.

Kikuchi M, Kawano K and Nitta K (1998). Calcium-binding and structural stability of echidna and canine milk lysozymes. *Protein Sci.* **7(10)**: 2150-5.

Klockars M and Reitamo S (1975). Tissue distribution of lysozyme in man. *J Histochem Cytochem*. **23(12)**: 932-40.

Kokoshis PL, Williams DL, Cook JA and Di Luzio NR (1978). Increased resistance to Staphylococcus aureus infection and enhancement in serum lysozyme activity by glucan. *Science.* **199(4335)**: 1340-2.

Lansbury, P. T., Jr (1999). Evolution of amyloid: what normal protein folding may tell us about fibrillogenesis and disease. *Proc. Natl. Acad. Sci. USA* **96**: 3342–3344.

Laschtschenko, P (1909). Uber die keimtotende und entwicklungshemmende Wirkung von Huhnereiweib. Z. *Hyg. Infekt. Krankheiten.* **64**: 419-426.

Lee-Huang S, Huang PL, Sun (1999). Lysozyme and RNases as anti-HIV components in beta-core preparations of human chorionic gonadotropin. *Proc Natl Acad Sci (USA).* **96(6)**: 2678-2681.

Lee-Huang, S., Huang, PL., Sun, YT., Huang, PL., Kung, HF and Blithe, DL (1999). Lysozyme and RNase as anti-HIV. Components in b-core preparations of human chorionic gonadotropin. *Proceedings of the National Academy of Science, USA.* **96**: 2678–2681.

Lemarbre, P., Rinehart, JJ., Kay, NE., Vesella, R and Jacobs, HS (1981). Lysozyme enhances monocyte-mediated tumoricidal activity: A potential amplifying mechanism of tumor killing. *Blood.* **58**: 994–999.

Lescar J, Souchon H, Alzari PM (1994). Crystal structures of pheasant and guinea fowl egg-white lysozymes. *Protein Sci.* **3(5)**: 788-98.

Maachupalli-Reddy, J., Kelley, B and DeBernardez-Clark, E (1997). Refolding of recombinant proteins. *Biotechnol. Prog.* **13**: 144–150.

Madhusudan, M and Vijayan, M. (1992): Additional binding sites in lysozyme - X-ray analysis of lysozyme complexes with bromophenol red and bromophenol blue. *Protein Engr.* **5**: 399-404.

Matsumura, I and Kirsch, JF (1996). Is aspartate 52 essential for catalysis by chicken egg white lysozyme? The role of substrate assisted hydrolysis. *Biochemistry*. **35**: 1881 – 1889.

Matthews, BW., Remington, SJ., Grütter, MG., Anderson, WF (1981). Relation between hen egg white lysozyme and bacteriophage T4 lysozyme: evolutionary implications. J Mol Biol. **147(4)**:545-58.

McClelland, DBL and Vanfurth, R (1975). In vitro synthesis of lysozyme by human and mouse tissues and leucocytes. *Immunology.* **28**: 1099-1114.

McKenzie, HA. and White, FH Jr (1991). Lysozyme and alpha-lactalbumin: structure, function, and interrelationships. *Adv. Protein Chem.* **41**: 173–315.

Mei, Yu, David, Irwin, M (1996). Evolution of stomach lysozyme: the pig lysozyme gene. *Molecular Phylogenetics and Evolution.* **5(2)**: 298-308.

Miranker AD and Dobson CM (1996): Collapse and cooperativity in protein folding. *Curr Opin Struct Biol*. **6**:31-42.

Mitraki, A & King, J. (1989). Protein folding intermediates and inclusion body formation. *BioTechnology*. **7**: 690–697.

Huang, J.B., Villemain, J., Padilla, R., Sousa, R (1999). Mechanisms by which T7 lysozyme specifically regulates T7 RNA polymerase during different phases of transcription. *J Mol Biol*. **293**:457–475.

Mandal, J., Kenneth L. Klotz., Shetty, J., Jayes, FL., Wolkowicz, MJ., Bolling, LC., Coonrod, SA., Black, MB., Diekman, AB., Haystead, TAJ., Flickinger, CJ and ,Herr, JC (2003). SLLP1, A Unique, Intra-acrosomal, Non-bacteriolytic, C Lysozyme-Like Protein of Human Spermatozoa. *Biol of Reprod*. **68**: 1525-1537.

Montagne, P., Cuilliera, ML, Mole, C., Bene, MC and Gilbert, F (1998). Microparticle enhanced nephelometric immunoassay of lysozyme in milk and other human body fluids. *Clin. Chem.* **44(8)**: 1610-1615.

Moore, WV and Leppert, P (1980). Role of aggregated human growth hormone (hGH) in development of antibodies to hGH. *J. Clin. Endocrinol. Metab.* **51**: 691–697.

Muller I, Subert N, Otto H, Herbst R, Ruhling H, Maniak M and Leippe M (2005). A Dictyostelium mutant with reduced lysozyme levels compensates by increased phagocytic activity. *J Biol Chem*. **280(11)**: 10435-43.

Newman J. (1995). How breast milk protects newborns. *Sci Am*. **273(6)**: 76-9.

Newman, J., Josephson, A., Cacatian, A and Tsang, A (1974). Preliminary communications. Spinal-fluid lysozyme in the diagnosis of central-nervous-system tumours. *Lancet.* **304**: 756-764.

Nitta, K. and Sugai, S (1989). The evolution of lysozyme and α – Lactalbumin. *Eur. J. Biochem.* **182**: 111-118.

Nitta, K., Tsuge, H., Shimazaki, K and Sugai, S (1988). Calcium binding lysozymes. *Biol. Chem. Hoppe-Seyler*. **369**: 671 -675.

Nitta, K., Tsuge, H., Sugai, S and Shimazaki, K (1987). The calcium-binding property of equine lysozyme. *FEBS Lett*. **223**; 405–408.

Onuchic JN, Nymeyer H, Garcia AE, Chahine J and Socci ND (2000). The energy landscape of protein folding: insights into folding mechanisms and scenarios. *Adv Protein Chem.* **53**:87-152.

Oberg, K., Chrunyk, B. A., Wetzel, R. and Fink, AL (1994). Nativelike secondary structure in interleukin-1 beta inclusion bodies by attenuated total reflectance FTIR. *Biochemistry.* **33**: 2628–2634.

Osserman, E (1967). Crystallization of human lysozyme. *Science.* **155**: 1536- 1567.

Osserman, E and Lawlor, D (1966). Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *J. Exp. Med.* **124**: 921-928.

Osserman, E., Klockars, M., Halper, J and Fischel, R (1973). Effects of Lysozyme on Normal and Transformed Cells. *Nature*. **243**: 331-339.

Pahud, J.J. and Widmer, F (1982). Calf rennet lysozyme. *Biochem. J.* **201**: 661-664.

Parry RM, Chandan RC and Shahani KM (1969). Isolation and characterization of human milk lysozyme. *Arch Biochem Biophys*. **130(1)**: 59-65.

Perraudin, J and Prieels, J (1982). Lactoferrin Binding to Lysozyme-treated *Micrococcus luteus*. *Biochim. Biophys. Acta*. **718**: 42-52.

Philips**,** D.C **(**1967). The hen egg white lysozyme molecule. *Proc. Natl. Acad. Sci.* **57:** 484–495**.**

Pirzkiewiez, D and Bruice, TC (1969). Interaction of cellodextrins with lysozyme: the necessity of the 2-acetamido group for binding and hydrolysis. *Arch Biochem Biophys.* **129(1**): 317-20.

Prager, EM and Jolles, P (1996). Animal lysozymes c and g: an overview. *EXS*. **75**: 9-31.

Prieur, DJ (1986). Tissue specific deficiency of lysozyme C in ruminants. *Comp. Biochem. Physiol.* **85B**: 349-353.

Pruzanski, W and Saito, S (1969). The diagnostic value of lysozyme (muramidase) estimation in biological fluids. *Amer. J. Med. Sci.* **258**: 405-414.

Przybycien, T., Dunn, J. P., Valax, P and Georgiou, G (1994). Secondary structure characterization of beta-lactamase inclusion bodies. .*Protein Eng.* **7**: 131–136.

Qasba, PK and Safaya, SK (1984). Similarity of the nucleotide sequences of rat alpha-lactalbumin and chicken lysozyme genes. *Nature.* **308***:* 377-380.

Ratner, RE., Phillips, TM and Steiner, M (1990). Persistent cutanous insulin allergy resulting from high-molecular-weight insulin aggregates. *Diabetes.* **39**: 728–733.

Reischl, U (2004). Purification and immunological characterization of recombinant antigens expressed in the form of insoluble aggregates (inclusion bodies). *Methods Mol. Med.* **94**: 213-219.

Richardson, RH and Brew, K (1980). Lactose synthase. An investigation of the interaction site of alpha-lactalbumin for galactosyltransferase by differential kinetic labeling. *J. Biol. Chem.* **255**: 3377–3385.

Rudolph, R. and Lilie, H (1996). In vitro folding of inclusion body proteins. *FASEB J.* **10**: 49–56.

Rudolph, R (1990). Modern Methods in Protein and Nucleic Acid Research. ed. Tschesche, H. (de Gruyter, New York), pp. **12**: 149–172.

Mine, S., Ueda, T., Hashimoto, T and Imoto, T (1997). Improvement of the refolding yield and solubility of hen egg-white lysozyme by altering the Met residue attached to its N-terminus to Ser. *Protein Engineering.* **10**: 1333-1338.

Sato, Y and Watanabe, K (1976). Lysozyme in hen blood serum. *Poult Sci.* **55(5)**: 1749-56.

Schwinte, P., Ball, V., Szalontai, B., Haikel, Y., Voegel, J and Schaaf, P (2002). Secondary structure of proteins adsorbed onto or embedded in polyelectrolyte multilayers. *Biomacromolecules.* **3**: 1135-1142.

Seyfert, HM (1999). Lactoferrin and lysozyme – two mammalian antibiotics. *Arch. Anim. Breed.* **42**: 22-27.

Shimizu, M., Losos, J and Gibbins, A (2005). Analysis of an approach to oviduct-specific expression of modified chicken lysozyme genes. *Biochem. Cell Biol.* **83**: 49-57.

Smith-Gill, S., Rupley, J., Pineus, M., Carty, R and Scheraga, H (1984). Experimental identification of a theoretically predicted "Left-Sided" binding mode for (GlcNAC)6 in the active site of lysozyme. *Biochemistry*. **23**: 993-1002.

Smith, SG, Lewis, M., Aschaffenburg, R., Fenna, RE., Wilson, IA., Sundaralingam, S. DI., Stuart, DI and Phillips,DC (1987). *Biochem. J.* **342**: 353–360.

Stewart, CB., Schilling, JW and Wilson, AC (1987). Adaptive evolution in the stomach lysozymes of foregut fermenters. *Nature.* **330**: 401-404.

Strynadka, NC and James, MN (1996). Lysozyme: a model enzyme in protein crystallography. *EXS*. **75**: 185-222.

Stuart, DI., Acharya, KR., Walker, NPC., Smith, G., Lewis, M and Phillips. DC (1986). Alpha-lactalbumin possesses a novel calcium binding loop. *Nature.* **324***:* 84-87.

Subhadra Priyadarshini and Vinod K Kansal (2002). Purification, characterization, antibacterial activity and N-terminal sequencing of buffalo-milk lysozyme. *J Dairy Res.* **69(3)**: 419-31.

Subhadra Priyadarshini and Vinod K Kansal (2003). Biochemical characterization of buffalo (Bubalus bubalis) milk lysozyme. *J Dairy Res*. **70 (4)**: 467-71.

Sudarshan, Kumar (2005). Molecular Cloning, Sequencing and Functional Analysis of Lysozymes. *Thesis*, NDRI, Karnal.

Sugai, S and Ikeguchi, M (1994). Conformational comparison between α-lactalbumin and lysozyme. *Adv Biophys.* **30**: 37-84.

Sun, HC., Xue, FM., Qian, K., Fang, HX., Qiu, HL., Zhang, XY and Yin, ZH (2006). Intramammary expression and therapeutic effect of a human lysozyme-expressing vector for treating bovine mastitis. *J Zhejiang Univ Sci B.* **7(4)**: 324–330.

Sun-Uk Choi, Hyun–Dong paik, Seung–Cheol Lee, Takuya Nihira and yong- Il Hwang (2004). Enchanced productivity of human lysozyme by pH – controlled batch fermentation of recombinant Saccharomyces cerevisiae. *Journal of bioscience and bioengineering*. Vol.98, No. **2**: 132-135.

Swanson, KW., Irwin, DM and Wilson, AC (1991). Stomach lysozyme gene of the langur monkey: tests for convergence and positive selection. *J. mol. evolution.* **33(5)**: 418-425.

Takumi Koshiba, Tomohiro Hayashi, Ishido Miwako, Izumi Kumagai, Teikichi Ikura, Keiichi Kawano, Katsutoshi Nitta and Kunihiro Kuwajima (1999). Expression of a synthetic gene encoding canine milk lysozyme in *Escherichia coli* and characterization of the expressed protein. *Protein Engineering.* **12(5)**: 429-435.

Thatcher, DR and Hitchcock, A (1994). Protein folding in biotechnology. *In: Pain, R.H. (Ed.), Mechanisms of Protein Folding. IRL Press, New York, pp.* 229–261.

Thakur A, Chauhan A and Willcox MD (1999). Effect of lysozyme on adhesion and toxin release by Staphylococcus aureus. *Aust N Z J Ophthalmol*. **27(3-4)**: 224-7.

Thornton, CA and Ballow, M (1993). Safety of intravenous immunoglobulin. *Arch. Neurol.* **50**: 135–136.

Valax, P and Georgiou, G (1993). Molecular characterization of beta-lactamase inclusion bodies produced in Escherichia coli. 1. Composition. *Biotechnol. Progr*. **9:** 539–547**.**

Vocadlo, DJ., Davies, GJ., Laine, R and Withers, SG (2001). Catalysis by hen egg – white proceeds via a covalent intermediate. *Nature.* **412**: 835-838.

Watanabe, M., Aizawa, T., Demura, M., andNitta K (2004). Effect of hydrostatic pressure conformational changes of canine milk lysozyme between the native, molten globule, and unfolded states. *Biochim Biophys Acta*. **1702(2)**: 129-36.

Weaver, LH., Grutter and Mathews, BW (1995). The refined structure of goose lysozyme and its complex with a bound trisaccharide show that the "goose type" lysozyme lack a catalytic aspartate residue. *J. Mol. Biol*. **245**: 54-68.

White, FH Jr., McKenzie, HA., Shaw, DC and Pearce, RJ (1988). Studies on a partially purified bovine milk Lysozyme. *Biochem Int*. **16(3)**: 521-8.

White, TJ., Mross, GA, Osserman, EF and Wilson, AC (1977). Primary structure of rat lysozyme. *Biochemistry.* **16**. 1430- 1436.

Won-Sik Kim, Heike Salm and Klaus Geider (2004). Expression of bacteriophage wEa1h lysozyme in Escherichia coli and its activity in growth inhibition of Erwinia amylovora. *Microbiology.* **150**: 2707–2714.

Xiaodong Cheng, Xing Zhangt, James W. Pflugrath, and F. William Studiert (1994). The structure of bacteriophage T7 lysozyme, a zinc amidase and an inhibitor of T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA*. **91**: 4034-4038.

Yao, XJ., Wainberg, MA and Parniak, MA (1992). Mechanism of inhibition of HIV-1 infection in vitro by purified extract of Prunella vulgaris. *Virology*. **187(1)**:56-62.

Zhu, K., Jin, H., He, Z., Zhu, Q and Wang, B (2006). A continuous method for the large-scale extraction of plasmid DNA by modified boiling lysis. *Nat. Protoc.* **1**: 3088-3097.