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A Review

Lysozyme in Livestock: A Guide to Selection for Disease Resistance: a Review

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Abstract

Lysozyme is a ubiquitous enzyme found in all major taxa of living organisms having diverse role starting from digestion to immune response. In livestock two major lysozyme types are present i.e. g-type (goose-type) and c-type (chicken type) classified depending on their origin, primary structure, three dimensional structure, biochemical as well as enzymatic properties. The course of this review covers brief introduction about the enzyme, its historical prospective, types and distribution, biochemistry and biological action with special reference to the antibacterial properties as well as the structural organization of concerned gene. It also covers the attempts to explore the role of lysozyme in disease biology, possibility of lysozyme as a conventional marker as well as the role of lysozyme gene as a candidate gene for future breeding for disease resistance in general and mastitis resistance in particular. The biotechnological approach to modulate the antimicrobial activity by controlling expression level of the lysozyme gene in native and recombinant form is also discussed.

Key words: Lysozyme, antibacterial activity, polymorphism, genetic selection, disease resistance, mastitis and association

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1. Introduction

The adverse effects of the indiscriminate use of antibiotics on the animals as well as environment encourage breeding of animals for resistance to various diseases in order to improve the economic viability in sustainable production system. For the success of selection experiments targeting disease resistance the choice of trait is very important. Unfortunately, the traits conferring disease resistance or reduced susceptibility are having low heritability, like in case of mastitis it is ≤ 0.1, (Gaunt *et al.*, 1980, Simianer *et al*., 1991) which makes the task more difficult. These traits are mostly quantitative traits and controlled by many genes (Gelderman, 1975), although major gene model suggests relatively larger contribution of a few genes (Lande, 1981). Exploring the complex nature of genetic control over immune responsiveness as well as disease resistance in one hand and need for genetic intervention at individual gene level for candidate gene approach in other hand emphasizes the interest on the genes mainly (i) which codes for pathogen receptors, (ii) which regulates phagocytic function as well as (iii) genes responsible for quality and quantity of antibody formation (Lewin *et al.* 1999). The choice of candidate genes depends on the nature of infection, type of pathogen and the host immune response to pathogens, involved in the biology of the disease. One such enzyme involved in immune system is lysoyme, which targets peptidoglycan, a unique and/or essential component of bacterial cell wall leading to its lysis at the time of phagocytosis. The present review covers brief description about lysozyme, the distribution in livestock and its scope to be utilized as a marker (both conventional as indicative of immune status as well as DNA marker for selection of animals of higher immune status), an immunoprotective agent as well as therapeutic agent against diseases in general and mastitis in particular.

2. Historical prospective

a. Discovery

Sir Alexander Fleming, recipient of Noble Prize in year 1945 in the subject Physiology and Medicine for discovery of Penicillin from mould *Penicillium notatum* also discovered lysozyme (Fleming, 1928). The lysozyme was first noticed during some investigation made on a patient suffering from acute coryza. The nasal secretion of the patient was cultured daily on blood agar plates and for the first three days of infection there was no bacterial growth with the exception of an occasional staphylococcal colony. From there Fleming's Remarkable bacteriolytic agent came to light. Since last 90 years this unique enzyme has become the centre of attraction for scientists due to several factors. It was the first protein found to contain together all the twenty usual amino acids (hen lysozyme) and the first enzyme to be submitted for a complete X-ray crystallographic analysis. It is also the first enzyme for which a detailed mechanism of action is proposed (Jolles and Jolles, 1984).

b. Origin and evolution

Lysozyme is an ancient enzyme whose origin goes back an estimated 400 to 600 million years. This was originally a bacteriolytic defensive agent and has been adapted to serve a digestive function on at least two occasions, separated by nearly 40 million years. The origin of the related goose type is distinct from the more common c-type. They share lower degree of homology in terms of amino acid sequence identity and yet they possess common secondary and tertiary structures. Lysozyme c gene also gave rise, after gene duplication 300 to 400 million years ago, to a gene that currently codes for alpha-lactalbumin, a protein expressed only in the lactating mammary gland. Alpha-Lactalbumin shares only 40% identity in amino acid sequence with lysozyme c, but it has a closer spatial structure and gene organization. Although structurally similar, functionally they are quite distinct. Specific amino acid substitutions in alpha-lactalbumin account for the loss of the enzyme activity of lysozyme and the acquisition of the features necessary for its role in lactose synthesis. (Qasba and Kumar, 1997)

3. Types, sources and abundancy

Lysozymes are enzymes of ubiquitous nature, occurring widespread in humans, animals, birds, invertebrates as well as bacteria, phages and plants (Table-1). They are found in various body fluids, external body secretions like tears, saliva, gastric juices, tracheal secretion and small amounts in internal secretions like serum, pleural fluid and CSF (Cerebrospinal fluid). These are classified in distinct sub-families based on their origin, amino acid sequence, and three dimensional structures (Jolles, 1996; Masschalack and Michiels, 2003). Although the similarity in primary structure between the lysozyme types is limited, their three-dimensional structures show striking similarities. HEWL is divided into two domains by a deep cleft containing the active site. One domain mainly consists of the β-sheet structure, while the other domain is more helical in nature. Similarly, the tertiary structure of GEWL is also a α/β structure with a pronounced active-site cleft separating a small β-strand domain from a larger α-helical domain (Weaver *et al*. 1995). Out of several types abundant in animal kingdom (Table 1), generally two types are found in livestock.

a. C-type lysozymes

Egg white is the richest source of lysozyme and the classical representative of the lysozyme family and is called *c*-type (chicken or conventional type) lysozyme. The archetype lysozyme, which has served as a model for studies on enzyme structure and function, is the c-type lysozyme from hen egg white (HEWL). C-type lysozymes are the major lysozymes produced by most vertebrates, including mammals. A BLAST search reveals that almost all available completely sequenced mammalian genome contain at least one putative c-type lysozyme gene.

b. G-type lysozymes

The *g*-type lysozyme stands for "goose type" lysozyme after 'Embden goose' (Canfield and McMurry 1967). It is remarkable that in the eggs of some bird species, g-type is the major lysozyme, while in others it is the c-type. Hikima *et al.* (2001) were the first to demonstrate the occurrence of g-

type lysozymes outside the class of birds. They reported a cDNA of g-type lysozyme in the Japanese flounder. A database search reveals additional g-type lysozyme homologues in other vertebrates including mammals, fish and amphibians.

Not only had the lysozyme varied among the species, but also within the body as per the tissue of origin. The protein and cDNA sequencing has proved that the s*Lys* (stomach lysozyme) differ in amino acid sequence from those found in kidney and granulocyte (e.g. conventional m*Lys*) by deletion of one Proline residue i.e. Pro^{103} (Jolles and Jolles, 1984; Jolles *et al.*,1990; Ito *et al.,* 1993; Steinhoff *et al.,* 1994).

Table 1: Types of lysozyme

Type of lysozyme	Source	Reference
c-type(Chicken type)	Chicken egg white	Chipman and Sharon, 1969
g-type(Goose type)	Birds	Prager and Jolles, 1996
h type (hevamine type)	Plants	Beintema and T.Van Scheltinga,1996
b type (barley type)	Plants	Beintema and T.Van Scheltinga,1996
i- type (invertebrate type)	Molluscs, insects	Jolles and Jolles, 1984
v-type	Virus	Losso *et al*., 2000
p- type	Rodents	Hammer and Wilson, 1987

4. Biochemical and biological action

There are different reported biochemical and biological properties of lysozyme like digestive, anti-bacterial, immune-modulating as well as therapeutic properties. Out of which the anti-bacterial properties are notable.

a. Biochemistry

Lysozyme is a short polypeptide (148 aa) of basic in nature. The molecular weight of lysozymes present in milk were reported to be 18kDa (Eitenmiller *et al.,* 1975) in bovine, 15kDa in human (Finkelstein and Finkelstein, 1982), 16kDa in bubaline species (Priyadarshini and Kansal, 2002a) and 14.3kDa for standard egg white lysozyme. As far as enzymatic action is concerned, lysozymes can be defined as 1,4-β-N-acetyl muramidases cleaving the glycosidic bond between the C-1 of N-acetyl muramic acid (MurNac) and the C-4 of N-acetyl glucosamine (GLcNac) in bacterial peptidoglycans (Phillips, 1966; Jeanloz *et al*., 1963). Some lysozymes also display a more or less pronounced chitinase activity and esterase activity (Jolles and Jolles, 1983). Li *et al.,* 2006 reported hen egg white lysozyme as an inhibitor of mushroom tyrosinase.

b. Digestive role

In some of the animals, lysozyme has been recruited as a digestive enzyme. In ruminants, higher level of lysozyme is produced in true stomach than that of monogastric animals. Gene duplications, as observed in the ruminants might be the reason for an increase in lysozyme production, and are thought to have facilitated the recruitment of lysozyme to the new function as a digestive enzyme, without loss of its function as an immune-related antimicrobial protein in other parts of the body. The disappearance of the acid-sensitive Asp-Pro bond usually present in lysozymes, together with a decrease in other acid labile amino acids (i.e. Asp, Asn or Gln residues) illustrates the adaption of resistance to an acidic medium. Moreover, these digestive lysozymes can function in this low pH compartment by virtue of their low pH optima of 4.8–5.2 for the ruminants, which needs regulatory and structural adaptations of the lysozyme protein.

c. Chitinase activity

C-type lysozymes can hydrolyse the β-(1,4)-glycosidic bond in a NAG-polymer, and are therefore said to have chitinase activity. Chitin is the main component of the exoskeletons of crustaceans, arachnids and insects. Since, in these animals, a role for lysozyme in the recovery of lesions in the chitinous body has been proposed (Li *et al*. 2005).

d. Immunological role

The roles of lysozyme in body's defense mechanism are abundant mainly against bacteria (cell wall) and chitin-covered pathogens. The natural substrate for lysozyme action is the peptidoglycan layer of the cell wall which forms a close network of the entire cell, giving the cell its shape and stability against cellular turgid pressure, hydrolysis of which leads to cell lysis. Thus, it is a unique but essential component of bacteria which is absent in eukaryotes and is an excellent target of innate immunity system. Thus lysozyme has got similar functions like PGN recognization molecules i.e CD14, Toll Like Receptor-2 (TLR-2), Nucleotide oligomerization domain (NoD)-containing proteins, and a family of peptidoglycon receptor proteins or PGRPs. (Dziarski and Gupta, 2005)

e. Preservative in animal feed

The preservative function of lysozyme was first observed in certain invertebrates who feed on blood. Though, blood is an enriched media for microbes, lysozyme might contribute to suppress the bacterial growth especially when it is stored before final digestion. HEWL is widely used as an antibacterial preservative in some industrially produced feeds. (Proctor and Cunningham, 1998).

5. Gene and genomic organization

a. Localization and structural organization

While most mammals possess lysozyme encoding gene (Swanson *et. al.,* 1991) being expressed in granulocytes and macrophages (mLys), duplications are wide spread throughout insects (Daffre *et. al.,* 1994 and Li *et. al.,* 2005), mouse (Hammer *et. al.,* 1987 and Cross *et. al.,* 1988), and rabbit (Camara *et. al.,* 1990). Even the genomic blot analysis revealed that there are about 10 lysozyme genes in the genome of true ruminants (where as most non-ruminant species posses a single gene) and at least 4 of bovine lysozyme genes are expressed in stomach (Irwin and Wilson, 1989). Irwin (2004) identified 2 new cow lysozyme cDNA sequences and showed that at least 4 different lysozymes are expressed in cows in nonstomach tissues and probably function as antibacterial defense enzymes. These four lysozyme genes are in addition to the four digestive lysozyme genes expressed in the stomach, yielding a number of expressed lysozyme genes in the cow larger than that found in most non-lysozyme-deficient mammals. The lysozyme genes being expressed primarily in cells of myeloid cell linage, specifically in macrophages and granulocytes, but their immuno-relevant products are found in secretion (Chung *et. al.,* 1988). In case of bovines the macrophage expressed lysozyme gene maps to chromosome No. 5q23 (Gallagher *et. al.,* 1993 and Brunner *et. al.,* 1994) whereas in bubaline species it is present at 4q23 (Ianuzzi *et. al.,* 1993). The basic gene structure among the vertebrate lysozyme is remarkably constant (Fig. 1 and 2). Aside of being segmented into four exons from chicken to man (Irwin *et. al.* 1996), all the lysozyme genes contain a pattern of introns very similar in size, if one ignores the number of retroposon elements. Bovine variant of the gene, which is expressed in granulocyte span about 12 kb of genomic DNA along with its promoter. Exon I of size 208 bp encodes complete 5'-UTR, signal peptide and first 27 amino acid residue of mature enzyme. Exon II of size 164 bp has been evolutionary conserved from mice to man. Similarly, Exon III extends the 78 bp in all vertebrate m*Lys*, three bp larger than that of s*Lys*. Exon IV of size 1163 bp encodes for the 21 C-terminal amino acids together with 3'-UTR segment of the mRNA (Steinhoff *et al.,* 1994). Two micro-satellites were also present, first within the intron II and second are present just 3' adjacent to exon III. The bovine m- and s- *lys* differ by less than 200 bp length. Again more than 80% of the intron sequences are conserved to a degree of 74% in between m- and s-lysozyme genes excluding retroposon elements (Henke *et al* 1996). Furthermore, more than 80% of the intron sequences are conserved to a degree higher than 74% in a comparison between the bovine m-Lys and s-Lys gene. The total gene size of lysozyme varies species to species. In ruminants like cattle, buffalo and sheep, cDNA length of this gene is 447 bp. However, in nonruminant, cDNA length varies from species to species. The total gene size and cDNA length of lysozyme in different species are listed in Table 2.

Table 2: Structural organization of macrophage expressed lysozymes in different species

Species	Gene Length (bp)	c DNA Length (bp)	Organization (Exons)	Reference
Human	5856	447	4	Wen and Irwin (1999)
Mouse	-	455	4	Irwin *et al.* (1996)
Rat	-	446	4	Yeh *et al.* (1993)
Pig	6304	441	4	Yu and Irwin (1996)
Chicken	-	443	4	Ruther *et al.* (1982)
Sheep	-	447	4	Wen and Irwin (1999)
Bovine	12039	447	4	Henke *et al.* (1996)
Buffalo	-	444	4	Sahoo *et al.* (2010b)

b. Scope as a candidate gene

As the heritability of the traits of disease resistance is very low, it is very difficult to improve them by traditional selection methods. The marker assisted selection employing DNA markers may aid to the traditional breeding technique in this aspect. Seyfert *et. al.* (1996) while defining lysozymes gene as a candidate gene for mastitis resistance felt the need of i) clearly defined breeding value for mastitis resistance and ii) resource families allowing the genomic mapping of potential candidate genes for this trait. This would require the demonstration that a variation in either quality or concentration of the enzyme correlated with the trait improvement. The genetic polymorphisms under-covering such a variation might be introgressed into elite dairy cattle population for future utilization as selection aids. The strategies should be based on one hand upon segregation analysis of gene variants with inherited parameters of udder health in population studies and selecting for improving gene function. Alternatively the gene can be hyper expressed as trans-gene in mammary gland of model animals (owing to their lower level of expression) under the control of strong lactation specific promoters. Working in this line Maga *et. al*. (2006) reported *in vivo* production of Human lysozyme (hLys) which altered the functional as well as physical properties of milk protein system and the lower Somatic cell count (SCC) suggested the improved udder health. For the efficient marker development the existence of genetically controlled variation as well as correlation of the trait of interest with the lysozyme activity is required. Thus it is an essential prerequisite to study the pattern of variation and the genetic variants responsible for that before utilizing those into the breeding programmes aimed to enhance disease resistance. Lie (1980) studied the genetic variation in the serum lysozyme activity in cattle and revealed that this trait in cattle is influenced at least at 2 different genetic levels. First in the basis of heritability estimates it was clearly observed to be affected by polygenes.

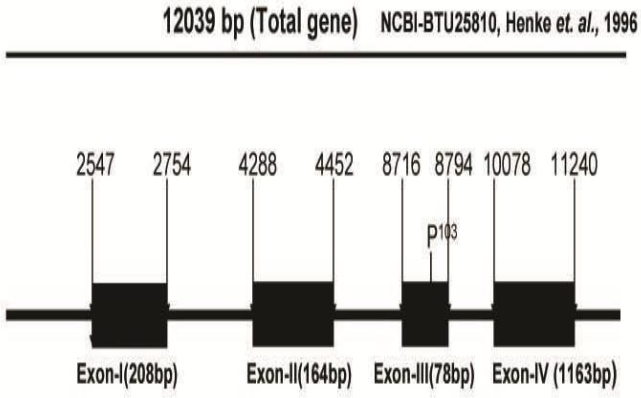

Fig. 1: Structural organization of bovine lysozyme gene.

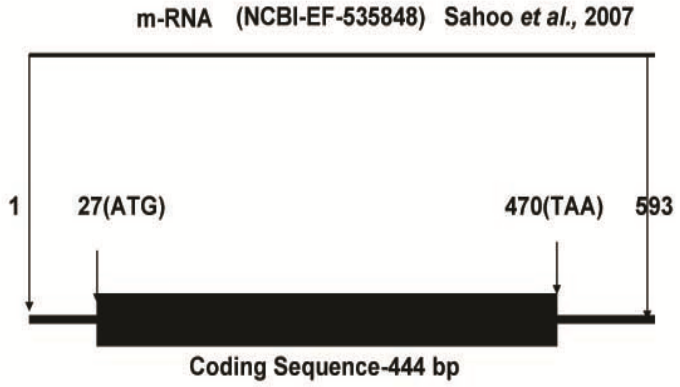

Fig. 2: m-RNA of bubeline lysozyme gene.

Secondly a possible lysozyme controlling major gene was occurring in the population at a very low frequency responsible for the existence of the animals of extremely high lysozyme activity

which can be targeted as a candidate gene. Dayal (2010) reported the differential expression profiling of serum lysozyme gene in Muzaffarnagri breed of sheep which revealed that the animal having AA genotype showed higher expression of serum lysozyme than the animal having AB, AC and BB genotypes.

c. Polymorphism and association studies

As the concentration and/or specific activity of lysozyme varies with species, breed and age (Sotirov *et. al.* 2006) and there is genetic and non genetic factors affecting the variation (Lie, 1980) careful study is highly essential before utilizing its potential for selection purposes. Under this search of variations the nucleotide sequence variation associated with the variation of lysozyme activity may aid to select animals of higher innate immunity. Davies and Maddox (1994) found $(TG)_n$ dinucleotide repeats in DGGE (Denaturing gradient gel electrophoresis) method in ovine lysozyme locus. They observed the data were consistent with 5 co-dominantly inherited alleles designated A to E. Weikard *et. al.* (1996) demonstrated $(TA)_n$ microsatellite within the intron-2 of the immunorelevant bovine lysozyme encoding gene was highly polymorphic. A total of 12 different alleles were observed (196-218 bp) from different bovine breeds. Zabolewicz *et. al.* (2002) genotyped 425 animals and noted that the alleles mLys-mic3 (62.0%), mLys-mic4 (24.5%) and mLys-mic2 (11.5%) were characteristics (frequently found) of Holstein friesian cattle and less frequently observed alleles, mLys-mic 10 (1.1%) and mLys-mic7 (0.9%) which were specific to Polish Black and White population. Pareek *et. al.* (2003) examined genetic polymorphism within the entire coding region and promoter region of mLys gene by comparative sequence analysis in bulls and two progenies exhibiting low serum lysozyme activity. However, two SNPs were detected in intron II and III of the gene at positions 8603 and 9963, PCR RELP assay was developed to detect C/T transition in intron-II creating a polymorphic Sau 3A restriction site. Prusinowska *et. al.* (2003) studied the different allelic variants of immunorelevant serum lysozyme gene (mLys-mic-7 and mLys-mic-3) showing co-segregation of high serum lytic power with mLys-mic-7 marker allele using two substrates *Micrococcus lysodiktycus* (ML) and p-Nitrophenyl-penta N-acetyl-β-chitopentaside (PNP-GlcNAc).

Analysis of restriction fragment length polymorphism (RFLP) of the lysozyme gene cluster was performed in a Norwegian bovine family segregating a single dominant Mendelian factor for high lysozyme activity in serum. (Olsaker *et. al*.,1994). Sigurdardottir *et. al*. (1990) studied genetic polymorphism of bovine lysozyme (LYZ) genes by analysing restriction fragment length polymorphism (RFLP). The analysis revealed three RFLP loci designated LYZ1, LYZ2 and LYZ2. Each system included two or three allelic variants. Evidence for close genetic linkage of the three loci was found. There was also significant linkage disequilibrium among the three loci in a sample of about 200 breeding bulls from one breed. No statistically significant association was found between LYZ RFLPs and breeding values of bulls for disease traits.

Our group has studied the immuno-relevant lysozyme gene (Sahoo, 2007) of *Bubalus bubalis* (Indian river buffalo) in four well known milch breeds with the objectives to identify single nucleotide polymorphic patterns and their effects on the somatic cell count and the serum lysozyme activity as well as to characterize the cDNA. A total of 280 riverine buffaloes maintained at different farms, were screened for SNP by SSCP technique at two separate genomic regions of length 276 bp (partial intron II, complete exon III and partial intron III) and 230 bp (partial intron III and partial exon IV) in length. The lysozyme gene in both the regions is found to be polymorphic. The milk somatic cell count was estimated by using Newman's Lampert stain. The mean somatic cell count in Murrah buffalo milk maintained at IVRI farm was found to be 1.25×10^5 cells per ml of milk. Although the mean values for SCC and serum lysozyme activity varies apparently, no significant differences among the group means of different genotypes have been observed (Sahoo *et al* 2010a). Upon sequencing, the 276 bp fragment was found to have differences in terms of nucleotides at 13 positions where as, in case of 230 bp fragment it was found at 4 positions among the

variants. In another study (Dayal, 2010) involving three Indian breeds of sheep viz. Chokla, Malpura, and Muzaffarnagri, two gene fragments i.e., 268bp fragment (partial promoter, exon 1 and partial intron1) and 275bp fragment (partial intron I, exon II and partial intron II) of macrophage expressed lysozyme gene were amplified and subsequently, the SSCP study was carried out to identify different allelic patterns and genotypes. Both fragments of serum lysozyme gene were found to be polymorphic in all the three breeds of indigenous sheep. Number of genotypes and alleles varied from breed to breed for both the fragments. All the six alleles were present in Chokla and Malpura where as only four alleles A, C, D and F were present in the Muzaffarnagri breed of sheep. In both fragments, A allele was pre dominant in all the three breeds of sheep. The least square analysis was done to study the association of breed as well as genotype with mean serum lysozyme activity. There was significant difference ($P \leq 0.05$) of serum lysozyme activity among the breeds. Among all the three breeds, Chokla showed highest mean serum lysozyme activity. The mean serum lysozyme activity in sheep breed was 2.68±0.09 µg/ml. Genotypes of first fragment showed significant ($P \leq 0.05$) association with mean serum lysozyme activity in Chokla breed of sheep where as genotype of second fragment showed significant associations ($P \leq 0.05$) with mean serum lysozyme activity in Malpura and Muzaffarnagri breeds of sheep. Nucleotide sequencing of samples showing different genotypic patterns showed differences at nucleotide level between different alleles. Different alleles of fragment 1 showed variations at 4 places; where as various alleles of fragment 2 showed variations at 8 places. Differential expression profiling of serum lysozyme gene in muzaffarnagri breed of sheep revealed that the animal having AA genotype showed higher expression of serum lysozyme than the animal having AB, AC and BB genotypes.

Olsakar *et. al.* (1994) reported high lysozyme activity locus in a Norwegian bovine family co-segregates wih RFLP sites with allelic bands of 10 kb and 5.9kb, with a lod score of 6.8 at a recombination frequency of 3.4% which can be used as a genetic marker for high lysozyme activity. Klussmann (1998) studied the association of somatic cell count with lysozyme gene polymorphism and did not get any significant result. Klossowska (1981a) studied the influence of udder health on the lysozyme activity of cow milk and found only in case of infection due to *streptococcus* and *staphylococcus* there was increase in lysozyme activity. He also reported significant positive correlation between milk lysozyme activity with that of serum and somatic cell count. (Klossowska 1981b)

6. Lysozyme in disease biology

a. As a disease marker

The lysozyme activity reflects the homeostatic expression of RES (Reticulo-endohelial system), which is one of the most fundamental defence mecganism against infection (Lie, 1980). Experimental findings have produced evidence for lysozyme being an index of macrophage functional status (Di Luzio, 1979). Recently Torsteinsdoffir *et. al.* (1999) described the serum lysozyme as a potential marker of monocyte/ macrophage activity in rheumatoid arthritis. The milk lysozyme activity has been suggested as a potential diagnostic marker of sub-clinical mastitis (Priyadarsini and Kansal, 2002b; Farid *et. al.*, 1984 and Grun, 1985). The bovine milk normally contains very low levels of lysozyme (i.e. 0.15 µg /ml). However, mastitic milk contains higher concentrations (1-25 mg/ml). Serra *et. al.* (2002) suggested the role of this milk protein as a new prognostic factor in patients with breast cancer in human beings.

b. Antibacterial activity

Vakil *et al.* (1969) found that several Gram +ve and Gram –ve bacteria were susceptible to different degree of purified lysozyme from bovine and human milk.The antibacterial role appears to be mediated through direct bacteriolytic action as well as via stimulatory effects on the phagocyte function of macrophage and PMN leucocytes (Klochars and Roberts, 1976). Different mechanisms are responsible for the bacterostatic, lytic and bactericidal properties of lysozyme. Lysozyme may act in association with complement system and lactoferrin which inhibits the growth of certain microorganism by chelating iron. Ogundele (1998) reported the anti-inflammatory activity of

human lysozyme and modulation of serum complement activation by an inhibition of PMN response towards complement derived chemotaxins. Leo *et al.* (1978) reported lysozyme dampens several responses of neutrophils to inflammatory stimulants.

Action against Gram +ve bacteria

The cell wall of Gram +ve bacteria is composed of thick layer of peptidoglycan with embedded chains of teichoic acids and lipoteichoic acids (Fig. 3). Lysozyme brings about the lysis of certain bacteria by altering the properties of the surface structures on the cell. Early observations of the microscopic sequence of changes affected by lysozyme from several different sources have shown that there is a marked swelling of the cells before cell lysis and this swelling is due to an alteration of the cell wall (Salton, 1957). In order to study the mechanism of action of lysozyme, he used three Gram +ve bacteria to show that lysozyme completely digested the bacterial cell wall. When *Micrococcus lysodeikticus, Sarcina lutea,* and *Bacillus megaterium* were digested with lysozyme, there was liberation of reducing and acetyl amino sugars (Salton, 1957). However, research has also shown that the killing mechanism of Gram +ve bacteria is independent of enzymatic activity but attributed mainly to the cationic and hydrophobic properties of lysozyme (Pellegrini *et al*. 1992). These studies have shown that enzymatic and lytic activity are not linked with each other as denatured lysozyme lacking in enzymatic activity was still able to inhibit bacterial growth (Pellegrini *et al.* 1992; Ibrahim *et al*. 2001).

Action against Gram -ve bacteria

The chemical composition of the cell walls of Gram negative bacteria differs from that of Gram positive bacteria. This is composed of two layers (Fig. 4). The inner layer is composed of a single layer of peptidoglycan without teichoic acid and an outer layer is composed of thick layer of lipopolysaccharide. Most gram-negative bacteria are not susceptible to the action of lysozyme alone because their outer membrane prevents access of the enzyme to the peptidoglycan layer (Salton, 1958). However, this barrier has been overcome in the innate immune systems of animals by the production of accessory antibacterial proteins which permeabilize the outer membrane, such as lactoferrin. In addition, some natural lysozymes as well as chemically or genetically modified hen egg white lysozyme (HEWL) have been reported to be active against gram-negative bacteria even in the absence of such permeabilizers (Callewaert *et. al.* 2008).

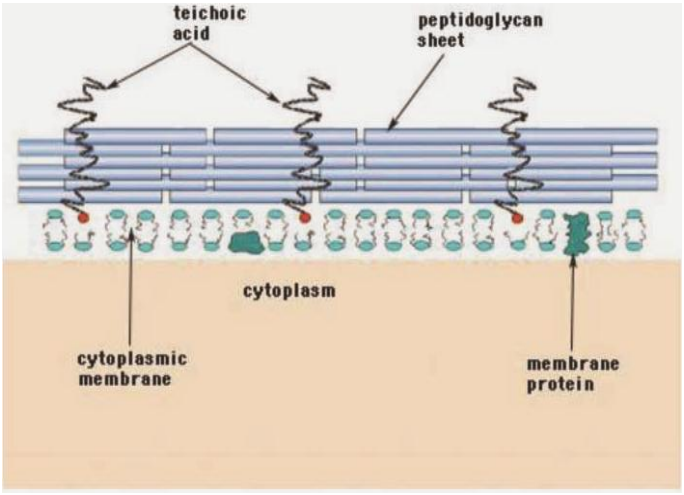

Fig. 3: Structure of the Gram-positive bacterial cell wall.

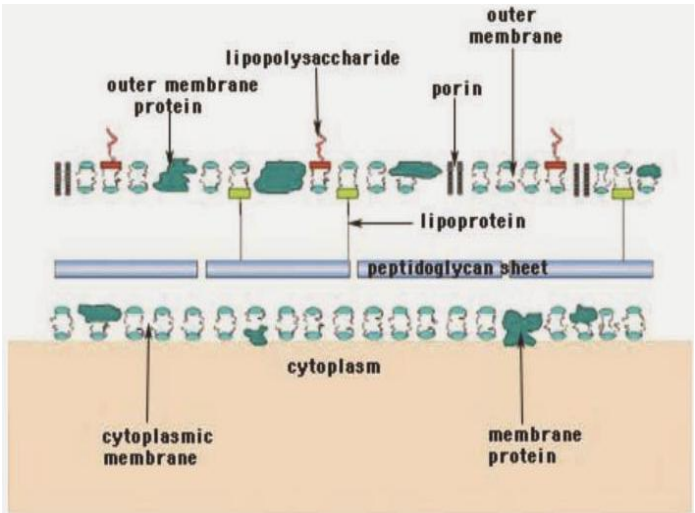

Fig. 4: Structure of the Gram-negative cell wall.

Early investigation with certain Gram negative bacteria showed that when isolated cell walls of certain Gram–negative bacteria were incubated with lysozyme, only small decrease in turbidity was detected. The soluble, non-dialyzable components which were released by lysozyme consisted of alanine, glutamic acid,

diaminopimelic acid (DAP) and glucosamine as the predominant substances, smaller amounts of muramic acid, and few other amino acids (Salton, 1958).

Recent studies showed that the action of lysozyme on Gram negative bacteria did not depend on its enzymatic activity but depend on its structural phase transition. A specific bacterial domain, namely residue 98-112, which is known to be involved in the antimicrobial action of lysozyme against Gram positive and Gram negative bacteria (Ibrahim *et. al*., 2001). It has also been demonstrated that the antimicrobial activity of lysozyme was independent of its muramidase activity (Ibrahim *et. al.,* 2001b). Heat-denatured hen egg white lysozyme (HEWL) was investigated for its bactericidal activity and it was found that after denaturation, there was an enhancement in the bactericidal activity towards Gram negative bacteria with a partially unfolded, enzymatically inactive and hydrophobic form of lysozyme (Ibrahim *et. al.,* 2001). The bactericidal activity was due to the membrane insertion of the dimeric form of lysozyme which led to membrane disruption. The bactericidal activity in this case could be uncoupled from the enzymatic activity (Ibrahim *et. al.,* 2001; Pellegrini *et. al*., 1992).

c. Immuno-modulation

Jolles (1976) suggested a possible role of lysozyme on immuno-stimulation. He suggested simple and incomplete digested or split products (from peptidoglycans) as well as similar compounds have a somewhat higher molecular weight. They belong to a group of naturally widespread substances which are able to exert an adjuvant or immno-stimulating activity with stimulation of production of antibody against variety of antigens, induction of delayed type of hypersensitivity to same or other antigens, enhancement of disease resistance against bacterial or viral infection and mitogenic activity etc.

d. Role in udder health

The enzyme lysozyme is one of the factors of the non-specific defense mechanism of the mammary gland. It represents a regular constituent of milk, which despite its very low content in milk determines the health condition of the udder and its defending ability against infectious agents (Grun, 1985). It is considered to be a constituent of primitive nonspecific defense mechanisms associated with the monocyte-macrophage system phylogenetically older than the more specific lymphocyte-plasma cell-immunoglobulin system. Lactoferrin, lysozyme and the lactoperoxidase-thiocyanate-peroxide-system are naturally occurring antimicrobial components of milk (Schonedf, *et. al.,* 2002). Klossowaka *et. al.* (1996) reported improvement of chronic mastitic cows by using lysozyme along with Ampicillin and Neomycin. However, it was pointed out that in most case molecular pathological studies have not yet been performed.

e. Other therapeutic role

Lysozyme was claimed to relieve rheumatic fever and had a therapeutic effect on rheumatoid arthritis. Some analgesic properties were also been reported (Di Pietro and Pellegris, 1963). Lysozyme almost exclusively from hen egg white has been used either alone or in addition to a series of other compounds such as various antibiotics (Tetracycline, Bacitracin), enzymes (amylase, papaine), vitamins etc. The presence of lysozyme was claimed (Saubusse, 1976) to be efficient in treatment of various bacterial, viral (Zona Herpes zoster), infectious colitis, various pains, allergies, inflammations as well as pediatrics (maternization of cow milk by addition of lysozyme). Oral Lysozyme supplementation in weaned piglets prevents diarrhoea even in the experimental challenge by Escherichia coli $K88^{+}$ (ETEC).

f. Lysozyme resistance in bacteria

In view of the widespread occurrence and effectiveness of lysozymes as antibacterial agents, it is not surprising that bacteria have in turn evolved mechanisms to evade or subvert this threat. Bacteria colonizing or infecting an animal host have developed various ways to overcome lysozyme action. A bacterial lysozyme resistance mechanism that has been known for long is peptidoglycan modification. However, a recently proposed mechanism being the production of lysozyme inhibitors which are under study. The only high affinity bacterial lysozyme inhibitor known thus far is produced only in few bacteria,

and this raised questions about their wider relevance in bacteria–host interactions. Callewaert *et. al*. (2008) reported the discovery of a novel and distinct family of bacterial lysozyme inhibitors that is widely distributed among the proteobacteria, including several major pathogens. The family comprises periplasmic as well as membrane-bound inhibitors, and both types contribute to lysozyme tolerance of bacterial cells. Interestingly, a gene encoding one of the newly identified inhibitors has been previously found to promote macrophage survival of *Salmonella typhi*.

g. Recent biotechnological approach to address disease problem

Mastitis is the most costly disease in the dairy industry, with over 1.7 billion dollars a year in losses in the US alone (Maga, 2005). Of all the bacteria that can enter the udder and cause mastitis, *S. aureus* is not only most prevalent, but also the most difficult to treat. Owing to the physiology of S. aureus, there is no effective treatment to completely get rid of infection. (Maga, 2005) The approach here is to find out the cause of variation in terms of quality of enzyme from the variation in the coding nucleotide sequence and amino acid sequence, and to utilize the type with better antimicrobial properties either in natural form or in recombinant form. Maga et. al. (2006) developed a line of transgenic goats as a model for dairy cow designed to express human lysozyme gene in the mammary gland. The milk from the transgenic animals had a lower somatic cell count with the level of milk production remaining constant. Tsuchiya et. al (2008) in 13th International Society for Infectious Diseases Conference, reported Production of Recombinant Porcine Lysozyme with the genetic engineering technique. This modified PLY gene (length: 484bp) was synthesized with 9 short DNA oligomers by SPR method and PCR extension, and expressed in insect cell line (expres SF+) using baculovirus expression system. The efficiency of recombinant PLY production was high (more than 50mg/L) in the expres SF+ cell culture fluid. The product was purified and analyzed its molecular weight by mass spectrometry and N-terminal sequence by peptide sequencer. The results proved that the expressed recombinant PLY was the same as the natural type. When having compared it with human lysozyme and chicken egg white lysozyme, PLY showed stronger lysing activity against M. lysodeikticus than human and chicken egg white lysozyme in the high salt or high pH conditions. Chang *et al.* (2006) reported a gene therapeutic strategy for the treatment of chronic bovine mastitis. They have developed a mammary specific vector containing human lysozyme (hLys) cDNA and kanamycin resistant gene (as marker) and conducted clinical trials. Clinical studies revealed that twice acupuncture of quarters with the vector had overt therapeutic effect on clinical and sub clinical mastitis previously treated with antibiotics, including disappearance of clinical symptoms and relatively high microbiological cure rates.

7. Conclusion and research prospective

Though lysozyme research crossed over 90 years several things are yet to be done to exploit this enzyme fully. As the lysozyme activity reflects the homeostatic expression of RES (Reticulo-endohelial system), being an index of macrophage functional status,the recent era of molecular biology/ biotechnology gives better opportunity to use it as genetic marker in the selection for innate immunity by screening the presence of genotypes with higher lysozyme activity as an indicator of better resistance to bacterial pathogens in the elite herd of animals. An alternative gene therapeutic approach is also possible for treatment of mastitis by hyper-expressing the transgene either native or in recombinant form to get the gene product of higher antibacterial activity. However, all this experiments are at the beginning stage and the research on this area is highly needed for a fruitful method for selection for disease resistance in livestock. Besides the above focal points, the following issues can be considered as promising research prospective in this area.

1. Although the role of lysozyme in antibacterial defence is now well established in some experimental models (e.g. using lysozyme knockout animals), a more detailed insight in the spatio-temporal expression of lysozyme and the

precise contribution of lysozyme to antibacterial defence in different tissues is lacking.

2. Lysozyme specific peptidoglycan fragments released by the action of lysozyme may have immune-modulatory action as well as role in inflammatory pathways by binding to molecular pattern receptors of the innate immune system. Detailed study in this regard may help to exploit this enzyme fully.

3. Lysozyme resistance mechanisms such as O-acetylation and N-deacetylation of peptidoglycan and the production of lysozyme inhibitors possibly allow bacteria (in particular, pathogens) to evade lysozyme antibacterial activity. The widespread occurrence of lysozyme inhibitors in bacteria is likely to reflect their functional importance in a wide range of bacteria–host interactions. They can be used as attractive novel targets for antibacterial drug development.

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