African Journal of Biotechnology Vol. 7 (25), pp. 4856-4867, 29 December, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Review

Antimicrobial activity of lysozyme with special relevance to milk

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Accepted 5 December, 2008

Lysozyme is a hydrolytic enzyme which has been purified from cells, secretions and tissues of virtually all living organisms and viruses. While this protein has been recognized to possess many physiological and functional properties, its high microbicidal activity remains, by far, the main virtue that explains the high attention of scientists and industrial stakeholders for its practical applications in medicine and food industry. Although the egg-white is the primary source for lysozyme production at industrial scale, other sources such as milk of mammals should not be overlooked, as they may contain lysozyme molecules with specific properties not present in the conventional egg-white lysozyme. This review discusses the antimicrobial activity of lysozyme with special emphasis on milk's lysozyme, and attempts to shed some light on the recent advances elucidating the mechanism of its antimicrobial activity against sensitive microorganisms as well as the means used by some bacteria to resist such an activity.

Key words: Lysozyme, antimicrobial activity, mechanism of action, resistance, milk.

INTRODUCTION

Milk is a complex medium containing a variety of nutrients, minerals, vitamins as well as other molecules of functionnal or bioactive properties. In particular, milk is rich in proteins that are classically grouped into two main classes: (i) major milk proteins including caseins (α, β and κ-caseins) and two whey proteins (i.e., α-lactalbumin and β-lactoglobulin), and (ii) minor milk proteins including lysozyme, lactoferrins, lactoperoxidase and immunoglobulins. Although the constituents represent only a minor fraction of milk proteins, they play an important role as first line defense due to their direct and indirect antimicrobial activity (Lonnerdal, 2004; Séverin and Wenshui, 2005; León-Sicairos et al., 2006) in addition to other important physiological and health promoting functions (Gorbenko et al., 2007).

Lysozyme is among the minor milk proteins that has attracted increased attention recently due to its potent antimicrobial activity against a wide range of microorganisms and hence potential in food preservation and safety. This review will focus on the antimicrobial activity of lysozyme with special reference to the milk of different mammals. The mechanisms of action and resistance of/to lysozyme will also be discussed.

LYSOZYME: A BACTERIAL CELL-WALL HYDROLASE (BCWH)

Also called N-acetylmuramidase or muramidase, lysozyme is a hydrolyse-type enzyme that catalyses the breakdown of peptidoglycan polymers of bacterial cell wall at the β1-4 bond between N-acetylmuramic (NAM) acid and N-acetylglucosamine (NAG) residues, thereby lysing sensitive bacteria. Lysozyme was first discovered by Flemming (1922) in the nasal mucus and subsequently purified from various plant, animal, microbial (bacteria, virus and fungi) materials (Masschalck and Michiels, 2003; Xue, 2004; Parisien et al., 2007).

However, the chicken egg white remains by far the richest source of this enzyme with a concentration ranging between 3400 and 5840 mg/L (Wilcox and Cole, 1957; Sauter and Montoure, 1972). Due to the wide variability in origin, and structural, antigenic, chemical and enzymatic properties of lysozyme molecules, they have been classified in different classes/types. The most studied and the best known is the conventional or chicken-type (i.e., c-lysozyme) with the lysozyme derived from the egg white of domestic chicken (*Gallus gallus*) as

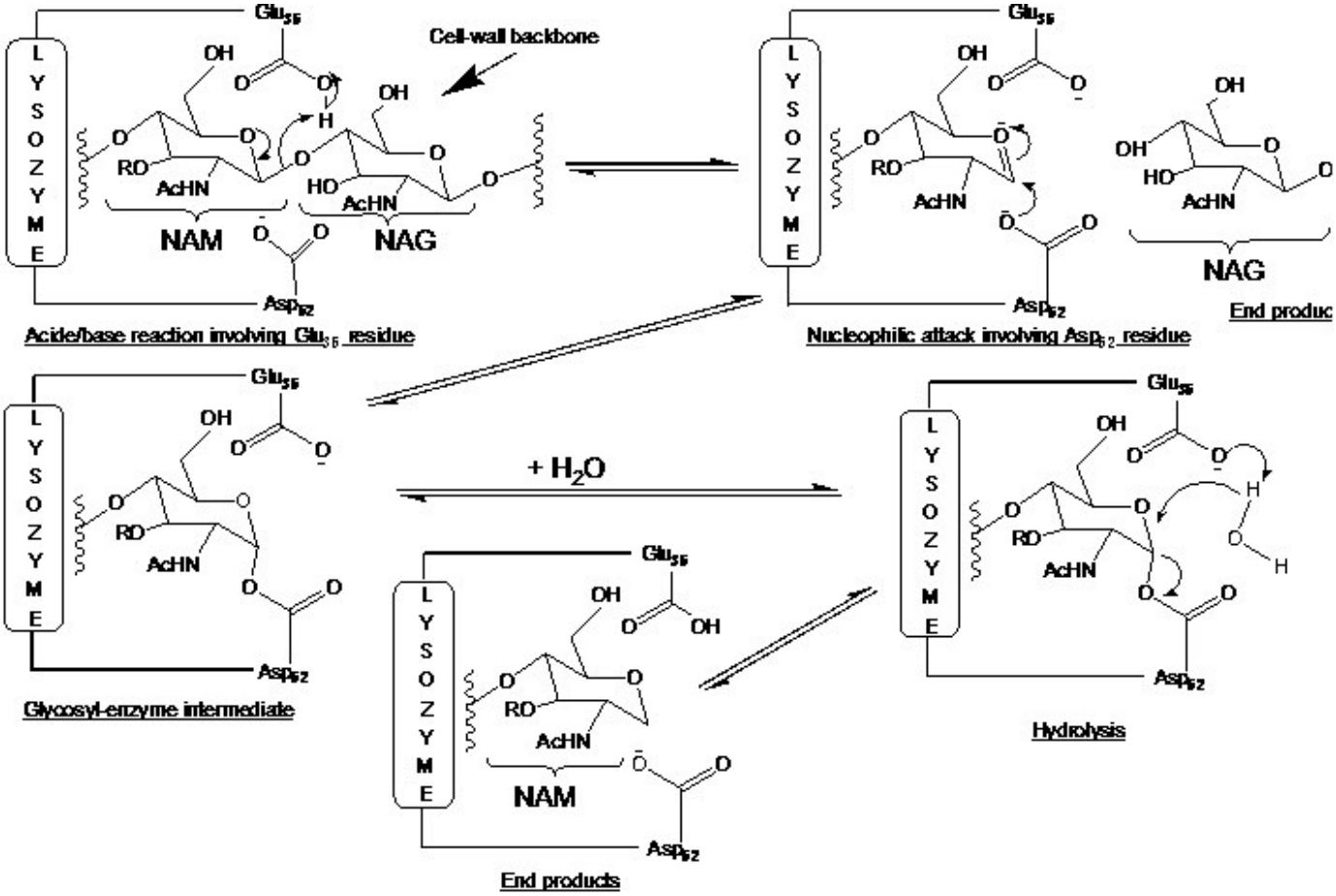

Figure 1. Hydrolytic mechanism of action of lysozyme on β1-4 linkages between N-acetylmuramic (NAM) acid and N-acetylglucosamine (NAG) residues of the bacterial cell-wall backbone. The mechanism shows the implication of Glu_{35} and Asp_{52} of chicken egg white lysozyme in the breakdown reaction (Vacadlo et al., 2001).

the prototype (Prager and Wilson, 1974; Kuroki et al., 1999). Although, c-lysozyme is typically found in the egg white of birds, it was also purified from various tissues and secretions of mammals including milk, saliva, tears, urine, respiratory and cervical secretions (Chandan et al., 1964; 1968; Halper et al., 1971; Jollès et al., 1990; El Agamy et al., 1996; El Agamy, 2000; Moroni and Cuccuri, 2001; Masschalck and Michiels 2003; Parisien et al., 2007). Other types of lysozyme are also known; and they include the *g*-type derived from the egg white of domestic goose (*Anser anser*), h-type lysozyme from plant, i-type from invertebrates, b-type from bacteria (*Bacillus*) and *v*-type from viruses (Meyer et al., 1946; Prager and Wilson, 1974; Sinnott, 1990; Heinz et al., 1992; Bachali et al., 2004; Xue 2004). Despite the variability in the amino acid composition and sequence of lysozyme molecules, amino acids of the catalytic centre of the active site are well conserved to keep the hydrolytic function of the enzyme (Prager et Wilson, 1974). In particular, glutamic acid and aspartic acid residues are directly involved in the breakdown of the glycosidic bond between NAG and NAM and their presence in the catalytic centre is thus crucial for the hydrolytic activity of the enzyme. Glutamic acid acts as a proton donor through the free carbonyl group of its side chain, while aspartic acid acts as a nucleophile to generate a glycosyl-enzyme intermediate, which then reacts with a water molecule to give the product of hydrolysis and release the enzyme unchanged (Vocadlo et al., 2000) (Figure 1). However, the amino acid sequence of known lysozymes reveals that aspartic acid is not consistently present in the active site of lysozyme molecules (Kuroki et al., 1999). In contrast, substitution of glutamic acid has resulted in a complete inactivation of the enzyme (Kuroki et al., 1999) confirming the critical role of this amino acid in enzymatic activity of lysozyme regardless of its origin or the class to which it belongs.

ANTIMICROBIAL ACTIVITY OF LYSOZYME

The antibacterial activity of milk lysozyme as part of the unspecific innate defence mechanism is well established. It acts either independently by lysing sensitive bacteria or as a component of complex immunological reactions to

enhance the phagocytosis of bacteria by macrophages (Varaldo et al., 1989). It thus contributes to the innate protection from microbial infections while still in the udder and delay milk spoilage after drawing (i.e., bacteriostasis period). The antimicrobial activity of lysozyme has extensively been demonstrated *in vitro* or in physiological fluids and secretions including milk, blood serum, saliva, and urine (Martinez and Carroll, 1980; Lee-Huang et al., 1999; Sexton et al., 1996). Although, lysozyme has been shown to have antimicrobial activities towards bacteria, fungi, protozoan and viruses (Reddy et al., 2004; Wang et al., 2005; Lee-Huang et al., 2005), it is essentially known for its antibacterial activity and has been used, on this basis, in food preservation.

Gram-positive bacteria: sensitivity and resistance to lysozyme

Antibacterial activity of lysozyme is essentially directed towards gram-positive bacteria, as their target cell-wall component (peptidoglycan) is freely accessible to the enzyme, contrary to that of gram-negative bacteria, which is shielded by the lipopolysaccharidic (LPS) layer of the outer membrane (OM). Nevertheless, recent studies suggest that resistance of bacteria to lysozyme is not exclusively related to the presence of the LPS layer.

The occurrence of resistant gram-positive bacteria indicates that the lack of the LPS does not expose *de facto* the bacterium to lysozyme hydrolysis (Hayashi et al., 1973; Kihm et al., 1994; Vollmer and Tomasz, 2000; Masschalck et al., 2002; Bera et al., 1007; Veiga et al., 2007). Conversely, the presence of the OM in gram-negative bacteria does not provide them with an absolute protecttion against the hydrolytic action of lysozyme; sensitive gram-negative bacteria have been described (Vakil et al., 1969; Ellison and Giehl, 1991; Pellegrini et al., 1992). In fact, the exact mechanism of lysozyme resistance is not fully understood and may vary according to the bacterial strain or species.

Various mechanisms of resistance in gram-positive bacteria have been suggested: (i) hindrance of lysozyme action by surface attachment polymers (e.g. capsular polysaccharides and teichoic acids), (ii) high degree of peptide cross-linking, (iii) *O*-acetylation of hexosamine residues of the cell-wall peptidoglycan, (iv) N-deacetylation of the acetamido group of the hexosamine residues, (v) incorporation of D-Aspartic acid in the bacterial peptidoglycan crossbridge as was demonstrated in *Lactococcus lactis* (Veiga et al., 2006) and (v) production of protein-inhibitors specific to lysozyme (Bera et al., 2007). However, the latter three mechanisms have attracted increased research attention in recent years, while there is a general agreement that surface attachment polymers and the degree of peptide cross-linking do not account for lysozyme-resistance.

Comparison of teichoic acid content in the cell-wall did not reveal significant differences between sensitive and resistant gram-positive bacteria; strains with high content of teichoic acid were shown to have similar or greater susceptibility to lysozyme than those devoid or with low content of this cell-wall component (Hayashi et al., 1973, 1973; Bera et al., 2007). Furthermore, *in vitro* removal of teichoic acid and/or capsular polysaccharides did not sensitize resistant strains of *Staphylococcus aureus* (Araki et al., 1972; Hayashi et al., 1973). As for capsular polysaccharides, they were shown to be degraded by lysozyme, since they share structural similarities with the cell-wall peptidoglycan including the presence of glycosidic bonds susceptible to interact with lysozyme (Salton, 1957; O'Riordan and Lee, 2004). On the other hand, studies have provided evidence that the modification of hexosamine residues of the glycan backbone, by *O*-acetylation or N-deacetylation, is the primary mechanism of resistance to lysozyme in gram-positive bacteria. Clarke and Dupont (1991) were the first to suspect a possible role of *O*-acetylation of the peptidoglycan muramic acid in lysozyme resistance. This observation was later substantiated by the characterisation of membrane-bound *O*-acetyltransferase A (OatA) that catalyses the insertion of acetyl group at the C_6-OH position of muramic acid in the cell-wall of resistant staphylococci (Bera et al., 2005; Veiga et al., 2007).

A survey on the prevalence of lysozyme resistance among staphylococci revealed that only pathogenic strains were resistant to lysozyme, and muramic acid of the peptiodoglycan of all resistant strains was *O*-acetylated, while all the non-pathogneic strains were sensitive and their muramic acid was not acetylated (Bera et al., 2006). Lysozyme-resistance was thus considered as a virulence factor directly related to the expression of *OatA* gene coding for the *O*-acetyltransferase A. Moreover, non-pathogenic lysozyme-resistant mutants could be produced by transformation of *oatA* gene from resistant *S. aureus* into sensitive *Staphylococcus carnosus*. Conversely, *oatA* gene was inactivated by deletion trials with a consequent sensitization of lysozyme-resistant strains, but these mutant strains have recovered their resistance upon complementation trials (Bera et al., 2007). These experiments (Bera et al., 2006; 2007) have provided an evidence for the role of *O*-acetylation of the peptidoglycan in lysozyme resistance in staphylococci suggesting that the same mechanism of resistance may prevail in other bacterial genera but is as yet to be demonstrated (Calleweart et al., 2008).

As for the modification of the glycan hexosamines by *N*-deacetylation, early observation that acetamido group of NAG is essential for lysozyme in order to react with the peptidoglycan (Phillips, 1967) provided a clue that such a modification may also play a role in lysozyme resistance among gram-positive bacteria. Subsequently, Araki et al. (1972) and Hayashi et al. (1973) have determined a high positive correlation between the content of *N*-unacetylated glucosamine residues in the cell-wall peptidoglycan and resistance to lysozyme; they could also convert *in vitro*, resistant peptidoglycan into a sensitive form by chemical

N-acetylation of its glucosamine residues. Vollmer and Tomasz (2000) have demonstrated that the formation of free amino groups (unacetylated) from NAG of the peptideglycan of lysozyme-resistant *Str. peumoniae* is catalysed by an innate peptidoglycan *N*-acetylglucosamine deacetylase A (pgdA) coded by *pgdA* gene, and that the inactivation of *pgdA* gene produced hypersensitive transformants with fully *N*-acetylated peptidoglycan.

Another mechanism of resistance to lysozyme has recently been described in group A streptococci, and consists in the production of an inhibitory protein that binds specifically to lysozyme acting as an inhibitory effector (Fernie-King et al., 2002). This protein was first designated as streptococcal inhibitory complement (SIC) as it had been known to act as an inhibitor of the complement system before its anti-lysozyme activity was elucidated. However, the SIC is not highly specific to lysozyme and binds to other components of the immune system (e.g., secretory leukocyte proteinase inhibitor and β-defensins) with higher affinity than to lysozyme (Fernie-King et al., 2002; 2007). Protein inhibitors more specific to lysozyme may be produced by gram-positive bacteria to resist the hydrolytic action of the enzyme, but remained to be identified and characterized. It is clear, therefore, that there is not only a single mechanism of resistance for all gram-positive bacteria, but one or more mechanisms may be used by specific strains or species. Deacetylation of the amino group of NAG residues appears to be a common mechanism of resistance in *Bacillus* and streptococci (Hayashi et al., 1973; Vollmer and Tomasz, 2000) while other bacteria (e.g., *S. aureus,* lactobacilli) would counteract lysozyme action essentially by means of *O*-acetylation (Hayashi et al., 1973; Bera et al., 2007).

Moreover, the above mechanisms may be modulated by other factors not directly involved in lysozyme resistance. For example, the presence of techoic acid or high degree of peptide cross-linking though shown not to have an intrinsic effect on lysozyme resistance, has enhan ced significantly the effect of *O*-acetylation on lysozyme resistance in *S. aureus* (Bera et al., 2007).

Gram-negative bacteria: sensitivity and resistance to lysozyme

Gram-negative bacteria are generally resistant to lysozyme due to their LPS layer acting as a physical barrier that prevents the access of lysozyme to the target peptiodoglycan. The prevalence of this mechanism of resistance is well established and has been extensively evidenced by the sensitization of gram-negative bacteria upon disruption or permeabilization of their LPS layer (Vaara, 1992; Masschalck and Michiels, 2003). However, the occurrence of gram-negative bacteria naturally sensitive to lysozyme (Repaske, 1956; Wolin, 1966; Ellison and Giehl, 1991; Pellegrini et al., 1992) suggests that LPS may not be the only protective means against lysozyme action, and that other mechanisms, not hindered by LPS, may exist. As a matter of fact, both assumptions have been scientifically substantiated (Monchois et al., 2001; Masschalck and Michiels, 2003; Callewaert et al., 2008). Besides the protective effect of LPS against the hydrolytic action of lysozyme, gram-negative bacteria have recently been shown to use another strategy involving specific protein-inhibitors with high affinity to lysozyme (Monchois et al., 2001). In the course of a systematic survey of *Escherichia coli* genes of unknown functions (i.e., ORFan genes), Monchois et al. (2001) identified a *ykfE* gene coding for a protein-inhibitor specific to the c-lysozyme, and the gene product has thus been designated "inhibitor of vertebrate lysozyme" (Ivy) (Swiss-Prot, accession number P45552). Ivy was further characterized as a homodimeric periplasmic protein with ~30 kDa molecular weight (i.e., 15 kDa each monomer) that binds strongly (dissociation constant of 1 nM) and specifically to c-lysozyme in a key-lock type interaction through a conserved CKPHDC protruding loop, thereby causing complete inactivation of lysozyme (Monchois et al., 2001). A survey on the prevalence of Ivy homologues among bacteria revealed that Ivy represents a family of protein-inhibitors occurring mostly in members of the Proteobacteria (gram-negative bacteria) of the beta and gamma divisions, and exceptionally in the alpha division (Abergel et al., 2007). In the gamma division, Ivy homologues were found in all members of the *Enterobacteriaceae* family except in *Salmonella*, but in all *Pseudomonadaceae*. In the beta division, Ivy homologues were present in all *Burkholderia* members, but only in one species of *Neisseriaceae* (Abergel et al., 2007). At the functional level, Ivy does not substitute the LPS as the primary mechanism of resistance to lysozyme but rather plays a subsidiary role, as it is expressed only when the outer membrane becomes porous as a result of exposure to damaging agents (Callewaert et al., 2005; Abergel et al., 2007). More recently, Callewaert et al. (2008) described a novel family of protein-inhibitors specific to c-lysozyme which is widely distributed among Proteobacteria, yet structurally and phylogenetically distinct from Ivy. This newly-discovered family of protein-inhibitors comprises periplasmic as well as membrane-bound lysozyme inhibitors that were accordingly named periplasmic lysozyme inhibitor of c-lysozyme (PliC) and membrane-bound lysozyme inhibitor of c-type lysozyme (MliC). PliC, a protein purified from the periplasmic extract of *S.* Typhimurium was found, by an iterative search for sequence homologies using appropriate searching protein databases, to be present in all *Salmonella* serotypes. On the other hand, MliC was isolated from *E. coli* and *Pseudomonas aeruginosa,* and characterized as lipoproteins anchored to the luminal face of the outer membrane (Callewaert et al., 2008). These newly identified protein-inhibitors share structural homologies with each other and with the COG3895 structural motif with unknown function that characterizes

hypothetical periplasmic proteins or lipoproteins of the proteobacteria, but they differ markedly from Ivy in their amino acid sequences and three-dimensional structure (Tokuda and Matsuyama, 2004; Narita and Tokuda, 2007; Callewaert et al., 2008). In addition, they are more specific to c-type lysozyme than Ivy which was shown to bind also to g-type lysozyme (Callewaert et al., 2005). It is worth mentioning, however, that amino acid sequences of the protein moiety differ among MliC molecules themselves depending on the producer micro-organism. For example, protein moieties of two MliC molecules purified from *E. coli* or *P. aerugionosa* were found to share only 38% sequence homology (Callewaert et al., 2008). Contribution of the novel lysozyme inhibitors (PliC and MLiC) to lysozyme resistance was further demonstrated in presence or absence of lactoferrin as a membrane-permeabilizing agent; however, PliC and MliC-deficient mutants of *Salmonella enteridis* and *E. coli*, respectively could resist lysozyme hydrolysis as long as their LPS layer is not damaged (in absence of lactoferrins) (Callewaert et al., 2008). Therefore, like Ivy, these protein-inhibitors would play a subsidiary role in lysozyme resistance and act only on strains with damaged outer membrane.

Apart from the production of inhibitory proteins specific to lysozyme, occurrence of natural sensitivity in gram-negative bacteria suggests that lysozyme may use other means than the hydrolytic activity to inhibit sensitive bacteria. In fact, an increasing body of evidence supports the existence of a non-enzymatic mode of action of lysozyme. Partially or completely denatured lysozyme having reduced or no enzymatic activity has been shown to retain the bactericidal activity against gram-positive bacteria and even extends it to gram-negative bacteria normally resistant to native lysozyme (Pellegrini et al., 1992; Ibrahim et al., 1993; 1996; 1996a; Düring et al., 1999; Touch et al., 2004).

Cell leakage without spheroblast formation or cell lysis upon exposure of sensitive bacteria to lysozyme indicates that the inactivation results from the perturbation of the selectivity of the plasma membrane rather than the degradation of the cell-wall peptidoglycan (Masschalck et al., 2002). The underlying explanation of the non-enzymatic mode of action of lysozyme is its cationic, lipophilic and hydrophobic nature allowing it to interfere with the selectivity of the plasma membrane in a similar way as other cationic antimicrobial peptides (Vaara, 1992; Hancock and Chapple, 1999; Stark et al., 2002; Jenssen et al., 2006). Nonetheless, in an earlier study Masschalck et al. (2001) have shown that lysozyme should retain, at least partially, its enzymatic activity in order to exert an antimicrobial effect, and that increased hydrophobicity only promotes the access of lysozyme to the target peptidoglycan in gram-negative bacteria. According to these authors, chemical or heat treatments induce an increase in hydrophobicity of lysozyme without completely inactivating it, which in addition to its cationic nature, helps it to partition into the LPS and thereafter hydrolyze the peptiodoglycan.

This was evidenced by spheroblast formation of gram-negative strains upon exposure to partially heat-denatured lysozyme along with high hydrostatic pressure. Conversely, other authors have demonstrated that the hydrolytic action is not required for the bactericidal activity of lysozyme; the increased hydrophobicity of heat-denatured lysozyme increases its binding capacity to the inner membrane of gram-negative bacteria causing cell leakage and ultimately cell death without need to degrade the cell-wall (Ibrahim et al., 1993; 1996; Touch et al., 2004). This is in agreement with the findings of Pellegrini et al. (1992) who showed, by an electron microscopic examination followed by immunogold labeling, that incubation of an *E coli* strain in presence of heat-denatured lysozyme results in gradual disintegration of the bacterial cytoplasm and the presence of lysozyme within the affected cytoplasm. As matter of fact, strategies to extend the spectrum of action of lysozyme to gram-negative bacteria through the increase in lysozyme hydrophobicity by heat or chemical denaturation, or through covalent attachment of fatty acids or C-terminal hydrophobic peptides, regardless of whether or not lysozyme retains its enzymatic activity, have been developed successfully (Masschalck and Michiels, 2003; Touch et al., 2004; Lua et al., 2005). The antimicrobial activity of non-hydrolytic cationic peptides derived from chicken egg white lysozyme (Pellegrini et al., 1997) or T4 lysozyme (Düring et al., 1999) against gram-positive and gram-negative bacteria provides further evidence for the non-lytic mode of action of lysozyme. In fact, Pelligrini et al. (1997) have found that a cationic pentadecapetides released from egg-white lysozyme by hydrolysis with clostripain possess [f(98-112); I_{98}VSDGNGMNAWVAWR_{112}] was highly bactericidal while devoid of any enzymatic activity. Furthermore, replacement of asparagine 106 by the positively charged arginyl residue has significantly increased the potency of the lysozyme-derived peptide in a similar manner as the peptide VLVLDTDYK [β-Lg f (92–100)] released by a tryptic digestion from bovine β-lactoglobulin (β-Lg) (Pelligrini et al., 2001), which is thought to act primarily on the plasma membrane by electrostatic interaction (Jenssen et al., 2006). Lysozyme has recently been shown to bind strongly to liposomes and form aggregates as a result of electrostatic and hydrophobic interactions with the synthetic phospholipid bilayer (Gorbenko et al., 2007), and these authors suggest that lysozyme would act on sensitive strains by lysing their cell-membrane according to the aggregation model described for alpha-helical cationic antimicrobial peptides (Brogden et al.,, 2005). Alpha helix is indeed the predominant form in the tertiary structure of lysozyme (Figure 2).

INHIBITORY ACTIVITY OF LYSOZYME AGAINST MICROORGANISMS OTHER THAN BACTERIA

In addition to bacteria, lysozyme has also been reported

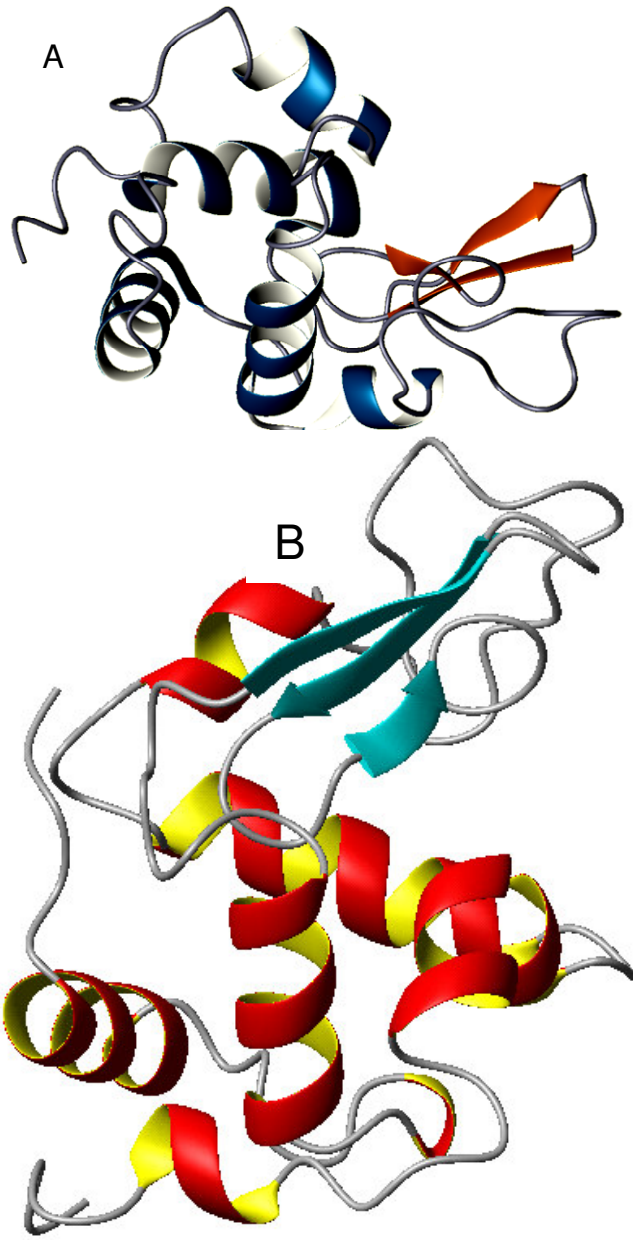

Figure 2. The tertiary structure of egg white lysozyme (A) and bovine milk lysozyme (B) showing the predominance of alpha-helix thought to be crucial for membrane-active antimicrobial peptides to permeabilize the microbial inner cell-membrane (Brogden, 2005; Jenssen et al., 2006). (A): After Biological Magnetic Resonance Data Bank; Website: www.bmrb.wisc.edu/.../Lysozyme/lysozyme2.html. (B): Kopp and Torsten (2004); drawn by using MolMol software (Koradi et al., 1996). NB: All known milk-lysozyme molecules contain alpha helices and beta sheets though at different locations and relative proportions.

to inhibit viruses (Cisani et al., 1984; Lee-Huang et al., 1999) and eukaryotic micro-organisms including parasites (León-Sicairos et al., 2006) and fungi (Wu et al., 1999; Razavi-Rohani et al., 1999; Knorr, 2004) despite the absence of typical peptidoglycan in their envelopes. However, the inhibition of yeast and mould has been explained by the presence of chitin as an important constituent of their cell-wall (Gow, 1994; Alsteens et al., 2008; New et al., 2008), and by the fact lysozyme also possesses a chitinase activity (Lundblad et al., 1979). Indeed, chitin has the same β-(1-4) glucosidic bonds as the bacterial peptidoglycan except that the bond links two N-acetylglucosamine residues instead of an N-acetylglucosamine and N-acetylmuramic acid. Furthermore, the inhibition of *Entamoeba histolytica* by lysozyme (Isibasi et al., 1982) was explained by the presence in its membrane of lipopeptidophosphoglycan which could react with the enzyme in a similar manner as the peptidoglycan (see León-Sicairos et al., 2006).

However, no clear explanation has so far been provided for the sensitivity of viruses to lysozyme. Cisani et al. (1984) suggested that the inhibition of herpes virus would rather be due to interference with antiviral activity because of the basic nature of lysozyme, than to the hydrolytic activity. Moreover, *in vitro* inhibition of human immunodeficiency virus (HIV) by lysozyme was attributed to the hydrolysis of viral polysaccharides and RNA transcripts, or genomic RNA (Lee-Huang et al., 1999). Therefore, lysozyme would not always act by its hydrolytic activity, but would inhibit the growth of some micro-organisms either by permeabilizing the plasma membrane and/or acting on intracellular components by virtue of its cationic hydrophobic nature, as has been described for a variety of antimicrobial peptides (Epand and Vogel, 1999; Reddy et al., 2004; Jenssen et al., 2006). In fact, at sublethal concentrations (ca, 10 µg/ml), lysozyme was shown to accumulate into the cytoplasm of *Candida albicans*, and reduce the production and activity of aspartyl proteinase (Sap), a putative virulence factor of the yeast indicating that lysozyme acts at the transcriptional or translational level of DNA expression, while at high concentrations, it induced a cell-swelling and invaginations near bud scars, suggesting that interference with the synthesis of cell-wall components may be alternative targets for this enzyme (Wu et al., 1999).

Non hydrolytic antimicrobial peptides have been shown to possess more than one mode of action, and to inhibit DNA expression and synthesis pathways such as those producing cell-wall components after translocation, into the cytoplasm of sensitive microorganisms (Wu, 1999; Brogden, 2005; Jenssen et al., 2006). Further research is though needed for a clearer understanding of the mechanism of action by which lysozyme acts against sensitive microorganisms in general, and eukaryotic and non-cellular microorganisms, in particular.

LYSOZYME IN MILK

Levels of lysozyme in milk

The milk of virtually all mammals contains lysozyme either as a free soluble protein or within leucocytes and

Table 1. Reported concentrations (mg/l) of lysozyme in the milk of different mammals.

Animal species	Average concentration	References
Human	400	Mathur, et al. (1990)
	320	Montagne et al. (1998)
	270-890	Montagne et al. (2001) and Chandan et al. (1968)
	224-426	Hennart et al. (1991)
Ass	1428	Salimei et al. (2004)
Mare	790	Jauregui-Adell (1975)
	1330	Sarwar et al. (2001)
Cow	0.13	Chandan et al. (1968)
	0.07	El Agamy et al. (1996)
	0.05 – 0.21	Piccinini et al. (2005)
Buffalo	0.0012*	Priyadarshini and Kansal (2003)
Ewe	0.1	Chandan et al. (1968)
Goat	0.25	Chandan et al. (1968)
Sow	6.8**	Schultz and Müller (1980)
Camel	0.6 – 6.5	Barbour et al. (1984)
	0.15	El Agamy et al. (1996)

*Authors reported 60 x 10^{-3} U/ml of milk using Sigma lysozyme (1 mg = 5 x 10^{4} units) as a standard; ** during the first 48 h after parturition; ND = not detected.

lysosomes (Ralph et al., 1976; Gupta et al., 1985;Lemansky and Hasilik, 2001). Although all milk lysozymes have been reported to belong to the c-type, they vary widely in terms of structure and physico-chemical properties such as the folding/unfolding status, ability to bind calcium ions, stability to heat and/or pH, the isoelectric point (Table 2). In addition, the concentration of soluble lysozyme in milk varies considerably from one species to another and within the same species depending on various factors such as the breed, stage of lactation, parturition, nutrition, udder health and season of the year (Blanc, 1982; Maroni and Cuccri, 2001; Priyadarshini and Kansal, 2003). Nonetheless, two main groups of milk can be distinguished on the basis of lysozyme content. A group comprising milks with exceptionally high levels of lysozyme averaging 200 to 1330 mg/L corresponding to ~2 to 7% of the total milk proteins, and another group of milks with low level of lysozyme (3000 to 6000 times less than the milks of the first group). Human, equine and canine milks are the main representatives of the first group while bovine, ovine and caprine milks (Table 1) represent the second group. In fact, the presence of lysozyme in some milk is controversial, and several authors have reported on the lack of lysozyme in bovine (Jollès and Jollès, 1961; Pahud and Widmer, 1982), swine (Chandan, 1968; Shahriar et al., 2006) and camel milks (Kappeler et al., 2003, El Hatmi et al., 2007). In contrast, other authors have confirmed its presence or have purified and characterized it in/from these milks (Chandan et al., 1964; Chandan et al., 1968; El Agamy et al., 1996; Allen at al., 2000; Parisien et al., 2007). Such controversy has been explained by the fact that lysozyme concentration in these milks is normally low and may fall, under certain conditions or periods of the year, to below the detection limit of the analytic methods used for lysozyme quantify-cation (Blanc, 1982; Montagne et al., 1998; Solaroli et al., 1993; Shahriar et al., 2006). Bovine milk has been reported to contain minute amounts of lysozyme (Blanc, and the level of the enzyme in porcine milk has been shown to be moderate at parturition and declines rapidly after the first 48 h to reach an undetectable level after 30 days post-partum (Schultz and Müller 1980). Therefore, lysozyme quantification in mature porcine milk may yield negative results depending on the sensitivity of the analytical technique used.

Regardless of the lactating species, it is generally admitted that the level of lysozyme is highest in colostrum and decreases in transitional or mature milk following a typical down-regulation of the expression of lysozyme genes (Barbour et al., 1984; Schultz and Müller, 1980; Montagne, 2001; Kappeler et al., 2003). Such a phenomenon has been considered as a natural means for lactating females to provide maximum protection to suckling offspring against microbial infections during the first days of parturition when their self-immunity is still immature. Nevertheless, exceptions to this tendency have been recorded. In human milk, for example, the evolution of lysozyme content has been described to follow three distinct phases: an initial decrease during the first few days after birth followed by a stabilization that may last several weeks, and then a steady increase to reach the highest level at the late lactation period (Montagne et al., 1998). On the other hand, Priyadarshini

Table 2. Main chemical properties of milk lysozymes of different mammals; chicken egg-white lysozyme properties are also given as a reference for the c-type lysozyme.

Origin	Class	MW (kDa)	IP	Number of amino acid residues*	Calcium binding sequence	Folding properties	Catalytic centre residues	Stability to heat treatment at neutral pH (% residual activity)	Reference
Camel milk	?	14.4	NA	NA	NA	NA	NA	75°C/30 min (56 %)	El Agamy et al. (1996)
Cow milk	c	14.4	10.2***	129	None	Two-state	Glu_{35} Asp_{53}	75°C/30 min (74 %) 75°C/15 min (25%)**	El Agamy (2000)
Ewe milk	c	16.2	NA	147	None	Two-state	Glu_{53} Asp_{21}	NA	Maroni et Cuccuri, (2001)
Human	c	15	11	130	None	Two-state		NA	Parry et al., (1960)
Goat	c	14.4		129	None	Two-state	Glu_{35} Asp_{53}	NA	Jolles et al. (1990)
Buffalo	c	16.0	NA	NA	None	Two-state	NA	75°C/30 min (81.7%) Pasteurisation (100%)	Elagamy (2000)
Mare	c	14.7	NA	129	81-92	Three state	Glu_{35} Asp_{53}	100°C/5min (70%) 82°C/2 min (101%)	Jauregui-adell (1974); Sarwar et al. (2001)
Canine	c	14.5	8.63	129	81-92	Three state	Glu_{35} Asp_{53}	NA	Grobler et al., (1994)
Egg-white	c	14.3	11	129	None	Two-state	Glu_{35} Asp_{52}	NA	Matagne and Dobson, (1998)

*Processed protein; **Percent loss of activity after heat treatment. ***From, Irwin (2004); NA = not available, IP = Isoelectric point.

and Kansal (2003) showed that lysozyme content in buffalo milk is not affected by the stage of lactation, as no significant difference in lysozyme content could be recorded between colstrum and mature milk. Moreover, lysozyme concentration may reach abnormally high levels in mastitis milk as a response to infections of the mammary glands regardless of the stage of lactation; a figure that has been considered as an indicator of the onset of clinical or subsclinical mastitis (Maroni and Cuccri, 2001).

Conflicting data concerning the level, and the presence or absence of lysozyme in camel milk are available in the literature. In an early study, Barbour et al. (1984) demonstrated the presence of lysozyme in the milk by nephelometric method using dead cells of *Micrococcus lysodeikticus* as a substrate and egg-white lysozyme as a standard. According to this study, a maximum concentration averaging 6.5 mg/L was recorded at parturition while mature milk contained significantly lower concentrations depending on the stage of lactation, with an overall average of 2.9 mg/L (i.e., whole period of lactation). The same authors showed that the level of lysozyme in camel milk decreases linearly throughout the lactation period according to the equation:

$$Y = -2.86X + 665.8$$

where: Y is the level of lysozyme at a given day after parturition; X is the stage of lactation (days after parturition) and -2.68 is the regression coefficient. Such a tendency implies that the level of lysozyme in camel milk decreases at a daily rate of 2.68 mg/L suggesting that the enzyme would remain detectable by the same method for more than 230 days after parturition.

Subsequent studies (El Agamy et al., 1992; 2000) confirmed the presence of lysozyme in camel milk but at significantly lower concentrations. Furthermore, the enzyme was reported to be purified successfully from camel milk and characterized at the structural level (Duhaiman, 1988; El Agamy et al., 1996). In contrast, Kappeler et al. (2003) did not detect the enzyme in mature camel milk (middle to late-lactation period) by using molecular biology techniques specific to the c-type lysozyme.

These findings were recently corroborated by El-Hatmi et al. (2007) who have monitored the changes in the composition of whey proteins of camel milk and colostrum over the first 8 days post-partum by using cation-exchange fast protein liquid chromatography for separation of the proteins followed by polyacrylamide gel electrophoresis for their identification. Therefore, further research is needed to provide a sound evidence for the presence or absence of lysozyme in camel milk. Studies on the genetic characterization of

putative encoding gene(s), sequence organisation and gene location by using sensitive molecular biology techniques (PCR, cloning, DNA or RNA hybridizations, etc.) are to be carried on different camel breeds and in various conditions (e.g., stage of lactation, season of the year and udder health status).

Antimicrobial activity of lysozyme in milk

Milk is a complex mixture of various salts and other constituents that influence the ultimate activity of lysozyme. Indeed, the inhibitory concentrations of lysozyme vary considerably depending on the target microorganism and ecological parameters in the reaction medium, and may be as low as 10 µg/ml (Masschalck et al., 2001), which is below the concentration of lysozyme in several mammalian milks (Table 2). In addition, the other antimicrobial proteins of milk (lactoperoxidase, lactoferrins, immunoglobulins, N-acetyl-glucosaminase, etc.) may positively or negatively affect lysozyme activity. Yet, the antimicrobial effects of different antimicrobial components of milk appear to add-up in a synergistic manner, as the total antimicrobial effect in milk was reported to be greater than the sum of the individual contributions of antimicrobial proteins (Clare and Swaisgood, 2000). In particular, the milk secretory Immunoglobulin A (sIgA) and lactoferrin were shown to significantly enhance lysozyme activity against trophozoites of *Entamoeba* histolytica (León-Sicairos et al., 2006). Furthermore, lysozyme was shown to be greatly influenced by the ionic strength and the concentration of metal ions in milk and in laboratory media (Priyadarshini and Kansal, 2003).

High concentrations (> 50 mM) of mono and bivalent cations such as Na^{+}, K^{+}, NH_4^{+}, Ca^{++} and Mg^{++} were shown to reduce lysozyme activity at various degrees, and such reduction was also dependent upon the nature of the salt to which the ions are conjugated. For example, sodium ions were more inhibitory when conjugated to citrate than when they were conjugated to chloride. Conversely, at lower concentrations (< 50 and mM of NaCl and other salts, respectively), lysozyme activity was stimulated (Priyadarshini and Kansal, 2003). The same study has demonstrated that lysozyme activity was reduced in presence of heavy metals such as Ni^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Fe^{3+} at 0.1 mM in the reaction mixture, with the Fe^{3+} being the most inhibitory to lysozyme which retained only 25.2% of its activity as compared to an iron-free control. The antagonistic effect of iron to lysozyme was also reported by León-Sicairos et al. (2006) regarding the inhibitory effect of the enzyme against *E. hystolytica*. In view of these data, and considering the moderate amounts of these constituents in milk of most mammals, it would be reasonable to expect that the mineral composition of milk would stimulate the antimicrobial activity of lysozyme, thereby reducing its minimal inhibitory concentration and hence allowing it to play an important role in the overall antimicrobial activity of milk.

CONCLUSION

Lysozyme is an iniquitous antimicrobial substance whose presence at variable levels in milk of different mammals is no more controversial; albeit not always detectable by the most commonly used techniques. Lysozyme appears to inhibit not only bacteria where the peptidoglycan layer is a major component of their cell-wall, but also viruses and eukaryotic microorganisms devoid of a typical peptidoglycan layer, suggesting that it acts by other mechanisms of action than the hydrolytic activity; the most probable of which is the interaction with the lipid layer of the inner membrane. Yet, microorganisms with natural resistance to lysozyme are common, and many mechanisms of resistance have been discussed.

At the practical level, lysozyme has found application in food preservation, and egg-while lysozyme is already used successfully as an antimicrobial in many foods, especially in cheese, and has had positive evaluations from international regulatory agencies such as the World Health Organization (WHO) and the Food and Drug Administration (Proctor and Cunningham, 1988). Furthermore, lysozyme has recently been introduced to the wine industry to control malolactic fermentation in wine and has been permitted to the level of 100 mg/L (Delfini et al., 2004; Pitotti et al., 199). However, egg-white lysozyme is so far the main source of lysozyme for food preservation due its high content in lysozyme and to the availability of industrially feasible and cost effective purification procedure from that source (Chiang et al., 2006).

Alternatively, a milk-based crude preparation of lysozyme may be envisaged, as milk represents a widely available and safe source. In addition such a crude preparation would have the advantage of being prepared at reduced cost while enhancing its activity by the presence of other antimicrobial milk proteins as contaminants.

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