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Advances in dairy Research

Research Article

Antibacterial Action 0f Dextran Conjugated Lysozyme against Bacteria Involved In Bovine Mastitis

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ABSTRACT

Mastitis is the bacterial-induced inflammation of cow's mammary glands which results in low quality and low yield of milk, and incurs substantial economic loss to dairy industry worldwide. Treatment is possible by long acting antibiotics, but milk from such cows is not marketable until drug residues have left the cow's system. Lysozyme is the enzyme that hydrolyses the β-glycosidic linkage of the outer peptidoglycan envelope of the cell wall of Gram positive bacteria, thereby destroying them. Antimicrobial action of lysozyme can be extended towards Gram negative bacteria by chemical modification. The aims of the present study were to conjugate chicken egg-white lysozyme with dextran and to assess the antibacterial properties of the lysozyme-dextran bioconjugate against causative agents of bacterial mastitis. Chicken egg white lysozyme was conjugated with dextran under mild Maillard-based conditions and the conjugated product purified and antibacterial activity studied (a 1:5 ratio of lysozyme: dextran, 60oC, RH79%). Degree of conjugation was followed by G 100 gel filtration chromatography and SDS-PAGE. Growth of bacteria was monitored by recording absorbance at 600 nm. The results showed that neither lysozyme nor the conjugate derivative have significant effect on the growth kinetics of Staphylococcus aureous, Listeria monocytogenes and Streptococcus agalactica. Bacillus cereus, on the other hand behaved entirely different. After 25 h the growth of this bacterium in the presence of lysozyme was reduced by 20% while the conjugated lysozyme lowered growth by 80% after 5 h. Conjugated lysozyme reduced the growth of E. coli, treatment by 50%. Growth of Klebsiella pneumonia decreased by 20% after 24 h in the presence of lysozyme and by more than 80% after 10 h in the presence of dextran-conjugated lysozyme. Taken together, the results of this study show that lysozyme-dextran conjugate may be a natural antimicrobial agent useful in pharmaceutical preparations for the treatment of bovine mastitis.

Keywords: Mastitis; Lysozyme; Dextran; Conjugation; Natural Antimicrobials

INTRODUCTION

Mastitis is an inflammatory reaction of the udder tissues due to the entering of microbes via the teat canal. Mastitis is potentially life threatening mammary gland infection with serious health and economic consequences. Farmers in developing countries are particularly concerned about bovine mastitis because it can result in low milk production, antibiotic residues in milk, increased somatic cell count, veterinary costs, shedding of bacteria and their toxins in the milk, culling or death of infected cows and serious zoonotic potential associated with it. Sanitization of the cow barns, suitable milking procedures and separation of infected cows from healthy animals are some of the steps that needs to be taken into account for prevention and control of mastitis. Treatment of mastitis is carried out by antibiotic administration such as penicillin injection in combination with sulfur drugs [1].

Mastitis is a multifactorial disease. Environmental and pathogenic factors affect the occurrence of mastitis. Therefore identification of causal factors for this disease is important for designing the prevention and treatment strategies [2].

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Received Date: July 30, 2021; **Accepted Date:** October 05, 2021; **Published Date:** October 14, 2021

Citation: Kuhi M, Aminlari M, Tavana M (2021) Antibacterial Action 0f Dextran Conjugated Lysozyme against Bacteria Involved In Bovine Mastitis. Adv Dairy Res 09:p311.

Bacteria are the most important causative agents of mastitis. Several different bacteria can infect the udder. The bacteria within the udder multiply producing inflammatory substances that can cause reduction in milk production and deteriorated milk quality. The predominant microbial contributor to mastitis are either direct bacterial pathogens such as Staphylococcus aureus, Streptococcus agalactiae and Corynebacterium bovis and the environmental bacterial pathogens including Escherichia coli, Streptococcus uberis, Streptococcus dysgalactiae, and other Gram-positive and catalase negative cocci (Dufour). In Canada, more than 16,000 mastitis-causing bacterial isolates were isolated from milk samples taken over a 2-year period (Dufour). Another study found that five species of bacteria accounted for nearly 80% of all mastitis diagnoses were Escherichia coli, Streptococcus uberis, Staphyfororcu aureus, Streptococcus disgalacuae, and Streptococcus agaJacJiae. The use of antibiotics is a key component in treating and preventing mastitis, however antibiotic residues in milk is a major concern for dairy farmers and industries as well as regulatory agencies and consumers since it can provoke allergic reactions in hypersensitive people and may result in appearance of resistant bacteria that do not respond to treatments commonly used for human and animal illnesses. The modem dairy industry's biggest challenge is to reduce antibiotic use. In addition, there is increasing interest by pharmaceutical manufacturers in developing antimicrobial agents that are perceived as more natural [4,5,6].

Lysozyme (EC 3.2.1.17, mucopeptide N-acetylmuramic hydrolase) is an enzyme that catalyses the hydrolytic cleavage of β-1, 4-glycoside bonds in the peptidoglycan of the bacterial cell wall. This enzyme has antibacterial activity only against Gram positive bacteria. Gram-negative are resistant to the action of lysozyme because of the presence of lipopolysaccharide layer in the outer membrane which serves as an additional barrier for lysozyme activity. The lysozyme activity can be extended towards Gram negative bacteria by using modifications leading to changes in the conformation of enzyme molecules [7].

Lysozyme has129 amino acid residues and is present in tear, saliva and other body fluids. Chicken egg white has a large concentration of lysozyme and is a suitable source for large scale isolation and purification [8].

Dextran is a branched polysaccharide that is used as a pharmacological agent because it has antithrombotic activity, is also used to reduce blood viscosity and in hypovolaemia to expand volume, and as lubricant in some eye drops. The polymer is formed by condensation of glucose units through α 1, 6 glycosidic linkages between glucose monomers, and branches from α-1, 3 linkages. Several species of certain lactic acid bacteria of the family lactobacillus, such as Leuconostoc mesenteroides and Streptococcus mutans, produced dextran from sucrose. Dextran-lysozyme conjugate have been reported to have excellent antimicrobial action against Gram negative bacteria such as E, coli [9-14].

The efficacy of lysozyme activity can be extended towards Gram-negative bacteria by producing derivatives through conjugation with different small and large molecules as ligand. The lysozyme conjugates have extended antimicrobial activity and improved functional properties such as solubility and heat stability. These lysozyme conjugates decrease Gram-negative bacteria both in culture and in foods, and they may serve as natural antimicrobial agents in food and pharmaceutical industries [15-20].

In this study, dextran-lysozyme conjugate was produced and its effect on bacterial mastitis isolates investigated.

MATERIALS AND METHODS

Materials

Dextran (molecular weight of 10000), Sephadex G-100, and Micrococcus lysodeikticus cell wall were obtained from Sigma Co., St Louis, MO, USA. Lysozyme was provided by Canadian Inovatech, Abbotsford, BC, Canada. A protein molecular weight marker was from Fermentas, Molndal, Sweden. Plate count nutrient agar and BHI broth were from Merck. All other chemical were reagent grade and were commercially available [21].

Preparation of Lysozyme-Dextran Conjugate

Lysozyme-dextran conjugate was prepared as described previously. Lysozyme and dextran in a weight ratio of 1:5 were dissolved in 0.1M sodium phosphate buffer (pH=7.4), mixed and lyophilized. The powder was heated at 60 ◦C under a relative humidity of 78.9% in the container saturated with KBr solution for one week. A control sample was included in which lysozyme was treated similarly in the absence of dextran. Gel permeation chromatography on a Sephadex G-100 column was used to separate the conjugated lysozyme from the un-reacted lysozyme. The gel filtration column (90×1.5 cm) was equilibrated and eluted with 0.05 M sodium phosphate, pH= 7.4. Fifty mg of lyophilized powder dissolved in 1.0 mL phosphate buffer centrifuged at 2500 × g for 10 min. The supernatant was applied to Sephadex G-100 column. Proteins were eluted by adding buffer to the column. The absorbance at 280nm was recorded to monitor the elution of proteins from the column. Fractions were pooled, concentrated by ultra-filtration and lyophilized. Fifty mg of unconjugated lysozyme was treated similarly [22].

Measurement of Protein Content

The soluble protein content of all samples was determined by the method of Lowery et al., (1951).

Electrophoresis

SDS slab polyacrylamide gel electrophoresis was described by Laemmli (1970). The separating gel was a 10% acrylamide gel. Loading buffer compromised 0.01 mol/L Tris-HCl, pH 6.8, 0.4% SDS, 100 g/L glycerol and 0.04 g/L bromophenol blue and 1 mg/mL protein. A gel with dimensions of 140 × 140 × 1 mm was made with 10% polyacrylamide in 1.2 mol/L Tris-HCl , pH = 8.8 and 3 g/L SDS. The stacking gel compromise of 0.03% acrylamide in 0.25 mol/L Tris-HCl, pH = 6.8 and 2 g/L SDS. The electrode buffer contained 0.025 mol/L Tris-HCl, 0.192 mol/L glycine and 1.5 g/L SDS at pH = 8.16. Electrophoresis was performed at a constant 25 mA current and gel was stained with 2.5 g/L Coommassie Brilliant Blue R-250

in 500 g/L acetic acid/250 g/L methanol and de-stained with 100 g/L acetic acid/ 70 g/L methanol[23].

Lysozyme Activity

Lysozyme activity was measured as described by Imoto and Yagishita (1971) using Micrococcus lysodeikticus cell walls assubstarte. Dried Micrococcus lysodeikticus cell walls was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) to give a final concentration of ~40 mg/100 mL) and diluted to a final volume of 30 mL with the same buffer. Lysozyme or modified lysozymes at a concentration of 1 mg/mL were dissolved in cold distilled water. Three mL Micrococcus lysodeikticus cell walls suspension and 0.1 mL enzyme solution were poured into a cuvette and absorbance at 450nm was recorded with time. The unit activity is defined as the rate of decrease in absorbance per minute at pH 7.0 and 25oC. All experiments were performed in triplicates [24].

Bacterial strains and culture conditions

Four Gram-positive strains (Listteria monocytogenes, Staphylococcus. aureus, Streptococcus agalacticae and Bacillus cereus) and two Gram negative strains (E. coli and Kelebsiella pneumonia) were from milk of cows with signs of clinical mastitis. Bacteria were incubated in BHI broth at 37 °C for 16 h and then decimally diluted to give a count of 106/CFU/mL based on a hematocytometer counting. All experiments were performed in triplicate[25].

Antibacterial activity

The antimicrobial assay of native lysozyme and dextran-conjugate lysozyme against Gram-positive and Gram-negative strains of bacteria was studied. Breifly, 0.5 mL of lysozyme or modified lysozymes solutions in 50 mM potassium buffer, pH=7.0, was added to 4.5 mL of the bacterial suspension in the same buffer to give a final lysozyme concentration of 400 µg mL-1. The mixture was incubated at 37 °C for 2 h. The lytic action of lysozyme and lysozyme-dextran conjugate against isolated bacteria was determined turbidometrically as described by Dalgaard et al., (1994). The increase in absorbance at 600 nm following addition of enzyme solution was monitored every hour up to 8 hours and after 24 hours incubation. All experiments were performed in triplicates [26-30].

Statistical Analysis

All data were analyzed by AS 11.5 and means comparison was performed by Duncan's multiple range test, The level of significance for all analyses was accepted as P<0.05.

RESULTS

Mixture of chicken egg white lysozyme and dextran were heated at 60◦C and 79% relative humidity for 7 days. This treatment resulted in pale brown protein powder which indicated progress of Maillard reaction. The result of gel filtration chromatography on a Sephadex G-100 column is shown in Figure 1. Chromatogram clearly shows that the conjugated lysozyme was eluted in the void volume and the unmodified lysozyme eluted which suggest conjugation with dextran has resulted in increase in the size and molecular weight of lysozyme, as expected. SDS slab polyacrylamide gel electrophoresis further confirms covalent attachment of dextran to lysozyme (Figure 2). A broad diffuse band in the electrophoretic patterns demonstrates production of modified lysozyme species, the molecular weight of which was higher than the band corresponding to the unmodified lysozyme.

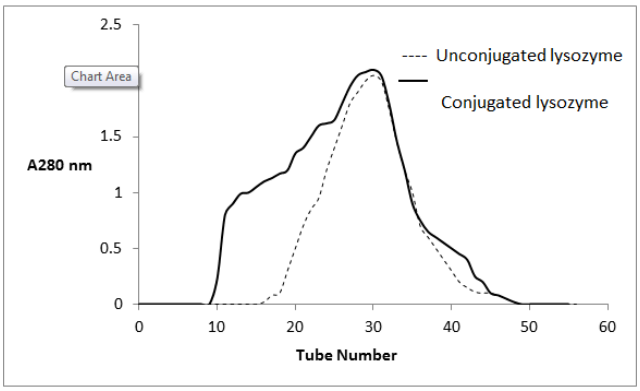


Figure1: Elution profiles of the unconjugated lysozyme and dextran-conjugated lysozyme on a Sephadex G-100 gel permeation chromatography column. Conjugated lysozyme was prepared under optimum condition (i.e. 60 ◦C, pH=7.00 79% RH, one week). The column was equilibrated and eluted with 0.05 M sodium phosphate, pH=7.4.

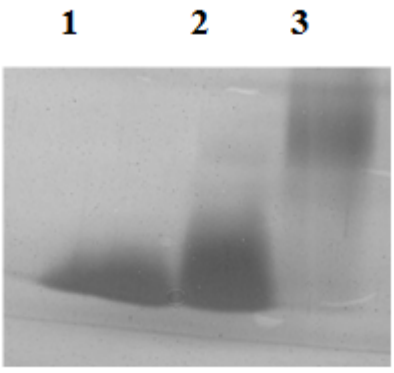


Figure2: SDS-PAGE of 1: Native lysozyme, 2: Unconjugated lysozyme and 3: dextran-conjugated lysozyme. Electrophoresis was performed on a 10% polyacrylamide gel, 20 µg protein per well.

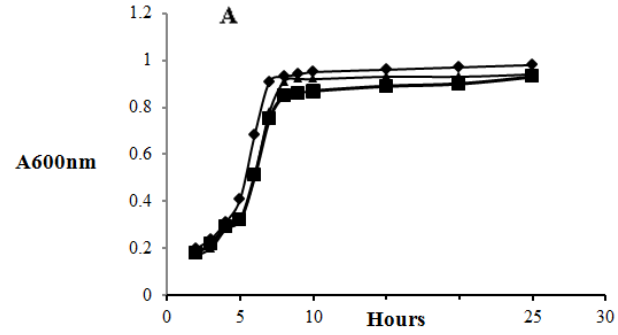
The antimicrobial effects of native and dextran-conjugated lysozyme against Gram positive and Gram negative strains of bacteria isolated from milk of cows with mastitis is shown in Figure 3. Figures 3A-C shows the effect of native and conjugated enzyme on Staphylococcus aureous, Listeria monocytogene and Streptococcus agalactica, respectively. No significant difference in absorbance at 600 nm between the control, native lysozyme and conjugated lysozyme at different time intervals was observed. In the case of Streptococcus agalactica (Figure 3C), the absorbance of native and conjugated enzymes, although not significant (P>0.050), was even greater than that of the control.

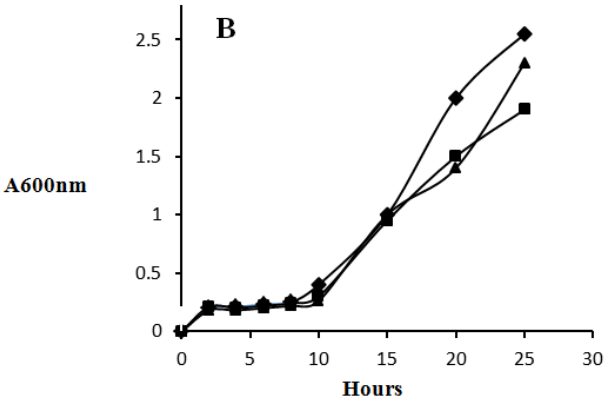
It appears that neither lysozyme nor the conjugate derivative have any effect on the growth kinetics of these three Gram positive species of bacteria. Bacillus cereus, on the other hand behaved entirely different. After 25 h incubation, the absorbance of the sample containing lysozyme was reduced by 20% while the conjugated lysozyme lowered the absorbance by 80% after 5 h, as compared with the control (Figure 3D).

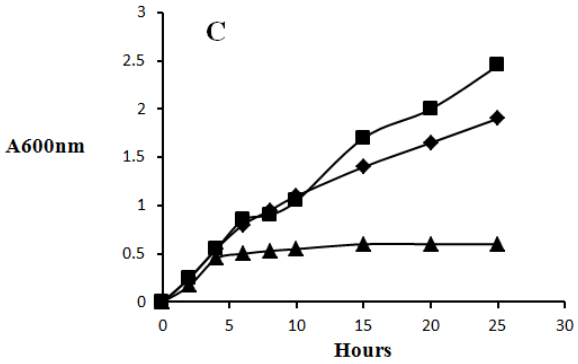
The growth of Gram-negative bacteria was significantly retarded by conjugated lysozyme. Figure 3E indicates that E. coli growth was not affected by native lysozyme but at all-time intervals dextran-conjugated lysozyme significantly reduced the growth of this species. In this species the absorbance at 600 nm decreased by 50% after 25 h incubation in the presence of dextran-conjugated lysozyme. Similar results were observed for Klebsiella monocytogenes, although some reduction was also observed for native lysozyme (Figure 3F).

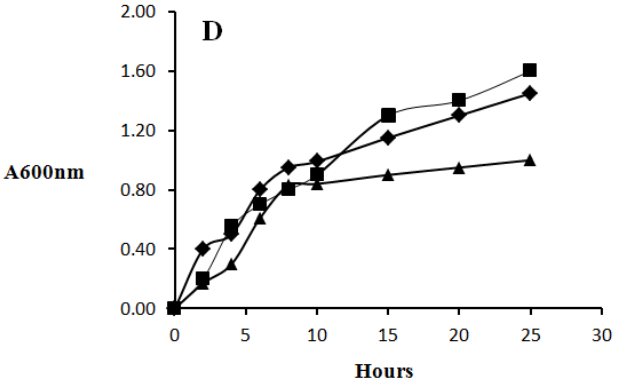
◆ Control, ■ Native lysozyme ▼ dextran-conjugated lysozyme

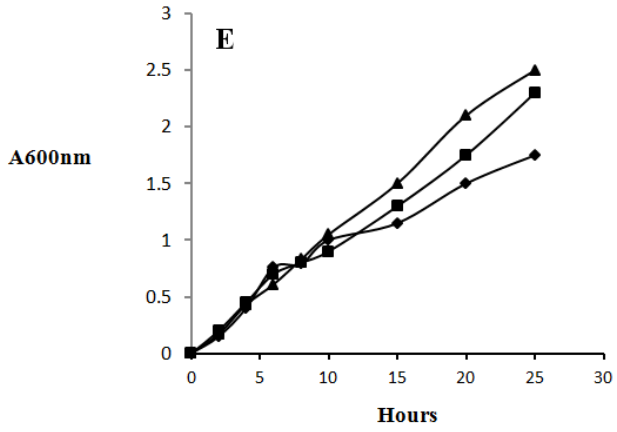
Figure3: Antimicrobial activity of lysozyme and dextran-conjugated lysozyme against Gram positive bacteria: A: Staphylococcus aureous, B: Listeria monocytogenes, C: Streptococcus agalactica, D: Bacillus cereus and Gram negative bacteria: E: E.coli, F: Kelebsiella pneumonia.

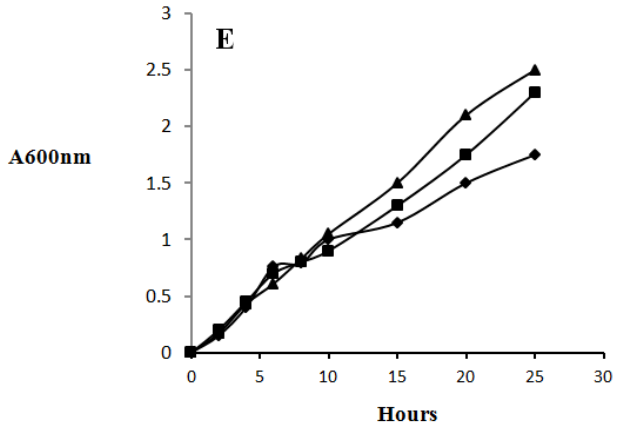












DISCUSSION

Pre-calving treatment with antibiotics is an attractive and applicable tool for control of mastitis. However, in recent years, appearance of antibiotic-resistant strains has resulted in less effective antibiotic treatment of mastitis. This condition has led to an increase in the search for alternative medicine such as natural antimicrobial agents. Preparation of new functional antimicrobials has recently received considerable attention. Lysozyme is an enzyme naturally present in biological fluids which acts in prevention of bacterial growth in foods of animal origin such as hen eggs and milk. It can be applied as preservative in foods that do not naturally possess it, and also may have therapeutic use against infectious diseases. Recent studies have shown that the bactericidal effect of this enzyme can be improved by chemical and enzymatic modifications.

In preparation of dextran-lysozyme conjugates, the εamino group of lysine residues and the N-terminal amino group of lysozyme molecules were allowed to react with the carbonyl group at the reducing end of dextran molecules under controlled temperature, pH and relative humidity. Formation of the dextran conjugated lysozyme was confirmed by SDS-PAGE (Figure 2). Appearance of diffused bands when lysozyme was conjugated with different polysaccharide has been reported by other investigators. Appearance of diffused band is indicates formation of multiple conjugated derivatives during the reaction of lysozyme with dextran. These multiple forms probably originate from the formation of molecules with different numbers of polysaccharides attached to each protein molecule. Lysozyme has seven free amino groups. It is therefore conceivable that these multiple derivatives belong to lysozyme with one or several dextrans molecules attached to amino groups of lysozyme.

Gram-positive bacteria, including Staphylococcus aureus, Enterococcus faecium, Streptococcus pneumoniae, Clostridium difficile, Bacillus cereus, Listeria monocytogenes, and others are pathogens that impact human and animal health, and the environment,. More than 16,000 mastitis-causing bacteria have been isolated from milk samples in Canada. The predominant contagious pathogens were Staphylococcus aureus, Streptococcus agalactiae, and Corynebacterium bovis, while Escherichia coli, Streptococcus uberis, and Streptococcus dysgalactiae were the major environmental pathogens. In our study the predominant species were Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Streptococcus agalactiae Escherichia coli and Kelebsiella pneumonia.

Rapid rise in antibiotic resistant have restricted therapeutic interventions for many strains of bacteria. Morbidity and mortality of bacterial infections have been increased due to the lack of suitable alternative antimicrobial strategies. An approach to find solution to antibiotic resistance is to develop antimicrobial agents that target target specific bacterial structural components.

Staphylococcus species are among few bacterial genera that are known to be completely lysozyme resistant. S. aureus is inherently resistant to egg-white lysozyme and other muramidase enzymes are needed for cell wall hydrolysis of this organism. This resistance has been attributed to the modification observed in muramic acid of the peptidoglycan of the cell wall of yhid species wherein an O-acetylation has been occurred at C6-OH.

Therefore, it is not surprising that derivatization with dextran did not increase activity against S. aureus. On the other hand, conjugation did significantly enhanced antibacterial activity against B. cereus, although presence of deacetylated glucosamine residues in peptidoglycan of some strains of this species of bacteria has been shown to contribute to resistance to lysis by lysozyme. However, lysozyme appears to rupture the polysaccharide chains of peptidoglycan in the cell wall of this bacteria and results in enhancement of sensitivity to autolysins. This phenomenon might explain the decrease in B. serious growth in the presence of native and conjugated lysozyme observed in this study (Fig.3 D).

The results of our study further demonstrate susceptibility of Gram negative bacteria to the lytic activity of the lysozyme-dextran conjugate. Treatment of E. coli with dextran-comjugated lysozyme resulted in a decrease of 50% growth compared with the lysozyme-treated bacteria after 24 h incubation. Growth of Klebsiella pneumonia decreased by 20% after 24 h exposure to lysozyme and by more than 80 % after 10 h exposure to the dextran-conjugated lysozyme.

In Gram-negative bacteria, the outer membrane of the cell wall is very sensitive to surface active agents. Improvement of emulsifying properties of lysozyme due to conjugation with different ligands have been reported in different studies. Therefore, the excellent emulsifying and surfactant properties of the conjugate might be the reason for the antimicrobial effects of lysozyme-dextran conjugate for the Gram-negative bacteria. The capacity of lipopolysaccharide (LPS) layer to bind and inhibit intrinsic lysozyme enzymatic activity might explain the inactivity of lysozyme against Gram negative bacteria. In an in vitro study it was shown that the product of the ORFan gene ykfE Escherichia coli strong inhibitor of C-type lysozyme, i.e. the chicken type lysozyme. The presence of this gene was believed to be one of the several factors that contribute to lysozyme resistance in E. coli.

CONCLUSION

The results of this study show that dextran conjugated lysozyme can be considered as an effective antibacterial agent in combating several types of Gram positive and Gram negative bacteria responsible for bovine mastitis. Large scale application of this compound may be applied for treatment of bacterial infections in farm animals. The observation that neither lysozyme nor the conjugated product had little effect on some staphylococcus and streptococcus species isolated from udder of cows with mastitis demands further studies to elucidate the mechanism of resistance and to develop new, more effective conjugated products of lysozyme.

REFERENCES

1. Aminlari, M, Ramezani R and Jadidi F. Effect of Maillard-based conjugation with dextran on the functional properties of lysozyme and casein. J Sci Food Agric.2005; 85: 2617– 2624.

2. Amiri S, Ramezani R and Aminlari M. Antibacterial activity of dextran-conjugated lysozyme against Escherichia coli and Staphylococcus aureus in cheese curd. J Food Prot.2008; 71: 411-415.
3. Ber HS, Jakob A, Vollmer W and Götz F. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol Microbiol.2005; 55: 778–787.
4. Bernkop-Schnürch A, Krist S, Vehabovic M and Valenta C. Synthesis and evaluation of lysozyme derivatives exhibiting an enhanced antimicrobial action. Europian Journal of Pharmacological Science. 1998; 6: 301–306.
5. Blake CCF, Koenig DF, Mair GA, North ACT, Phillips DC and Sarma VR. Structure of hen egg-white lysozyme: a three-dimensional Fourier synthesis at 2 Angstrom resolution. Nature.1965; 206: 757– 761.
6. Bradley AJ. Bovine mastitis: An evolving disease. Vet J.2002; 164 116-128.
7. Branen KJ and Davidson PM. Enhancement of nisin, lysozyme and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. Int J Food Microbiol.2004; 90: 63 -73.
8. Cegielska-Radziejewska R, Lesnierowski G, Szablewski T and Kijowski J. Physico-chemical properties and antibacterial activity of modified egg white—lysozyme. EEur Food Res Technol.2010; 231: 959–964.
9. Cunningham FE, Proctor VA and Goetsch SJ. Egg-white lysozyme as a food preservative: an overview. Worlds Poult Sci J.1991; 47: 141– 163.
10. Da Silva Freitas D and Abrahão-Neto J. Biochemical and biophysical characterization of lysozyme modified by PEGylation. Int J of Pharmacol. 2010;392: 111–117.
11. Dalgaard P, Ross T, Kamperman L, Neumeyer K and McMeekin TA. Estimation of bacterial growth rates from turbidimetric and viable count data. Int J Food Microbiol.1994; 23: 391-404.
12. Dayan AD . Allergy to antibiotic residues in food: Assessment of the risk to man. Vet Microbiol. 1993;35: 213-226.
13. Deckers D, Masschalck B, Aertsen A, Callewaert L, Van Tiggelen CGM, Atanassova M and Michiels CW. Periplasmic lysozyme inhibitor contributes to lysozyme resistance in Escherichia coli. Cell Mol Life Sci. 2004;61: 1229–1237.
14. DePamphilis ML. (1971). Dissociation and reassembly of Escherichia coli outer membrane and lipopolysaccharide and reassembly onto lagellar basal bodies. J Bacteriol. 105: 1184-1199.
15. Dewendy JM, Maes L, Raynaud JP, Blanc F, Scheid JP, Jackson T, Lens S and Verschueren C. (1991). Risk assessment of antibiotic residues of beta-lactams and macrolides in food products with regard to their immunoallergic potential. Food Chem Toxicol. 9: 477-483.
16. Dohoo IR, Martin SW and Stryhn H. In: Veterinary Epidemiological Research, AVC in Charlote Town, Prince Edward Island, Canada. 2003; Pp 1-26.
17. Dufour S Labrie J and Jacques M. The Mastitis Pathogens Culture Collection. Microbiol Resour Announc. 2019; 8:e00133 19.
18. Fair RJ and Tor Y. Antibiotics and bacterial resistance in the 21st century. Perspect Medicin Chem.2014; 6: 25-64.
19. González RN and Wilson DJ. Mycoplasmal mastitis in dairy herds. Veterinary Clinics of North America - Small Animal Practice.2003; 19: 199–221.
20. Heinze T, Liebert T, Heublein B and Hornig S.Functional polymers based on dextran. Adv Polym Sci.2006; 205: 199–291.
21. Ibrahim HR, Kato A and Kobayashi K. Antimicrobial effects of lysozyme against gram-negative bacteria due to covalent binding of palmitic acid. J Agric Food Chem.1991; 39: 2077–2082.
22. Ibrahim HR, Hatta H, Fujiki M, Kim M and Yamamoto T. Enhanced antimicrobial action of lysozyme against Gram-negative and Gram-positive bacteria due to modification with perillaldehyde. J Agric Food Chem.1994; 42: 1813–1817.
23. Ibrahim, H.R., Yamada, M., Matsushita, K., Kobayashi, K. and Kato, A. Enhanced bactericidal action of lysozyme to Escherichia coli by inserting a hydrophobic pentapeptide into Its C terminus. J Biol Chem. 1994;269: 5059-6331.
24. Ibrahim H, Aoki T and Pellegrini A. Strategies for new antimicrobial proteins and peptides: Lysozyme and aprotinin as model molecules. Curr Pharm Des. 2002;8: 671–693.
25. Imoto T and Yagishita K. A Simple activity measurement of lysozyme. Agric Biol Chem. 1971;35: 1154- 1156.
26. Jamali H, Barkema HW, Jacques M, Levallée-Bourget E, Malouin F, Saini V, Stryhn H and Dufour S. Invited review: Incidence, risk factors, and effects on clinical mastitis recurrence in dairy cows. J Dairy Sci. 2018;101: 4729–4746.
27. Koshani R, Aminlari M, Niakosari M, Farahnaky S and Mesbahi GR. Production and properties of tragacanthin-conjugated lysozyme as a new multifunctional biopolymer. Food Hydrocolloids. 2015;47: 69-78.
28. Lewis SL. (2010). Medical Surgical Nursing (8th ed.). ISBN 978-0323079150.
29. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227: 680-685.
30. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. (1951). Protein measurement with the Folin phenol reagent. J Biol Chem.1951; 193: 265–75.