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Application of Lactoperoxidase System in Fish and Food Products: A Review

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Abstract: Milk is known to contain proteins (e.g. lactoferrin, lactoperoxidase, immunoglobulins) and free peptides having specific non-nutritional physiological functions. Lactoperoxidase (LP), is undoubtedly important in the case of the human infant, but it potentially has greater significance and functional role in milk industry. LP, a non-haem iron-binding glycoprotein, is a peroxidase enzyme secreted from mammary, salivary and other mucosal glands. The lactoperoxidase system (LPS) plays an important role in the innate immune system by killing bacteria in milk and mucosal secretions hence augmentation of the LPS may have therapeutic applications. Furthermore, addition or augmentation of the lactoperoxidase system has potential applications in controlling bacteria in food and consumer health care products. Though the most extensively suggested industrial application of the LPS in food production is for the preservation of raw milk during storage and transportation to the site of plants, additional novel applications of the LP system are being considered. In the present article, mechanisms of LPS's action and its potential biological functions in food systems were illustrated.

Key words: Lactoperoxidase · LPS · Mechanism · Antibacterial property

INTRODUCTION

There are many processes in food industry whereas enzymes are used and these mainly include improvement of extractions, bioconversions and synthesis, changes in functionality, reduction in viscosity and flavour modification [1]. Nowadays, consumers demand 'natural' foods that contain no or reduced use of chemical additives. As a result, there has been a great interest in naturally produced antimicrobial agents [2]. In this respect, emerging preservation techniques, such as lactoperoxidase system (LPS) have received particular attention [3-5].

LPS is a naturally occurring antibacterial system in milk, which is activated by means of increasing the concentrations of two components or activators (hydrogen peroxide and thiocyanate), reacting with each other [3]. This reaction is catalysed by the enzyme lactoperoxidase which is naturally present in milk and leads to the formation of antimicrobial compounds [4]. So in warm climates whereas the refrigeration equipment is not accessible, a LPS can be applied in combination with conventional preservation treatments at sub-lethal levels to inhibit pathogenic microorganisms. The direct effect of LP system action on cell is the membrane damage resulting in loss of pH gradient, K^+ leakage, an inhibition of transport of solutes, such as amino acids and glucose [6]. Observations from laboratory and field studies indicated that the LPS does not induce any significant adverse effects on the chemical, physical or sensory characteristics of raw milk and processed dairy products. The LPS of raw milk preservation is currently the only approved method of raw milk preservation, apart from refrigeration [7].

Fish from catch to consumption, are prone to contamination with several types of microorganisms. Chilling and mechanical refrigeration are not adequate enough to protect the fishery product against the action of microbial spoilage [8]. Consequently, there is a need to overcome the problem of fish deterioration in the time from capture to processing and marketing. Integration of various techniques like use of food preservatives with refrigeration [9] could provide additional advantage in controlling spoilage microflora of fish [10].

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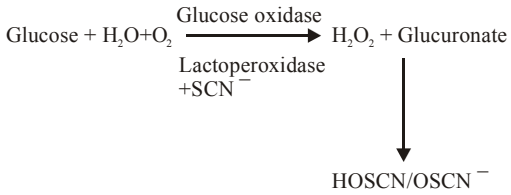
Lactoperoxidase System: The lactoperoxidase/ thiocyanate/ hydrogen peroxide system is an indigenous antibacterial system in milk and human saliva [11]. Lactoperoxidase catalyzes the oxidation of thiocyanate by hydrogen peroxide, yielding short live oxidation products, principally the hypothiocyanate ion, though sulfurdicyanide (HO_3SCN) and cyanosulphurous acid (HO_2SCN) have also been suggested [12]. These ions in turn react with the bacterial cytoplasmic membranes, as well as impair the function of metabolic enzymes, hence exert anti-microbial effect [13, 14]. The overall reaction when the source of hydrogen peroxide is glucose oxidase, is as follows:



Antimicrobial activity occurs in the lactoperoxidase system (LPS) when the lactoperoxidase enzyme (LP), the thiocyanate ion (SCN^-),and hydrogen peroxide are present, thus acting as a natural inhibitor in milk [15] and affecting a great number of Gram-positive [16-18] and Gram-negative [18-20] microorganisms. The extent of the inhibitory effects will be related to the bacterial species present and the temperature of the food.

Constitutive Components of the LPS

The Lactoperoxidase Enzyme: The peroxidase isolated from milk or lactoperoxidase is one of the most abundant milk enzymes in natural form and it represents approximately 1% of milk proteins. Lactoperoxidase (LP, E.C.1.11.1.7) is a glycoprotein consisting of a single peptide chain with a molecular weight of 78, 431 Dal. It has 15 half-systemic residues and a much higher isoelectric point (pH 9.2) than most of the other whey proteins. The enzyme contains a haeme structure, with 1 iron molecule per mole of lactoperoxidase. The conformation of the protein is stabilized by a strongly chelated calcium ion [21]. LP play important roles in strengthening the innate immune system by catalyzing the conversion of halide or thiocyanate ions into potent ions that are toxic to pathogens. These peroxidases are widely present in mammalian systems [22]. LP is an oxido-reductase secreted into milk and plays an important role in protecting the intestinal tract of the newborn infants against pathogenic microorganisms [12]. LP is present in a variety of secretions including tears, saliva and milk and, as is demonstrated in this issue, airway surface fluid [23]. There are particular interests to use LP as antibacterial agents in cosmetics, ophthalmic solutions, dental and wound treatment and as antitumor and anti viral agents [21].

Thiocyanate Ion Source (Sodium Thiocyanate): The thiocyanate ion (SCN^-) is widely distributed in animal tissues and secretions, including the mammary, salivary and thyroid glands and in the stomach and kidneys [24] and in fluids such as synovial, cerebral, cervical and spinal fluids, lymph and plasma [15]. The thiocyanate concentration varies according to animal species [25], breed and lactation cycle [26], season of the year [27] and feed [28]. The SCN^- comes from glucosinolates (from vegetables such as cabbage, kale, brussel sprouts, cauliflower, turnips and rutabaga) and detoxifuncation of the cyanogenic glocosides (which are also found in cassava, potatoes, mize, millet, sugar cane, peas and beans). The levels in these foods are higher than those proposed for use in the lactoperoxidase system (5-40 ppm). The practical use of the method consequently requires addition of some thiocyanate to ensure that a level necessary to achieve the desired effect is present in the milk [29]. In human body fluids, levels typically range from 10 to 200 ppm [15, 30] and in bovine milk from 1 to 10 ppm [15]. The thiocyanate ion has been shown to have toxic effects at high levels, with excessive intake interfering with iodine metabolism and hence thyroid function [31]. However, results from clinical experiments have clearly demonstrated that milk treated according to this method will not cause any interference of the iodine uptake of the thyroid gland, neither in persons with a normal iodine status nor in cases of iodine deficiency [29].

Hydrogen Peroxide (H_2O_2): The third component, hydrogen peroxide is not normally detected in raw milk [32, 33]. H_2O_2 is the only approved additive for the preservation of milk in the absence of refrigeration. It can be produced in endogenous form by the polymorphonuclear leukocytes in the process of phagocytosis [34] and by numerous microorganisms (e.g. *Lactobacilli, Streptococci* and *Lactococci*) under aerobic condition to active the LPS [4]. It can also be produced by H_2O_2 generator system, such as sodium percarbonate, the oxidation of ascorbic acid, oxidation of glucose by glucose oxidase (the enzyme glucose oxidase is currently listed as an approved processing aid) oxidation of

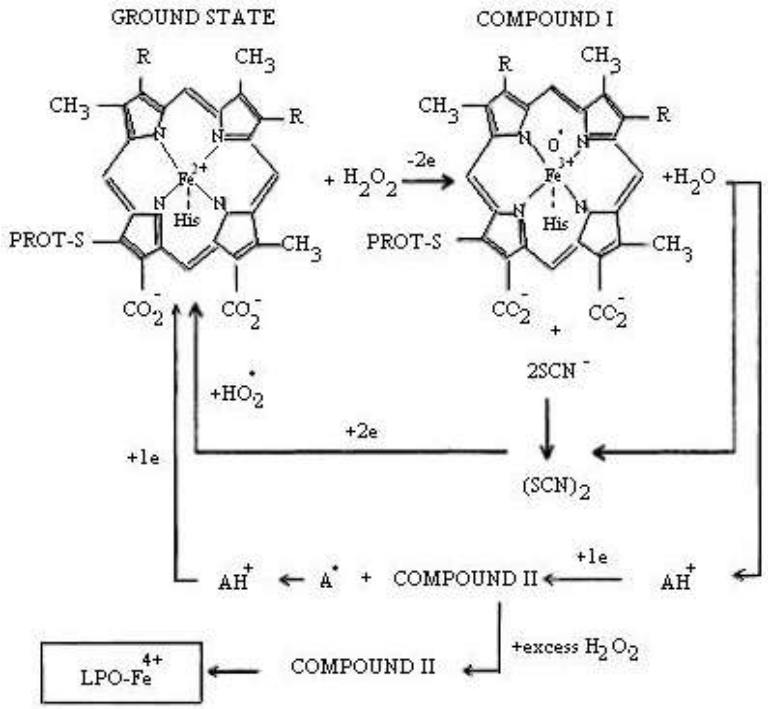


Fig. 1: Pathways in the lactoperoxidase-catalysed reaction mechanism

hypoxanthine by xanthine oxidase and the manganese-dependent aerobic oxidation of reduced pyridine nucleotides by peroxidase action [4]. Hydrogen peroxide is highly toxic for mammalian cells. However, at low (100 milimol or less) concentrations and in the presence of LP and SCNK, mammalian cells are protected from this toxicity [33].

Mechanisms of Action: The LP enzyme catalyses the peroxidation of thiocyanate and some halides (I_2, Br_2 but not Cl_2) to generate products which kill or inhibit the growth of many species of microorganisms. The reaction mechanisms are very complex [35].

According to the study of Kussendrager and van Hooijdank [36], the first step in the enzymatic mechanism is the initiation reaction of the resting LP (Fe^{3+}) to its ground state, using H_2O_2, according to $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO°_2$, followed by the propagation reactions, as illustrated in fig. 1 [37]. The superoxide radical $HO°_2$ plays an important role in termination of the catalytic reactions to the resting LP [38]. The propagation reactions include the conversion of LP from the ground state into the so-called compound I state by reaction with H_2O_2. At low SCN^- (<3 milimole) and halide concentrations, compound I reacts with one-electron donors that are present (proteins peptides etc.) to form compound II, that is continuously reduced to the ground state at a low rate. At an excess of H_2O_2 (>0.5 milimol) compound II may react to form compound III, leading to a ferrylperoxidase adduct and to irreversible inactivation of LP. The agent that oxidises SCN_2 or halides is compound I [37].

In general, peroxidation of H_2O_2 by LP can occur through three different cycles, resulting in divergent antimicrobial activities as follows [37]:

- In the presence of sufficient oxidizing halide or SCN^- as 2-electron donor for compound I, giving optimal activation of LP.
- In the presence of insufficient halide or SCN^- of appropriate redox potential, resulting in dominating 1-electron donors and accumulation of compound II and reversible inactivation of LP.
- In the presence of an excess of H_2O_2 resulting in the formation of compound III, associated with irreversible inactivation of LP [34].

A proposed reaction scheme for the LP catalysed oxidation of SCN_2, resulting in short-lived oxidation products, being responsible for the antimicrobial activity [36]. The major intermediate oxidation product of the LP catalysed oxidation of SCN^- is the hypothiocyanite ion ($OSCN^-$) [38-40] which is in

equilibrium with hypothiocyanous acid (HOSCN) and at the pH of maximal LP activity (pH 5.3) their amounts are equal. Both forms exert antibacterial activity but there is evidence that the uncharged HOSCN is more bactericidal. The stability of hypothiocyanite, $OSCN^-$, is affected by many factors, such as pH, light, metal ions (Fe, Ni, Cu, Mn etc.), glycerol and ammonium sulphate as well as by the presence and removal of LP [41]. Other short-lived intermediate compounds include thiocyanogen ($(SCN)_2$), cyanogen thiocyanate (NC-SCN), cyanosulphurous acid (HO_2SCN) and cyanosulphuric acid (HO_3SCN) which may be formed in varying amounts depending upon reaction conditions [33].

The oxidation of sulphydryl (SH) groups of microbial enzymes and other proteins is considered to be the key to the antimicrobial action of the LPS. This activity can be inhibited by reducing agents containing SH groups such as cysteine, glutathione, mercapto-ethanol, dithiothreitol and sodium hydrosulphite, either by direct binding to the haem group or by scavenging $OSCN_2$. HOSCN and $OSCN_2$ appear not to oxidise SH groups of milk proteins such as â-lactoglobulin [37]. The structural damage of microbial cytoplasmatic membranes by the oxidation of SH-groups results in leakage of potassium ions, amino acids and peptides into the medium and subsequently uptake of glucose, amino acids, purines, pyrimidines in the cell and synthesis of proteins, DNA and RNA is also inhibited [15, 42].

Thermal Inactivation of the LPS: The kinetics of thermal inactivation of the lactoperoxidase enzyme are well established [6, 34, 37, 43, 44]. In practical terms, batch pasteurization (e.g. 65°C/30 minutes) has little effect on enzyme activity, HTST pasteurization (72°C/15 seconds) results in retention of approximately 70% lactoperoxidase while treatment at 80°C or more (including conventional or UHT sterilization) leads to complete destruction of the enzyme.

Microbiological Effect of the LPS: The LPS could elicit bacteriostatic and/or bactericidal activity on a variety of susceptible microorganisms including bacteria, fungi and viruses. The molecular mechanism (s) of such inhibitory effects depend on the type of electron donor, test media, temperature and pH and could range from oxidative killing to blockage of glycolytic pathways or interference in cytopathic effects [12].

Antibacterial Effect: Different groups of bacteria show a varying degree of sensitivity to the LPS. Gram-negative, catalase-positive organisms, such as pseudomonas, coliforms, salmonellae and shigellae, are not only inhibited by the LPS but also, depending on the medium conditions (pH, temperature, incubation time, cell density) may be killed [19]. Gram-positive, catalase negative bacteria, such as streptococci and lactobacilli are generally inhibited but not killed by the LPS [45]. This difference in sensitivity to the LPS can probably be explained by the differences in cell wall structure and their different barrier properties [15, 37]. The inner membrane of Gram-negative bacteria appears to be more extensively damaged by LP-treatment than is that of Gram-positive species [46].

Many bacteria are mesophilic, growing best at temperatures of 30°C to 35°C. However, psychrotrophic and psychrophilic bacteria can grow at low temperatures, with some strains capable of surviving and growing at temperatures down to 0°C. Listeria monocytogenes is an example of pathogenic bacteria that can grow at very low temperatures. However, in products such as milk that have a diverse microflora, it would normally be outgrown by the psychrotrophic spoilage bacteria, such as members of the genera Pseudomonas, Bacillus and Micrococcus. Several studies [4, 12, 14, 18, 23, 30, 34] indicated that activation of the LPS can delay grow of psychrotrophic bacteria and thus delay food spoilage for several days compared to what can be achieved with refrigeration alone.

Antifungal Effect: Antifungal activity of the LPS has investigated by several researchers [4, 12, 25, 34, 37, 47, 48]. Popper and Knorr [48] in a work on antifungal activity of the LPS (with glucose oxidase as source of H_2O_2) in salt solution and in apple juice on *Rhodutorula rubra, Saccharomyces cerevisiae, Mucor rouxii*, *Aspergillus niger* and *Byssochlamys fulva* were observed that all tested organisms in both media were affected by LPS. The yeast strains were found to be least stable while *B. fulva* was most resistant. However, a combination of LP (5 U/ml) with glucose oxidase (0.5-1 U/ml) caused total inactivation of this mould in salt solution within 2 h. The LPS also showed antifungal activity in apple juice at acid pH (3.2), although its effectiveness was reduced. In this medium, *B. fulva* was inactivated by LP (20 U/ml) and glucose oxidase (1 U/ml) within 4 h. Strains of *R. rubra* and *S. cerevisiae* were also inhibited in apple juice by LP (5 U/ml) and glucose oxidase (1 U/ml). In other study, Seifu *et al*. [34]

declared that LPS in goat milk inhibit growth and proliferation of many fungal species viz., *Aspergillus flavus*, *Penicillium chrysogenum, Trichoderma* spp., *Alternaria* spp. and *Claviceps* spp., *Corynespora cassicola*, *Phytophthora meadii* and *Corticium salmonicolor;* however, *Candida albicans* and *Pythium* spp. were not affected by the this system. The ability of the LPS to degrade aflatoxin in the presence of sodium chloride (225 μM) and H_2O_2 (50 μM) has been also reported by these investigators.

Antiviral Effect: It is declared that LPS has antiviral activity and may assist to prevent of viral diseases. Shin *et al*. [50] reported the potential of oral administration of LPS to attenuate pneumonia in influenza-virus-infected mice through the suppression of infiltration of inflammatory cells in the lung. Seifu *et al*. [34] also reported that LP and glucose oxidase are virucidal to HIV-1 in the presence of sodium iodide, as assessed by the loss of viral replication in a syncytium-forming assay or by the inhibition of cytopathic effects on infected cells. These *in vitro* findings demonstrate that the LP/H_2O_2/halide system provides potent virucidal activity against HIV-1.

Application of LPS in Food Industry

Application of LPS in Fish and Meat Industry: There are lots of documents for the use of lactoperoxidase and sodium thiocyanate as processing aids applied to a broad range of foods including meat, fish, vegetable, fruit, milk and their products. However, extension of LPS to non-dairy foods requires the lactoperoxidase to be added, as it will not be naturally present in the food. Treatment with LPS may inhibit bacteria present on product. The ions generated by activation of LPS damage bacterial membranes and impair metabolic enzymes. However, since bacterial membranes associated with the cell walls of the various bacterial species are differ, LPS may show variable effects related to which bacterial species are present in the food being treated. As one of the antibacterial effects is to impair metabolic enzymes, these effects may be manifest only when bacteria are growing. If the bacteria are not actively metabolising at the time of treatment with LPS, the antibacterial effects will be lessened. Activity against cold tolerant bacteria such as *Listeria monocytogenes* is thus more pronounced than other bacterial species, when LPS is applied to chilled meat [24]. Many unified factors such as temperature, moisture, atmospheric oxygen, endogenous enzymes, light and particularly microorganisms influence the freshness and keeping quality of fish and meat products. Food Standards Australia New Zealand Act 1991 approved LPS as a processing aid (natural antimicrobial agents) in meat and its products that could contribute to a hurdle system to minimize the risk to consumers of pathogens. Hurdles are factors, conditions or processing steps that limit, retard or prevent microbial growth and/or reduce the microbial load but which cannot by themselves keep microbiological hazards under control. This definition can be applied to LPS. It is important to note that LPS will reduce but not eliminate pathogens present on the meat surface and that these pathogens will not always be present as the meat industry is actively engaged in a number of strategies to minimize carcase contamination [24].

Antimicrobial effects of LPS alone or in combination with other antibacterial component such as nisin were surveyed by many researchers [3-5, 10, 12, 25, 36, 50]. Elotmani and Assobhei [10] reported that while nisin inhibited only gram-positive bacteria, LPS inhibited all strains studied. Furthermore, they found that combination of nisin (100 IU ml^{-1}) and LPS (10 level) was significantly more effective than LPS or nisin alone against all strains, excepting Aeromonas salmonicida subsp. salmonicida and Vibrio alginolyticus. So, combination of nisin and LPS could be of great interest as biopreservatives for fish and fish products.

Application in Milk and Dairy Industry: Milk is the nutrient-rich liquid contains considerable amounts of lactose, unsaturated fat, protein and minerals. Raw milk may subject to microbial contamination during milking processes especially in hand-milking procedures. Contaminating organisms may multiply rapidly and turn milk into an unsuitable foodstuff either for processing or human consumption. Therefore, milk has to be quickly refrigerated after milking. However, this requires particular equipments which are not widely available, especially in small-scale dairy production and processing systems in developing countries. In these conditions, it would be valuable to have access to a method, other than mechanical refrigeration, for retarding bacterial growth in raw milk during gathering and transportation to the site of dairy processing plant. To solve this problem, an alternative method to increase the storage stability of milk at high ambient temperatures has been developed [32, 51, 52].

Table 1: Extension of milk keeping quality by the LPS at different temperatures

Temperature (°C)	Time (hours)	Reference
31-35	4-7	Ponce *et al.*, 2005
30	7-8	CAC,1991b
25	11-12	CAC,1991b
20	16-17	CAC,1991b
15	24-26	CAC,1991b
4	5-6 days	Zapico *et al.*, 1995; Lin and Chow, 2000

The LPS elicits antimicrobial activity against a wide variety of milk spoilage and pathogenic microorganisms including bacteria, HIV-1 virus, moulds, yeasts, mycoplasma and protozoa. The LPS effectiveness is depend on milk temperature and enhances with lower milk temperature (Table 1). This allows sufficient time for the milk to be transported from the collection point to a processing centre without refrigeration [7]. These centres must be equipped with proper facilities for cleaning and sanitising the vessels used to hold and transport milk. This dairy centres should set up appropriate control methods to monitor usage of the method, raw milk quality and quality of the milk prior to processing. The use of the lactoperoxidase method does not exclude the necessity of pasteurization of the milk before human consumption. Neither does it exclude the normal precautions and handling routines applied to ensure a high hygienic standard of the raw milk [29].

Future Trends: Because LPS has a broad activity spectrum, it can be used as an interesting additional hurdle to improve the safety of food preservation. However, the possible toxicological effect of LP-system and/or its constituent components should be surveyed. As an example, it has been recognized that the thiocyanate ion can interfere with iodine uptake. Furthermore, human health risks (acute or chronic) associated with either the short or long-term exposure to LP-system treated milk or its derived milk products (such as reduced iodine metabolism) either in general or to the more vulnerable sectors of society (e.g. children, HIVAIDS affected households, etc.) should be considered. Although a reduction in folate levels in milk may occur as a result of LP-s treatment, milk is not considered to be a significant dietary source of folate and the overall dietary impact is not considered important [7].

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