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Chapter 7

An Important Milk Enzyme: Lactoperoxidase

Zeynep Koksal, Ilhami Gulcin and Hasan Ozdemir

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Abstract

It has been acknowledged since ancient times that milk and dairy products have a vital role in nutrition and contribute considerably to human health. Because of its content, Because of its content, it has many important effects on the life that include immunoglobulins, enzymes, hormones, growth factors, antibacterial agents, fat acids, vitamins, and minerals. Approximately 70 indigenous enzymes have been reported in normal bovine milk, including lactoperoxidase. Lactoperoxidase LPO is a natural constituent of milk, saliva, and tears. It also exists in all mammary milk. LPO is an iron heme group basic glycoprotein, with a molecular weight of approximately 78 kDa. The LPO enzyme catalyzes the H_2O_2 +SCN^- → $OSCN^-$ + H_2O reaction. Hydrogen peroxide and hypothiocyanate are indispensable for antimicrobial activity. The biological significance of LPO is involved in the natural host defense system against pathogenic microorganisms.

Keywords: milk, protein, lactoperoxidase, enzyme, LPO system, LPO inhibition

1. Introduction

1.1. Peroxidases

Peroxidases (POD: H_2O_2-Oxydoreductase E.C.1.11.1.7) are oxidoreductase enzymes, which catalyze the reactive oxygen species generated during metabolism, and are converted into harmless molecules [1]. These exhibit antioxidant characteristics and catalyze the oxidation of organic and inorganic substrates with hydrogen peroxide being the electron acceptor [2]. Those enzymes are present in eukaryotes, prokaryotes, and photosynthetic cells [3, 4].

LPO is generally found in mammalians such as human [5, 6], bovine [7], buffalo [8], goat [9], sheep [10], llama, cow, camel, and mice milk [6, 11], saliva [12], tears [13], and mammary, salivary, and lachrymal glands [6, 14].

Peroxidases are frequently used in the studies of metabolic reactions, enzymatic functions, protein structures [15] and in clinical diagnoses, microanalytic applications, and the food and drug industry [16, 17]. Mammalian POD enzymes are localized in milk, saliva, and tears as lactoperoxidases (LPO) [18] and in leukocytes and platelets as myeloperoxidases [6].

Prosthetic groups of peroxidase are protoheme and are connected to the apoprotein loosely in contrast to many hemoprote [14]. Firstly, protein portion is synthesized in the organisms bearing peroxidases. However, enzymes are not functionally active. The enzyme gets activated by both apoprotein and hem groups [3, 19]. The general formula of the reaction catalyzed by peroxidases is shown below [3].

$$ROOH + AH_2 \rightarrow ROH + H_2O + A$$

The H_2O_2 formed during the metabolism having oxidizing property must be quickly removed. The catalase and peroxidase enzymes exhibiting antioxidant properties play this role in cells [20]. The amount of hydrogen peroxide in the cells is removed by catalase in peroxisomes. In other parts of the cells, the peroxidase enzymes utilize various aromatic components as the substrate to [21] neutralize H_2O_2 [22].

The characteristics of peroxidase enzyme that is isolated from milk are similar to animal and human peroxidases [23]. It displays 55, 54, and 45% similarity with myeloperoxidase (MPO), eosinophilperoxidase (EPO), and thyroidperoxidase (TPO), respectively. Following xanthine oxidase, lactoperoxidase is the most common enzyme in milk and it is also commonly present in whey which is the liquid remaining after milk has been curdled and strained. Each lactoperoxidase enzyme contains an iron molecule. The conformation of the protein is stabilized by a chelated calcium ion [24–26].

The main function of the LPO enzyme is to catalyze the oxidation of thiocyanate with H_2O_2 to hypothiocyanite having antimicrobial activity [27, 28].

$$SCN^- + H_2O_2 \xrightarrow{LPO} OSCN^- + H_2O$$

The combined action of these three components, which constitute the 'lactoperoxidase system', was defined by Reiter and coworkers [29–31]. The biological significance of the system is protection of the lactating mammary gland and the intestinal tract of newborn infants, thereby providing a natural host defence system against invading microorganisms [23]. The LPO catalyzes the oxidation of some halides (I_2 and Br_2 but not Cl_2) to yield the most oxidizable form of I^-, that is, I_2 [26].

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$$H_2O_2 + 2ABTS^{-+} \xrightarrow{LPO} 2ABTS^{+} + 2H_2O$$

The LPO-H_2O_2-SCN^- system shows bacteriostatic effect and thus prevents bacterial growth and development, whereas the LPO-H_2O_2-I_2 system shows bactericidal potency thus killing bacteria. Both SCN^- and I_2 have shown strong bactericidal effect when present within the system [32, 33].

1.2. Lactoperoxidase enzyme

Lactoperoxidase (LPO, E.C. 1.11.1.7) is one of the crucial enzymes in milk with oxidoreductase activity. The peroxidase isolated from milk was given the name lactoperoxidase [23] and was the first enzyme reported to be found in milk [34]. The main function of the enzyme is to catalyze the oxidation of molecules in the presence of hydrogen peroxide and to help production of products with a wide antimicrobial activity. Pseudohalogens, thiocyanates, or halogens should function as second substrates for the enzyme to exhibit such antimicrobial effects [23, 29].

The LPO system shows a significant protective effect in bovine milk. The activation of the system depends on the concentration of the two reactants, thiocyanate and hydrogen peroxide. In the presence of hydrogen peroxide, the system catalyzes the transformation of thiocyanate into hypothiocyanate, which has an antibacterial nature [35–37]. The end products of these compounds are oxidized and hence are safe for human health.

Many studies show that this system destroys several bacterial and fungal strains [38–42]. Lactoperoxidase has a broad antifungal activity [43, 44]. Mastitis is a bacterial inflammation in mammals. The effects of different concentrations of thiocyanate-H_2O_2 medium on several antibacterial and antifungal strains were studied to solve this dairy industry issue [45, 46]. They are capable of reducing bacterial growth by damaging the cell membranes and inhibiting activities of several cytoplasmic enzymes.

The LPO enzyme, a glycoprotein consisting of 8–10% carbohydrate, comprises a chain containing 612 amino acids. It consists of a single polypeptide chain of molecular weight approximately 78 kDa [47]. It is a basic protein containing heme as its prosthetic group with an isoelectric pH value of 9.2 [24–26]. Furthermore, it is very active in acidic pH [48]. It is fairly voluminous as a LPO molecule [49]. The Ca^{2+} ion stabilizes the enzyme. The Ca^{2+} ion disappears under pH 5.0 and thus reduces the stability of the enzyme [26].

The biocidal activity of the LPO results from the products of the chemical reactions that it catalyzes. Hypothiocyanate, which is the main product of the reaction, interacts with the thiol groups of various proteins, which is critical for the survival of pathogens. The impact of LPO on bacteria results from the oxidation of sulfhydryl. The oxidation of the -SH groups makes the bacterial cytoplasmic membrane lose its ability to transport glucose, potassium ions, amino acids, and peptides [45].

The biological significance of this enzyme results from the fact that it has a natural protection system against the invasion of microorganisms. Besides this antiviral effect, it is report-

ed that it protects animal cells against various damages and peroxidative effects [23, 29–31]. Lactoperoxidase is a significant agent of the defense system against pathogen microorganisms from the digestive system of neonatal babiess. The LPO enzyme functions as a natural compound of the non-immune biological defense system of mammals and it catalyzes the oxidation of the thiocyanate ion into the antibacterial hypothiocyanate [52].

Although peroxidases have similar catalytic mechanisms, they are distinguished for their ability to oxidize halides and pseudohalides. For example, only myeloperoxidase is able to oxidize bromine, iodine, and chlorine at neutral pH. Lactoperoxidase, on the contrary, can only oxidize iodine and thiocyanate but it either cannot or can hardly oxidize brome under the same conditions (**Figure 1**) [53].

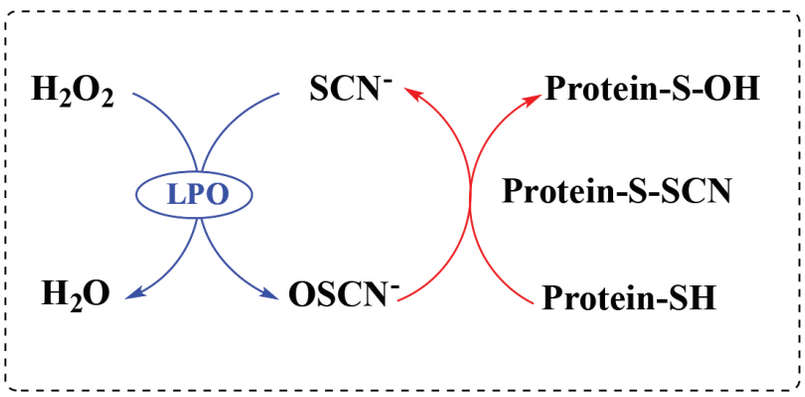

Figure 1. The activation mechanism of the peroxidase enzyme.

In the first step of this mechanism, the peroxidase enzyme reacts with one equivalent of the peroxide to form an Fe (IV)-containing compound I that is the porphyrin cation radical. In the second step, the cation radical takes a proton of the substrate and reduced (substrate)-Fe (IV) form, the substrate becomes radical to lost a proton. The compound II takes a proton from the substrate and return the first reducing form. Also radicalic substrate which are formed with interacting each other are polymerization [54].

The LPO enzyme is covalently bound to the heme group proteins, with the bond occurring between the hydroxyl group of the heme group and the carboxyl group of the protein [14]. Approximately 10% of the molecule is composed of carbohydrates and the molecule contains five main potential glycosylation regions and 15 semi-cysteine residues [11, 23, 45]. The heme group at the catalytic center is protoporphyrin IX, which is covalently bound to the polypeptide chain along with the disulfide bridge. The iron compound, which is a part of the heme group, constitutes 0.07% of LPO. The calcium ion is tightly bound to the enzyme, which ensures the molecular conformation and structural integrity of the enzyme [55].

1.3. Lactoperoxidase system in milk

The activation of the natural antibacterial system has been adopted for protecting raw milk. This system is defined as the lactoperoxidase/thiocyanate/hydrogen peroxide (LPO) system.

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The antibacterial effect of the system on milk is based on the oxidation of SCN^- ions catalyzed by the lactoperoxidase enzyme in the presence of H_2O_2. The short-lived components, $OSCN^-$ ions, generated in this oxidation reaction have bacteriostatic effect [56, 57]. The system is recommended in developing countries where there are no sufficient cooling facilities for collection of raw milk and their transportation to processing centers [57].

1.4. Antibacterial effect of LPO

LPO oxidized the -SH groups of the enzymes in bacteria such as hexokinase and glyceraldehyde-3-phosphate dehydrogenase and tends to lose biological functions of these enzymes. As a result, the bacterial cytoplasmic membranes are damaged structurally, and glucose, purine, pyrimidine, and amino acid uptake as well as protein, DNA, and RNA synthesis are blocked. Thus, bacteria growth and proliferation is prevented (**Figure 2**) [58].

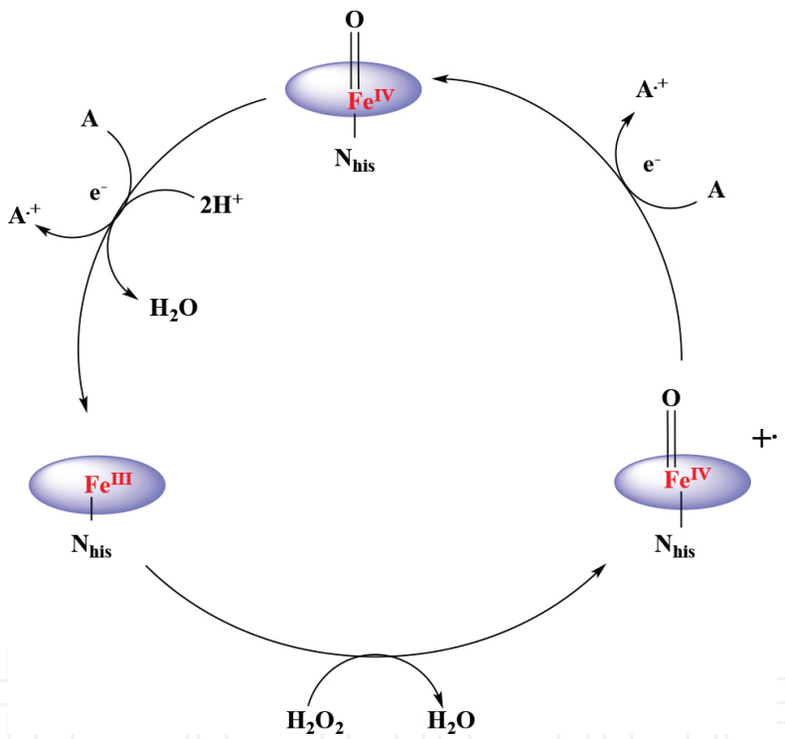

Figure 2. Antibacterial effect of the lactoperoxidase enzyme.

1.5. Lactoperoxidase system and health

Milk and milk products area rich sources of mineral, protein, vitamin, nutritional elements [56, 57]. Lactoperoxidase showed antifungal activity in apple juice and salt solution. In vitro findings also show that the LPO/H_2O_2/halide system has a strong virucidal activity against HIV-1 [59].

In conditions of iodine deficiency, the level of SCN ions in milk plays a vital role in thyroid function. The studies showed that when milk containing 19 ml/L thiocyanate ion and iodine

at 0.1 mg/l concentration is consumed, the thyroid functions of patients with iodine deficiency did not have any negative symptoms [60].

1.6. Determination of the lactoperoxidase activity with spectrophotometer

The measurement of the activity is based on the oxidation of 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) chromogenic substrate by H_2O_2 and observation of the increase in the absorbance caused by the resultant colored compound at 412 nm and pH: 6.0 [61]. For determination of LPO activity with spectrophotometric assay, 2.8 mL of ABTS (1 mM) and 0.1 mL of H_2O_2 (3.2 mM) were pipetted in the spectrophotometer tube of 3 mL. Enzyme solution of 0.1 mL was added and the tube was turned upside down before it was placed in the spectrophotometer. The increase in the absorbance was observed against blank at 412 nm for 3 min and recorded every 60 sec. Phosphate tampon (0.1 M) at pH: 6.0 was used as blank, instead of the enzyme, while all other solutions were used at the same rates. The following formula was used to determine the activity:

$$A = \varepsilon.b.c \qquad c = A/\varepsilon.b \qquad V = c.D_f$$

A : Absorbance (absorbance read at the end of 1 minute)

b : Path length (1 cm)

c : Concentration (μmol/mL)

ε : Extinction coefficient (32400 $M^{-1} \times cm^{-1}$)

D_f : Dilution coefficient

V : Velocity of the reaction (μmol/mL.min.)

1.7. Unit of enzyme activity

One LPO unit (EU) is defined as the amount of LPO that catalyzes the oxidation of l μmol of substrate (ABTS) per min at 20°C [52].

1.8. Substrates

2,2'-azino-bis(3-etilbenztiazolin-6-sülfonik asit (ABTS) [62], p-phenylenediamine [63], pyrogallol, guaiacol, catechol, phenols, aromatic amines, ascorbates, epinephrine, and tetramethylbenzidine [6, 14, 47, 52, 61].

1.9. Purification procedures

To date, several chromatographic methods have been reported about purification and characterization of the LPO enzyme from bovine milk [14]. For example, CM-Cellulose [64], CM-Sephadex ion-exchange chromatography [46, 64], Sephadex G-100 gel filtration chroma-

tography [6, 64], hydrophobic affinity chromatography on Phenyl-Sepharose CL-4B [5], and Toyopearl-SP cation exchange chromatography [64] are among the methods used in the purification of LPO enzyme from bovine milk. LPO was purified in one stage using the affinity technique and sulfanilamide was used as the ligand [65].

1.10. Kinetic studies

The K_m and V_{max} values are suitable parameters for kinetic studies. K_m is a substrate concentration at which half of the enzyme active sites are filled. V_{max} is an expression of the catalytic activity of the enzyme.

To find K_m and V_{max} values for LPO, activity was measured at 20°C, 412 nm, at pH 6.0, for five different substrate concentrations. For this purpose, generally 0.2–1.5 mL volume from the stock solution of the substrates was used. The total volume was made up to 2.8 mL with a buffer solution and then 0.1 mL enzyme and 0.1 mL H_2O_2were added. The K_m and V_{max} values were calculated from the Lineweaver-Burk graph [65, 66].

1.11. Procedure of enzyme inhibition

The K_i and IC_{50} values show the inhibitory effect on enzyme. The K_i and IC_{50} values depend on the inhibitory mechanism. IC_{50} is the inhibitor concentration required for 50% inhibition and K_i value is the constant.

To investigate the inhibitory effects of some inhibitors on LPO and to determine the IC_{50} values, the LPO activity was measured in the presence of five different concentrations of inhibitor**.** For example, the experimental procedure for inhibitor: A control sample without inhibitor was taken as 100% and an activity-[Inhibitor] plot was drawn. To determine the K_i, three different concentrations were used for x inhibitor. ABTS was also used as a substrate at five different concentrations. Lineweaver-Burk plots (1/V-1/[S]) were obtained for inhibitor; the K_i and the inhibition type were calculated from these plots [65].

1.12. Selection of the LPO inhibitors and the ligand

The inhibitors of the LPO enzyme were identified in studies on the enzyme [52, 67]. Non-selective monoamine reuptake inhibitors consist of opipramol, lofepramime, dibenzepin, protriptyline, melitracen, butriptyline, dimetacrine, dosulepin, and quinipramine; selective serotonin reuptake inhibitors include alaproclate and etoperidone; non-selective monoamine oxidase inhibitors comprise moclobemide, toloxatone, and isocarboxazid; and other antidepressants are viloxazine, minaprine, bifemelane, oxaflozane, and medifoxamine (**Table 1**).

Many inhibitors such as sulfanilamides [65], propofol and derivatives [68, 69], some anesthetic drugs [70], some bacteria [46], some phenolic acid compounds and phenolics [71, 77], avermectins [73], adrenaline, melatonin, serotonin and norepinephrine [45, 73, 74], fungi and bacteria [58], antibiotics [75], hydrazines [52], and some thiocarbamide compounds [67] are assayed and reported as a LPO inhibitor in the literature.

Inhibitor	IC_{50}	K_i	Inhibition type	Publication
L-Adrenaline	34.5 mM	2.26 mM	Noncompetitive	Sisecioğlu et al. [74]
Ceftazidime pentahydrate	0.048 mM	0.018 ±0.0035 mM	Competitive	Sisecioğlu et al. [76]
Prednisolone	0.053 mM	0.019 ±0.0005 mM	Competitive	
Amikacin sulfate	0.26 mM	0.04 ±0.015 mM	Competitive	
Ceftriaxone sodium	0.29 mM	0.10 ±0.055 mM	Competitive	
Teicoplanin	1.016 mM	0.13 ±0.022 mM	Competitive	
Melatonin	1.46 mM	0.82 ±0.28	Competitive	Sisecioğlu et al. [75]
Serotonin	1.29 mM	0.26 ±0.04	Competitive	
Norepinephrine	67.2 mM	62 mM	Noncompetitive	Sisecioğlu et al. 2010b
L-Ascorbic acid (Vitamin Q)	2.03 mM	0.508 ±0.257 mM	Competitive	Sisecioğlu et al. [43]
Menadione sodium Bisulfate (Vitamin K3),	0.025 mM,	0.0107 ±0.0044 mM,	Competitive	
Folic acid	0.0925 mM	0.0218 ±0.0019 mM	Competitive	
2,6-Dimethylphenol	836.67 nM	4442 nM		Koksal et al. 2014
2,6-Di-Tbutylphenol	10 nM	9 nM	Competitive	
Di(2,6-Dimethylphenol)	6.86 nM	0.53 nM.	Competitive	
Di(2,6-Di-Tbutylphenol)	185 nM	48.33 nM	Competitive	
Di(2,6-Diisopropylphenol)	154 nM	19.33 nM	Competitive	
Sulphanilamide	0.84 nM	3.57 nM	Competitive	Atasever et al. [65]
Emamectin benzoate	4.33 μM	6.82±2.60 μM	Competitive	Koksal et al. [73]
Eprinomectin	16.90 μM	4.80±1.95 μM	Competitive	
Moxidectin-Vetranal	99.00 μM	61.31±9.89 μM	Competitive	
Abamectin	138.60 μM	103.73±34.03 μM	Competitive	
Doramectin	173.20 μM	80.14±29.38 μM	Competitive	
Ivermectin	231.00 μM	519.97±47.62 μM	Noncompetitive	
Caffeic acid	393.61 nM	430.033±79.04 nM	Competitive	Gulcin et al. [72]
Ketamine	0.29 mM	0.019 ± 0.031	Noncompetitive	Ozdemir et al. [70]
Bupivacaine	0.155 mM	0.015 ±0.021 mM	Noncompetitive	

Table 1. Inhibitors of lactoperoxidase enzyme.

1.13. Concentration

LPO is the second most abundant whey enzyme in bovine milk, [31, 77] and its concentration is approximately 30 mg/L [23]. The peroxidase activity in cow milk is 20 times richer than in human milk and contains 1.2–19.4 units/mL LPO [78]. The mean LPO enzyme activity varies

in different species, for example, 1.4 units/mL in cow, 0.34–2.38 units/mL in lamp, 1.5–4.45 units/mL in goat, 0.794 units/mL in buffalo, 22.0 units/mL in pig, and 0.06–0.97 units/mL in human [78].

1.14. Application fields

There is a growing interest in the purification of LPO with increasing applications. The LPO system's natural biological functions are preferred against antimicrobial chemicals. Lactoperoxidase has many fields of application. It is widely used especially in milk-processing facilities in the milk industry [79]. LPO is used in milk and cheese to reduce the microflora [23]. The LPO enzyme derived from various animal sources has a significant role in the suppression of bacterial growth and helps bacterial inhibition. Inhibition of bacterial growth by the bovine LPO is attributed to the peroxidase system, which contains H_2O_2 and thiocyanate [9]. The antimicrobial effect of the LPO system occurs naturally in milk. LPO has a bacteriostatic effect on gram-positive and gram-negative bacteria. The antibacterial studies on the LPO enzyme purified from camel milk show that the LPO-thiocyanate and peroxide system leads to significant inhibition of pathogenic bacteria.

Most popular applications of the system include food production for preservation of raw milk, pasteurized milk, and cheese milk during storage and/or transportation to the processing plants. The system can be used to avoid the suppression of acidity in yoghurt. In the absence of refrigeration, LPO system is preferable [80] and the system can be used to extend the shelf-life of pasteurized, raw, and cheese milk [79, 81]. This is used for the preservation of emulsions and cosmetics.

The LPO system can ensure an extensive spectrum of antimicrobial properties against bacteria and yeasts when it is composed of LPO, H_2O_2, SCN^-, and I_2. Hence, it is preferred in oral care products and cosmetic preservation [82, 83]. The system is used against fish pathogenic bacteria in aquaculture with strong bacterial effects.

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