



# Molecular and Functional Properties of Milk - MFPM

## Group 05 project: Milk Enzymes - Membrane Associated Enzymes

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Link to Git repo.: [https://github.com/DanishUnicorn/mfpm\\_group\\_05](https://github.com/DanishUnicorn/mfpm_group_05)



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# Chapter 1

## Membrane-Associated Enzymes

### 1.1 Introduction

Many enzymes have been identified in milk fat globules (MFGs) and extracellular vesicles (EVs), with advancements in proteomic techniques continuously expanding this knowledge. However, many of these enzymes are present in low abundance and often originate from endoplasmic reticulum (ER), Golgi membranes, or cytosolic remnants. A large proportion remains inactive in milk due to the absence of relevant substrates or a suitable environment. This chapter focuses on sulfhydryl oxidase, catalase, lactoperoxidase (LPO), xanthine oxidoreductase (XOR),  $\gamma$ -glutamyltransferase (GGT), and 5'-nucleotidase, discussing their relevance in mammary gland biology, milk integrity, and physiological function upon consumption [2].

### 1.2 Classification of Mechanisms of Enzymes

Sulfhydryl Oxidase (EC 1.8.3.2) is an enzyme that helps form chemical bindings in proteins by catalysing the oxidation of protein thiols (SH-groups) to form disulphides (-S-S-) while simultaneously reducing oxygen to hydrogen peroxide. In milk, both metal-dependent and flavin-dependent sulfhydryl oxidase types have been identified, where the flavin-dependent sulfhydryl oxidase is the most common in bovine milk [2].

Catalase (EC 1.11.1.6) is an enzyme found in almost all living organisms exposed to oxygen. It catalyses hydrogen peroxide to water and oxygen.



From the given material, it has not been possible to detect catalase in milk 1.1. When catalase is detected in milk, it is due to somatic cells or contamination, such as when the cow has mastitis [2].

Lactoperoxidase (EC 1.11.1.7) is a heme containing peroxidase enzyme. Found in large quantities in bovine milk (30mg/L) where it functions as an antimicrobial defence by oxidizing thiocyanate to hypothiocyanite (se formlen). That is why the amount of lactoperoxidase is higher if the if the cow has mastitis. Has an antimicrobial activity against both Gram-positive and Gram-negative bacteria. When it bounds to calcium it is very heat stable up to 80°C [2]. Oxidation of thiocyanate to hypothiocyanite can be seen in equation 1.2.



**Table 6.1** Selected list of enzymes reported to be associated with milk fat globules (MFG) or extracellular vesicles (EV)

Enzyme	Enzyme number	UniProt/ gene name	Presence by proteomics in bovine/human MFG and/or EV	Comments
Sulphydryl oxidase	EC 1.8.3.2	F1MMU52/QSOX1	MFG <sup>a,b</sup>	Flavotype
Catalase <sup>c</sup>	EC 1.11.1.6	P00432	n.d.	From somatic cells/ microorganisms
Lactoperoxidase <sup>c</sup> (LPO)	EC 1.11.1.7	P80023	MFG <sup>a,b,d,e</sup> , EV <sup>b</sup>	Abundant in skim milk and whey
Xanthine oxidoreductase (XOR)	EC 1.17.3.2 XO EC 1.17.1.4 XD	P80457	MFG <sup>a,b,d,e,f</sup> , EV <sup>b,g</sup>	Associates with butyrophilin/ adipophilin
$\gamma$ -Glutamyltransferase (GGT)	EC 2.3.2.2 (EC 3.4.19.13)	G3N2D8 (human P19440)	MFG <sup>a,b</sup> , EV <sup>b</sup>	Heterodimer
5'-Nucleotidase	EC 3.1.3.5	Q05927	MFG <sup>a,d</sup> , EV <sup>b</sup>	Ecto-5'-nucleotidase
Alkaline phosphatase	EC 3.1.3.1	P09487 (tissue non-sp)	MFG <sup>a</sup> , EV <sup>b</sup>	Described in Chap. 4
Lipoprotein lipase		P11151	MFG <sup>a,b,c</sup> , EV <sup>b</sup>	Described in Chap. 10

<sup>a</sup>Liao et al. (2011)<sup>b</sup>Reinhardt et al. (2013)<sup>c</sup>Suggested not to be classified as a "true" MFG or EV enzyme<sup>d</sup>Fong et al. (2007)<sup>e</sup>Ma et al. (2019)<sup>f</sup>Reinhardt and Lippolis (2006)<sup>g</sup>Admyre et al. (2007)

Figure 1.1: Table 6.1 from the book with the selected enzymes in the chapter [2].

Xanthine Oxidoreductase (XOR) (EC 1.17.3.2) is an enzyme that has been identified in both skim milk and cream phospholipid membranes. It is released from the Milk Fat Globule Membrane (MFGM) during phase inversion. Significant amounts can be removed through washing with non-ionic detergents or high salt concentrations. XOR is classified as a peripherally associated membrane protein with both soluble and insoluble properties, due to its ability to associate with membranes while also being extractable under certain conditions. It has been detected in extracellular vesicles (EVs) and MFGs in both human and bovine milk. Proteomic studies have further confirmed the presence of XOR in extracellular vesicles (EVs) and milk fat globules (MFGs) in both human and bovine milk [2].

$\gamma$ -Glutamyltransferase (EC 2.3.2.2) catalyses transfer of  $\gamma$ -glutamyl groups from glutathione to amino acids, peptides, or water. Found in cell membranes and has been suggested to aid amino acid supply for milk protein synthesis. Primarily located in skim milk rather than MFGM (74.3% vs 6.6%) [2].

## 1.3 Role of Enzymes in Milk and Dairy Products

In the following section, we will briefly discuss the effects of membrane-associated enzymes on milk and dairy products, focusing on their role in protein modification and lipid metabolism through oxidative reactions [2].

### Sulphydryl Oxidase

Sulphydryl oxidase forms disulfides by catalysing the oxidation of protein thiols, while simultaneously reducing oxygen to hydrogen peroxide [2]. It has been suggested that sulphydryl oxidase may contribute to the oxidation process by inhibiting the formation of sulfur-containing compounds, such as methyl ketones and aldehydes, which are responsible for the “cooked” and “flat” flavours in UHT milk [2].

### Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) is found in MFGs and EVs and has been proposed as a protein marker due to its association with microRNAs. Under aerobic conditions, XOR exhibits antimicrobial effects through the production of superoxide and peroxynitrite. Additionally, XOR generates hydrogen peroxide, which serves as a substrate in the LPO system.

### $\gamma$ -Glutamyltransferase

$\gamma$ -glutamyl is transferred from glutathione to amino acids by  $\gamma$ -glutamyltransferase (GGT). This enzyme is primarily located in skim milk, where 74.3% of its activity is found. Only 6.6% is associated with MFGM, suggesting that GGT has various roles in milk biochemistry [2]. The optimal pH for GGT is 8.5–9.0, and it has been suggested as an indicator for thermal processes at 70–80°C due to its heat stability [2].

### 5'-Nucleotidase

5'-Nucleotidase are enzymes in MFGM that is suggested to play a role in milk secretion. It are found in large quantities in human milk but are lower in bovine milk [2]. It is hypothesized that large quantities in human milk helps break down nucleotides into absorbable nucleosides for newborns, as they have limited ability to digest nucleotides [2].

## 1.4 Summary of Recent Scientific Studies

To limit the scope of this report, it was decided to investigate one enzyme from the given literature [2]. Lactoperoxidase was selected due to its antimicrobial properties and a relevant scientific study by Aouadhi et al. (2024) was identified and used in current section.

Lactoperoxidase (LPO) combined with hydrogen peroxide and thiocyanate forms a well described system called the Lactoperoxidase System (LPS), known for its antimicrobial properties through production of hypothiocyanite which impairs protein functions in organisms like *E. coli*, yeasts, and moulds [1]

A recent study by Aouadhi et al. (2024) investigated the impact of combining the LPS system with nisin and/or heat treatment to enhance the microbial safety and shelf life of raw milk products through decrease of microbial load. Nisin was chosen as it increases membrane permeability thereby facilitate transport of

hypothiocyanite through membranes to impair cellular proteins. In contrast, heat treatment reduces the initial microbial load, enhancing the system's efficacy.

As noted in [2], the LPS system remains inactive in bovine milk due to low concentrations of hydrogen peroxide and thiocyanate. Aouadhi et al. (2024) confirmed this and activated the system by adding hydrogen peroxide and thiocyanate to the raw milk. Following activation, nisin was added in different concentrations, and samples were incubated at 25°C for 72 hours. Microbial analysis following the experiment revealed:

- 6-log reduction in total mesophilic bacteria
- 5-log reduction in coliforms
- 4-log reduction in yeasts and moulds

These reductions were achieved with 7 mg/L SCN, 15 mg/L hydrogen peroxide, and 50 IU/mL nisin after 72 hours of incubation at 25°C.

With the optimal concentrations an experiment was prepared including pasteurization. The LPS system was activated for 2, 4 and 6 hours before pasteurization at 63°C for 30 minutes and subsequent incubation at 4°C and 25°C for 96 hours. All samples were analysed for total bacterial count, coliforms, yeasts and mould. Results for coliforms are shown below in figure 1.2:

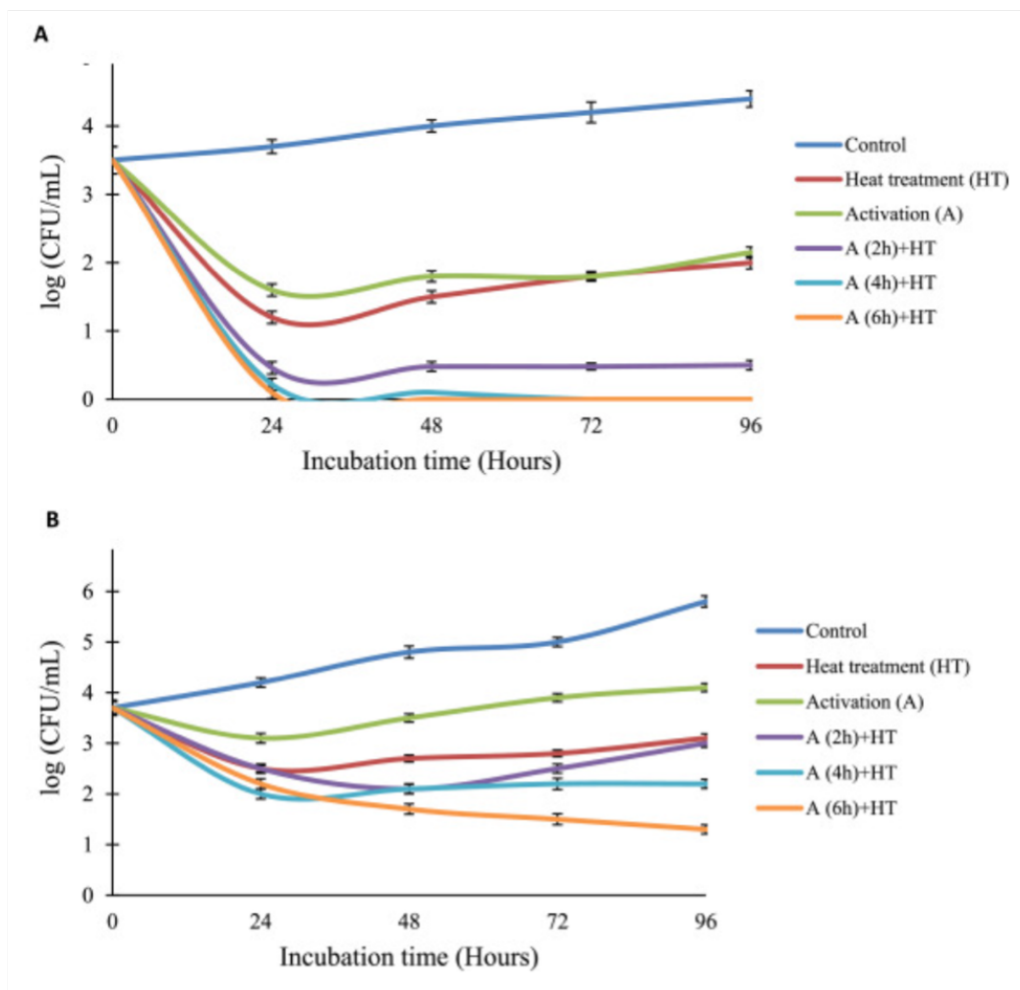


Figure 1.2: Evolution of total coliforms in raw cow milk according to different treatments at 4°C (A) and 25°C (B) for 96 h [1]

From 1.1, it is evident how the concentration of coliforms steadily increases in the control sample, incubated without any treatment. In contrast, the heat-treated sample show an initial decrease in coliforms, followed by an increase after 24 hours of incubation at both temperatures (4°C and 25°C). Samples where LPS was activated before heat treatment shows the greatest log reduction in coliforms when stored at 4°C for 96 hours. Although a significant reduction is also observed at 25°C, the effect is less pronounced compared to storage at 4°C, highlighting the impact from incubation temperature on microbial growth.

Identical results were obtained for yeasts, and moulds with complete destruction at 4°C with 4 and 6-hour LPS activation before heat treatment.

The article concludes that LPS activation cycles of 4 and 6 hours before heat treatment (pasteurization) show promising results in reducing the microbial load of milk and thereby the quality. This while utilizing an already present enzyme in milk, LPO, combined with a food grade approved preservative nisin.

## 1.5 Conclusion

The enzymes examined in the book chapter contribute significantly to milk stability, flavour, and safety by various means. Sulphydryl oxidase and XOR impact oxidative stability and antimicrobial defence, while GGT and 5'-nucleotidase influence milk protein utilization. The research from Aouadhi et al. (2024) demonstrates the potential of LPO activation in microbial control, offering a natural and effective strategy for improving dairy product shelf life. Continued research of these enzymes can lead to improved processing techniques and functional dairy applications.

# Bibliography

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