IMPACT OF COMMERCIAL STABILIZERS ON PROTEASE-INDUCED MILK DETERIORATION

Lucas F. Almeida[1*], Isabel F. Vaz[1], Solimar G. Machado[2], Antônio F. de Carvalho[2] & Gabriela P. Maitan-Alfenas[1].

[1] Department of Biochemistry and Molecular Biology, Federal University of Vicosa, Vicosa, Brazil.
[2]. Department of Food Technology, Federal University of Viçosa, Viçosa, Brazil.
** Corresponding author's email address: lucas.filipe@ufv.br*

ABSTRACT

Milk is susceptible to spoilage by psychrotrophic bacteria like Pseudomonas, causing economic losses due to the formation of a compact gel from peptides and hydrolyzed proteins. The enzyme AprX, a heat-resistant extracellular metalloprotease, accelerates age gelation by hydrolyzing caseins and it remains active even after UHT treatments. Thus, methods like additives seem to be effective in slowing the gelling process by protease activity, though further research is needed for commercial-scale application. This study aimed to understand the effects of some milk stabilizers on AprX activity. Enzymatic activity was measured using the Azocasein method and partial purification was confirmed by SDS-PAGE. The effects of commercial stabilizers on enzyme activity were tested at 0.05% concentration. Hydrolysis products were analyzed using RP-HPLC to assess the degree of proteolysis in milk treated with stabilizers and AprX. Results showed no difference between skimmed or whole milk for inducing AprX production, but the minimal growth medium is preferable. Stabilizers do not directly affect the enzyme but reduce its activity in the milk matrix. Hydrolysis effects were observed, with casein levels decreasing over time even with additives. Further research is needed to understand the long-term effects of ions on milk stability.

Keywords: Metalloprotease thermoresistant. *Pseudomonas fluorescens*. Milk spoilage. Proteolysis. Additives.

1 INTRODUCTION

Milk is a rich source for the growth of microorganisms, primarily psychrotrophic bacteria such as the genus Pseudomonas. This microbiological contamination represents substantial economic losses for the dairy industry, as it leads to the spoilage of milk and its derivatives[1,2]. The main effect observed is the formation of a compact gel composed of peptides and hydrolyzed milk proteins.

Age gelation is an irreversible condition, described as coagulation with the formation of lumps, occurring after storing the product for weeks to months[1]. AprX has been reported to promote and accelerate age gelation by mediating the hydrolysis of α, β, and κ-caseins. This enzyme, an extracellular metalloprotease, is known for its heat resistance, maintaining its activity even after undergoing UHT treatments and milk pasteurization. While heat treatments typically used in the dairy industry effectively destroy psychrotrophic microorganisms, their extracellular proteases generally remain unaffected.

As a result, AprX spoilage is mainly controlled by implementing good hygiene practices and maintaining low storage temperatures. However, it is necessary to study new methods to develop efficient treatments for AprX inactivation. Some alternatives to explore include the use of new temperature or time conditions, such as innovative steam injection, high-pressure homogenization, ultrasound, pulsed electric fields, and chemical additives[1]. Additionally, further research is needed to explore the feasibility of applying some of these methods to commercial-scale dairy processing, considering issues related to costs, infrastructure, and the addition of operational units.

Additives such as phosphates and citrates, commercially used as stabilizers, can negatively affect the action of AprX in milk and delay the gelation process, preventing the premature loss of product quality. The mechanism by which this works is not yet fully understood, but these additives may act as inhibitors within the milk matrix, creating a barrier that interferes with the interaction with protein micelles. Moreover, they represent a relatively simple method already commonly used in the industry. Therefore, this study aimed to evaluate the production of enzymes in the presence of whole and skimmed UHT milk and to study the effect of ions on the reduction of proteolytic activity in the dairy matrix.

2 MATERIAL & METHODS

Production of Cell-Free Enzyme Extract:

The production of the enzyme extract was evaluated for the extracellular protease activity, AprX, from *Pseudomonas fluorescens* 07A grown in skim and whole UHT milk and minimal media (KH_2PO_4 7 g/L, K_2HPO_4 2 g/L, $MgSO_4$ 0.2 g/L, $(NH_4)_2SO_4$ 0.1 g/L, and glycerol 4% (v/v)) containing 2% (v/v) of skim and whole UHT milk. All growth substrates were inoculated with $3.55x10^7$ CFU/mL and incubated at 28 °C for 72 hours. Under sterile conditions, aliquots were taken at 0, 6, 12, 24, 36, 48, 60, and 72 hours, centrifuged at 10,800 g for 30 minutes, and the supernatant was frozen for later analysis. The experiment was performed in biological triplicates.

Enzymatic Activity and Total Protein Assays:

Proteolytic activity was measured using the Azocasein colorimetric method with three technical replicates. In microtubes, 50 µL of the supernatant from each extract was added to 250 µL of 2% (w/v) azocasein diluted in 120 mM Tris-HCl buffer, pH 8.0, homogenized, and then incubated at 37 °C for 1 hour. After this time, 600 µL of 20% (w/v) TCA was added as a stop reagent, and the mixture was centrifuged for 10 minutes at 10,000 g. Reaction controls included a blank containing water and substrate and another microtube containing substrate, stop reagent, and enzyme extract before incubation. Absorbance was read in a cuvette at 366 nm. One enzyme unit was defined as the amount of enzyme capable of causing a 0.01 change in absorbance. Total proteins were quantified using the Bradford method[3], and the concentration was confirmed by SDS-PAGE[4].

Partial Purification of the Enzyme Extract:

The extract was purified using a 30 kDa membrane by centrifugation at 4,000 g for 1 hour at 4 °C. The filtrate was stored at -20 °C for subsequent tests with stabilizers. Partial purification was confirmed by the application of 15 µg of protein in SDS-PAGE[4].

Interference Effect Test with Commercial Stabilizers:

The test was performed by measuring enzymatic activity, using the crude and partially purified extract, in the presence of additives commercially used as milk stabilizers at a concentration of 0.05 % (w/v). The stabilizers used were polyphosphates, pyrophosphates, tripolyphosphates, orthophosphates, and trisodium citrate, all solubilized in reaction buffer. The results were expressed as relative activity concerning a control without the additive. ANOVA test with significance below 0.05% and Tukey's test were applied to evaluate statistical differences.

Incubation of UHT Milk with Stabilizers and AprX:

Firstly, Santa Clara skimmed UHT milk powder was previously analyzed to account for mesophilic aerobic bacteria, mesophilic aerobic spores, and thermophilic aerobic spores, ensuring no microbiological contamination. After, to simulate real conditions, reconstituted skimmed milk was added with additives separately and inoculated with 116 U/mL of AprX after 40 minutes. The bottles were subjected to heat treatment, equivalent to UHT, for 8 minutes and 45 seconds at 94 °C, then immediately cooled in an ice bath. Bottles without the enzyme and another without the additive were used as reaction controls. The experiment was conducted under aseptic conditions for 5 days under temperature control, 25 °C. The entire experiment was performed in triplicates, and aliquots were collected at 0, 2, 6, 12, 24, 48, 72, 96, and 120 hours. The results were presented as relative activity, measured using the Azocasein method, and compared to the control at time zero. ANOVA test with significance below 0.05 % and Tukey's test were applied to evaluate statistical differences.

Measuring Degree of Hydrolysis of Milk by RP-HPLC:

The hydrolysis products of milk incubated with stabilizers and AprX were evaluated by reverse-phase high-performance liquid chromatography (RP-HPLC). The samples were centrifuged at 10,000 g for 20 minutes and filtered through a 0.45 µm membrane before injecting 20 µL into the chromatograph with an Aeris™ Widepore 3.6 µm XB-C18 (250 x 4.6 mm) column. The run was performed with a flow rate of 1.1 mL/min, a column oven temperature of 40 °C, with detection at a wavelength of 214 nm, and an elution gradient starting at 72 % phase A (0.1% trifluoroacetic acid in deionized water) and 28 % phase B (0.1% trifluoroacetic acid in acetonitrile) to a ratio of 48:52 % phase A and phase B over 20 minutes. Analytical curves with external standards of α-, β-, and κ-casein, α-lactalbumin, and β-lactoglobulin were constructed by linear regression, and the results were expressed in g/L.

3 RESULTS & DISCUSSION

Both skimmed and whole milk could induce the production of AprX, but the enzymatic activity was higher in pure milk compared to the minimal medium. Whole milk exhibited greater enzymatic activity than skimmed milk up to 24 hours, but thereafter, skimmed milk was found to be a more efficient inducer of AprX production compared to whole milk. The highest activity in skimmed milk was 10,131.56 (±391.57) U/mL and 5,786.89 (±295.41) U/mL in whole milk at 60 and 36 hours, respectively. The *aprX* gene is part of the aprX-lipA2 operon, regulated by a single promoter located upstream of *aprX* gene[5]. Additionally, the production of extracellular protease and lipase by *P. fluorescens* strains is inhibited by iron and influenced by temperature, meaning that medium composition, time and temperature of incubation impact its production[6]. Milk is a homogeneous mixture of various substances, including sugars, proteins, salts, and vitamins, some in emulsion, others in suspension, or completely in solution. However, the fat concentration, which varies between 3.2 and 6%, is the component that fluctuates the most, influenced by cattle breeding and production factors[7]. Therefore, as expected, whole milk has higher concentrations of fat and minerals than skimmed milk, potentially affecting positively the production of AprX in the first hours of inoculation. Although enzymatic activity on skimmed milk substrate was lower than whole milk up to 24 hours, it was observed that *P. fluorescens* 07A produced much more pigment in the presence of skimmed milk than whole one. The skimmed milk was completely deteriorated and had turned fluorescent green at 24 hours. This fluorescent pigment is a siderophore, which acts as an iron-chelating molecule and imports this mineral into the cell, being essential for the regulation of *aprX* expression and bacteria growth among Pseudomonas genera[8]. Thus, this may contribute to higher activity on this substrate from the first day of microbial growth.

Among the minimal growth medium, the induction of whole milk was more effective for the first 24 hours, leading to the trend of pure milk. AprX reached a maximum of 12,60.33 (±45.73) U/mL in the minimal medium with 2% skimmed milk and 1,098.44

(±14.30) U/mL in whole milk at 36 and 48 hours, respectively. Thus, the minimal medium with skimmed milk was chosen to produce AprX from an inoculum of *P. fluorescens* 07A. Although pure milk as a culture medium led to higher protease production, it was not the best option for the partial purification step of the extract. Milk is a matrix rich in soluble proteins and other macromolecules, so the specific activity was about 10 times lower in milk than in the minimal growth media with traces of milk. Therefore, more efforts would be necessary to purify and concentrate the extract from AprX produced in the pure milk growth medium. In the partially purified extract, a lower band profile was observed compared to the crude extract on SDS-PAGE, with the presence of AprX band at approximately 49 kDa. The specific activity also confirmed the purification, increasing from 22.37 (±0.83) U/mg in the crude extract to 39.03 (±0.96) U/mg in the partially purified extract.

Both the crude extract and the partially purified extract were evaluated in the assay with interferents. The relative activity values ranged from 88.55 (±2.08) % to 103.87 (±5.29) %, but there was no statistically significant difference compared to the control without the additive. However, a new assay was conducted with the extracts diluted 5 and 20 times for the partially purified and crude extracts, respectively, to evaluate the effect on a lower enzyme load. After all, the remaining concentration in commercial milk that leads to a long gelation process is much lower than that used in these assays. Nevertheless, no significant decrease in enzymatic activity was observed, indicating that the stabilizers do not seem to directly affect AprX activity. Instead, they may stabilize the casein micelles in milk and prevent the enzyme from acting on its natural substrate.

The enzymatic assay conducted with the supernatant of reconstituted milk in the presence of the stabilizers showed that the control without ions always exhibited the highest relative activity. While sodium citrate, tripolyphosphate, and orthophosphate showed 49.86 (±5.42), 47.30 (±9.04), and 43.04 (±6.63) % activity at 24 hours, respectively. This corresponded to an average reduction of over 50 % in AprX activity, and no milk gelation was observed up to 24 hours. The other phosphates also showed reductions in enzymatic activity, but these were less than 30 %. This reduction was statistically significant compared to the control, but at 120 hours, all activity decreased and stayed identical. Previously microbiological analyses ruled out the possibility of external interferents, aside from the inoculated enzyme extract.

Since the enzymatic colorimetric method has limitations, the degree of proteolysis was evaluated by RP-HPLC. It was noticed that more peaks, related to hydrolysis products, were present in samples from the control with AprX and without additives. Generally, no considerable changes in β-casein were observed among the samples within 24 hours; however, the content of κ- and α-casein decreased over time. The α-casein experienced the most significant reductions in peaks, especially in the control without stabilizers. Larger reductions in α-casein were expected, as it constitutes most casein micelles[7], making it the predominant substrate. No changes were observed in α-lactalbumin and β-lactoglobulin in the presence of AprX.

4 CONCLUSION

This work showed a potential alternative to control the gelation process of stored milk. Although the methodology still requires some fine adjustments, such as the amount of enzyme that leads to age gelation, either this method is sufficient or needs to be combined with another.

REFERENCES

[1] ZHANG, C.; BIJL, E.; SVENSSON, B.; HETTINGA, K. 2019.The Extracellular Protease AprX from Pseudomonas and Its Spoilage Potential for UHT Milk: A Review. Compr Rev Food Sci Food Saf. 18 (4). 834–852. https://doi.org/10.1111/1541-4337.12452.

[2] POLÊTO, M. D.; ALVES, M. P.; LIGABUE-BRAUN, R.; ELLER, M. R.; DE CARVALHO, A. F. 2019. Role of Structural Ions on the Dynamics of the Pseudomonas Fluorescens 07A Metalloprotease. Food Chem. 286. 309–315. https://doi.org/10.1016/J.FOODCHEM.2019.01.204.

[3] BRADFORD, M. M. A. 1976. Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal Biochem. 72 (1–2). 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

[4] BLUM, H.; BEIER, H.; GROSS, H. J. 1987. Improved Silver Staining of Plant Proteins, RNA and DNA in Polyacrylamide Gels. Electrophoresis. 8 (2). 93–99. https://doi.org/10.1002/ELPS.1150080203.

[5] MAIER, C.; HUPTAS, C.; VON NEUBECK, M.; SCHERER, S.; WENNING, M.; LÜCKING, G. 2020. Genetic Organization of the AprX-LipA2 Operon Affects the Proteolytic Potential of Pseudomonas Species in Milk. Front Microbiol. 11. https://doi.org/10.3389/fmicb.2020.01190.

[6] ALVES, M. P.; SALGADO, R. L.; ELLER, M. R.; DIAS, R. S. 2018.Oliveira de Paula, S.; Fernandes de Carvalho, A. Temperature Modulates the Production and Activity of a Metalloprotease from Pseudomonas Fluorescens 07A in Milk. J Dairy Sci.101 (2). 992–999. https://doi.org/10.3168/JDS.2017-13238.

[7] HORVATH, A.; FUXREITER, M.; VENDRUSCOLO, M.; HOLT, C.; CARVER, J. A. 2022. Are Casein Micelles Extracellular Condensates Formed by Liquid-Liquid Phase Separation? FEBS Lett. 596 (16). 2072–2085. https://doi.org/10.1002/1873-3468.14449.

[8] VINDEIRINHO, J. M.; SOARES, H. M. V. M.; SOARES, E. V. 2021. Modulation of Siderophore Production by Pseudomonas Fluorescens Through the Manipulation of the Culture Medium Composition. Appl Biochem Biotechnol. 193 (3). 607–618. https://doi.org/10.1007/S12010-020-03349-Z/FIGURES/5.

ACKNOWLEDGEMENTS

The authors thank the Research Support Foundation of the State of Minas Gerais (Fundação de Amparo à Pesquisa do Estado de Minas Gerais-FAPEMIG APQ-01251-22), National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq), Higher Education Personnel Improvement Coordination (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and ICL Group for the financial support of the work.