

MAURA PINHEIRO ALVES

**PURIFICATION, CHARACTERIZATION AND EVALUATION OF THE
DIFFERENTIAL EXPRESSION OF PROTEASES PRODUCED BY
*PSEUDOMONAS FLUORESCENS***

Dissertation thesis presented to
Universidade Federal de Viçosa as part of
the requirements for the PostGraduate
Program in Food Science and Technology
to obtain the title of Doctor Scientiae.

VIÇOSA
MINAS GERAIS – BRASIL
2017

Ficha catalográfica preparada pela Biblioteca Central da Universidade
Federal de Viçosa - Câmpus Viçosa

T

A474p
2017
Alves, Maura Pinheiro, 1988-
Purification, characterization and evaluation of the
differential expression of proteases produced by *Pseudomonas
fluorescens* / Maura Pinheiro Alves. – Viçosa, MG, 2017.
xv, 91f. : il. (algumas color.) ; 29 cm.

Orientador: Antônio Fernandes de Carvalho.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Leite. 2. Microbiologia. 3. Tecnologia de Alimentos. 4.
Pseudomonas fluorescens. I. Universidade Federal de Viçosa.
Departamento de Tecnologia de Alimentos. Programa de
Pós-graduação em Ciência e Tecnologia de Alimentos. II. Título.

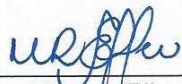
CDD 22 ed. 637.1

MAURA PINHEIRO ALVES

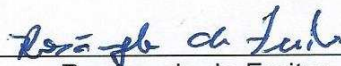
**PURIFICATION, CHARACTERIZATION AND EVALUATION OF THE
DIFFERENTIAL EXPRESSION OF PROTEASES PRODUCED BY
*PSEUDOMONAS FLUORESCENS***

Dissertation thesis presented to
Universidade Federal de Viçosa as
part of the requirements for the
PostGraduate Program in Food
Science and Technology to obtain
the title of Doctor Scientiae.

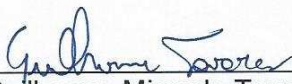
APROVED: February 17, 2017.



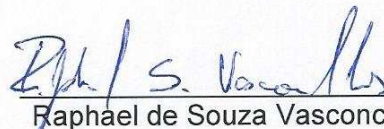
Monique Renon Eller
(Co-advisor)



Rosângela de Freitas
(Co-advisor)



Guilherme Miranda Tavares



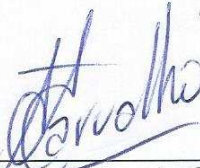
Raphaél de Souza Vasconcellos



Evandro Martins



Cláudia Lúcia de Oliveira Pinto



Antônio Fernandes de Carvalho
(Advisor)

I dedicate to the loves of my life
My parents, Eny and Mauro, and my husband, Luiz.

Acknowledgments

To God, for my life, family and for the providence.

To Universidade Federal de Viçosa and to Department of Food Technology, for the opportunity, material and structure support.

To CAPES, for the financial support to this research.

To my advisor, professor Antônio Fernandes de Carvalho, for the advice, trustworth, motivation, for the transmitted knowledgments and mainly for all the words of incentive.

To my co-advisor, professor Monique Renon Eller for the teachings, motivation, friendship, attendance and assistance in carrying out each stage of this work.

To my co-advisors, professor Ítalo Tuler Perrone and Dr. Rosângela de Freitas, for their attention and knowledge.

To professors Guilherme Miranda Tavares, Raphael de Souza Vasconcellos and Dr. Evandro Martins and Dra. Cláudia Lúcia de Oliveira Pinto for the availability and advice.

To Rafael Salgado, for the valuable suggestions in the realization of each stage of this work and for friendship.

To professor Maria Cristina Dantas Vanetti (Laboratory of Microbiology of Food of the Department of Microbiology of the UFV) for available the bacterial culture used in this research.

To Núcleo de Análise de Biomoléculas of the Universidade Federal de Viçosa, for providing the facilities for the conduction of part of the experiments, and to Pedro Vidigal, Edvaldo Barros and professor Humberto Ramos, for assisting the experimental design for sequencing.

To Laboratory of Molecular Immunovirology, for providing the facilities for the conduction of the Real Time PCR experiments, and to Roberto Dias, for the suggestions and contributions.

To the colleagues of the Inovaleite Laboratory, for the encouragement and the harmonious conviviality.

To my parents, Eny e Mauro, for all love, care, support, dedication and example of life.

To Luiz, for making my days more joyfull, for the understanding, love and companionship.

And to my loves Cecília, Ana Luiza, João Gabriel, Tiago, Murilo and Otávio for making my life happier.

Anyway, to all who stood by my side and who contributed to this realization, my thanks!

TABLE OF CONTENTS

LIST OF ABBREVIATIONS	viii
LIST OF FIGURES	ix
LIST OF TABLES	xi
ABSTRACT	xii
RESUMO	xiv
1. GENERAL INTRODUCTION	1
2. CHAPTER 1: LITERATURE REVIEW	4
PROTEOLITIC ACTIVITY OF PSYCHOTROPHIC BACTERIA IN MILK AND DAIRY PRODUCTS	5
Abstract.....	5
System of refrigeration of raw milk.....	5
Psychrotrophic microorganisms in milk.....	7
Adaptation of psychrotrophic microorganisms at low temperatures	9
Proteolysis in the dairy industry	10
Proteolytic enzymes in milk.....	12
Proteolytic enzymes from psychrotrophic bacteria.....	14
References.....	17
3. CHAPTER 2: CHARACTERIZATION OF THE PROTEASE FROM <i>PSEUDOMONAS FLUORESCENS</i> 07A	25
CHARACTERIZATION OF A HEAT-RESISTANT EXTRACELLULAR PROTEASE FROM <i>PSEUDOMONAS FLUORESCENS</i> 07A SHOWS THAT LOW TEMPERATURE TREATMENTS ARE MORE EFFECTIVE IN DEACTIVATING ITS PROTEOLYTIC ACTIVITY	26
Interpretive Summary: Protease characterization and control of milk degradation	26
Abstract.....	27
Introduction	28
Material and methods	29
<i>Bacteria and Culture Conditions</i>	29
<i>Proteolysis Assay</i>	29
<i>Protease Purification</i>	30
<i>Protease Characterization</i>	31

<i>Effect of Temperature and pH on Enzyme Activity</i>	31
<i>Effect of Inhibitors and Metallic Ions on Enzyme Activity</i>	31
<i>Enzyme Thermostability</i>	32
<i>Protease Sequencing Using MALDI TOF/TOF</i>	32
<i>Identification of the Gene Encoding the Extracellular Protease on the Bacteria P. fluorescens 07A Genome</i>	33
Results	34
<i>Protease Purification</i>	34
<i>Effect of Temperature and pH on Enzyme Activity</i>	35
<i>Effect of Inhibitors and Metallic Ions on Enzyme Activity</i>	36
<i>Protease Thermostability</i>	38
<i>Identification of the Gene Encoding the Extracellular Protease on the Bacteria P. fluorescens 07A Genome</i>	40
Discussion	42
Conclusions	47
Acknowledgments	48
References	49
Supplemental Material	55
4. CHAPTER 3: PRODUCTION AND ACTIVITY OF PROTEASES FROM PSEUDOMONAS FLUORESCENS 07A IN MILK	61
TEMPERATURE MODULATES THE PRODUCTION AND ACTIVITY OF PROTEOLYTIC ENZYMES FROM PSEUDOMONAS IN DAIRY	62
Abstract	62
Introduction	64
Material and Methods	65
<i>Protease purification</i>	65
<i>SDS-PAGE and zymogram</i>	66
<i>Activity Assay</i>	67
<i>Protease expression in milk</i>	67
<i>Total RNA extraction and cDNA synthesis</i>	68
<i>Real-time PCR</i>	68
<i>Statistical analysis</i>	68
<i>Protease production in LB</i>	69
<i>Proteolysis assay</i>	69
Results	70
<i>Protease expression and activity in milk</i>	70

<i>Protease production in LB</i>	72
<i>Proteolysis assay</i>	73
Discussion.....	76
Conclusions	80
References.....	82
Supplemental Material	86
5. GENERAL CONCLUSION AND PERSPECTIVES	89

LIST OF ABBREVIATIONS

ANOVA - Analysis of variance

AMBIC - Ammonium bicarbonate

BSA - Bovine serum albumin

CFU/mL - Colony forming unit for milliliter

DTT - Dithiothreitol

E-64 - Trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane

EDTA - Ethylenediaminetetraacetic acid

HTST – High temperature and short time

IAA – Iodoacetamide

MALDI-TOF - Matrix Assisted Laser Desorption Ionization - Time of Flight

MS - Mass Spectrometry

LB – Luria Bertani medium

LTLT – Low temperature and long time

PMSF - Phenylmethylsulfonyl fluoride

PNQL - National Program of Quality Improvement of Milk

RT-qPCR - Real time quantitative polymerase chain reaction

SD - Standard deviation

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TYEP – Tryptone, yeast extract and phosphate medium

UHT - Ultra high temperature

LIST OF FIGURES

Chapter 2: Characterization of the protease from *Pseudomonas fluorescens* 07A

Figure 1. SDS-PAGE (A) and zymogram (B) of the proteolytic fractions in the purification steps 35

Figure 2. Effect of temperature (a) and pH (b) on the activity of the extracellular protease from *Pseudomonas fluorescens* 07A 36

Figure 3. Effect of Mn^{+2} on enzyme thermostability. The protease was incubated at 80 °C for 5, 10, 15 and 20 min (\diamond) in the absence of manganese and (\square) in the presence of 5 mM Mn^{+2} 40

Figure 4. Sequencing and identification of the protease by a proteogenomics approach 41

Figure S1. Pairwise sequence comparisons between the proteases of *Pseudomonas fluorescens* strains 60

Chapter 3: Temperature modulates the production and activity of proteolytic enzymes from *Pseudomonas* in dairy

Figure 1. Relative expression of protease (a) and enzymatic activity (b) by *P. fluorescens* 07A at 4, 10 e 25 °C 72

Figure 2. Zymogram of protease production by *P. fluorescens* 07A in Luria Bertani after 12, 24, 36 and 48 h of incubation at 4, 10 and 25 °C 73

Figure 3. Biological assay – Profile of casein fractions in reconstituted skim milk at days 0, 1, 2, 3, 5 and 7..... 75

Figure S1. Biological assay – visual effects of treatments on reconstituted skim milk at day 7 87

Figure S2. Growth curve of *P. fluorescens* 07A in reconstituted skimmed milk at 4, 10 °C and 25 °C 88

LIST OF TABLES

Chapter 2: Characterization of the protease from *Pseudomonas fluorescens* 07A

Table 1. Effect of inhibitors on protease activity..... 37

Table 2. Effect of metallic ions on protease activity..... 38

Table 3. Thermostability of the extracellular protease from *Pseudomonas fluorescens* 07A..... 39

Table S1. Functional annotation of genes predicted from a contig of the genome of *Pseudomonas fluorescens* 07A 58

Chapter 3: Temperature modulates the production and activity of proteolytic enzymes from *Pseudomonas* in dairy

Table S1. Biological assay - Percentage of degradation of casein fractions of reconstituted skim milk at day 7 86

ABSTRACT

ALVES, Maura Pinheiro, D.Sc., Universidade Federal de Viçosa, February, 2017. **Purification, characterization and evaluation of the differential expression of proteases produced by *Pseudomonas fluorescens*.** Adviser: Antônio Fernandes de Carvalho. Co-advisers: Italo Tuler Perrone, Monique Renon Eller and Rosângela de Freitas.

The milk contaminating psychrotrophic bacteria produce thermo resistant proteases that hydrolyze milk caseins, resulting in loss of milk quality and yield of dairy products. Studies involving the characterization of these enzymes and the knowledge about the conditions that influence their production and activity are essential for preventing these problems. This work aimed to understand the activity and production of the extracellular protease produced by *Pseudomonas fluorescens* 07A strain. This enzyme showed to be a metalloprotease inhibited by Cu^{2+} , Ni^{2+} , Zn^{2+} , Hg^{2+} , Fe^{2+} and Mg^{2+} , but induced by Mn^{2+} and has a molar mass of 49,486 kDa. Partial sequencing of the proteic structure of this enzyme by mass spectrometry allowed the identification of the gene that encodes this protein in the genome of *P. fluorescens* 07A. The enzyme has 477 amino acids and highly conserved Ca^{2+} and Zn^{2+} -binding domains, indicating that Ca^{2+} , the main ion in milk, is also a cofactor of this enzyme. The enzyme activity is maximum at 37 °C and pH 7.5, but it maintains more than 40% activity when subjected to 100 °C for 5 min in ideal medium and only 14 to 30% in milder heat treatments, which may cause significant problems in the conditions normally used for the processing and storage of milk and dairy products. Low relative expression of protease was observed after 12 h of incubation at 25 °C, when the bacteria is in logarithmic growth phase, compared to its expression in refrigeration temperatures of 4 and 10 °C, when the bacteria is still in lag phase. Protease production significantly increased ($P < 0.05$) after 24 h at 25 °C and remained constant up to 48 h, when the bacteria remained at stationary phase, indicating that this enzyme could be produced as an adaptive strategy of the bacteria. The casein fractions of reconstituted skim milk were completely degraded

as by *P. fluorescens* 07A, the purified protease or the bacterial extract within seven days of incubation at 25 °C, and to a lesser extent at 10 °C for milk inoculated with the bacteria. Heat treatment at 90 °C for 5 min inactivated the purified enzyme and inhibited its activity in the bacterial extract. This work allowed understanding the biochemical and biological characteristics of the extracellular protease produced of *P. fluorescens* 07A strain, as well as the conditions that influence its production and activity in milk. These results can help the dairy industry in the search for alternatives for the processing of dairy products to control production and activity of these proteases in milk.

RESUMO

ALVES, Maura Pinheiro, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Purificação, caracterização e avaliação da expressão diferencial de proteases produzidas por *Pseudomonas fluorescens*.** Orientador: Antônio Fernandes de Carvalho. Coorientadores: Italo Tuler Perrone, Monique Renon Eller e Rosângela de Freitas.

As bactérias psicrotróficas contaminantes do leite produzem proteases termo-resistentes que hidrolisam as caseínas do leite, resultando em perda de qualidade de leite e rendimento de produtos lácteos. Estudos envolvendo a caracterização dessas enzimas e o conhecimento das condições que influenciam sua produção e atividade são essenciais para a prevenção destes problemas. Neste trabalho, uma protease extracelular produzida pela cepa *Pseudomonas fluorescens* 07A foi purificada para estudos visando o entendimento de sua atividade e produção em diferentes condições. A enzima é uma metaloprotease inibida por Cu^{2+} , Ni^{2+} , Zn^{2+} , Hg^{2+} , Fe^{2+} e Mg^{2+} , mas induzida por Mn^{2+} e possui massa molar de 49.486 kDa. O seqüenciamento da enzima por espectrometria de massa permitiu identificar o gene que codifica esta protease extracelular no genoma da cepa *P. fluorescens* 07A. A enzima possui 477 aminoácidos e domínios altamente conservados de ligação a Ca^{2+} e Zn^{2+} , indicando que Ca^{2+} , o principal íon no leite, é também um co-fator dessa enzima. Sua atividade é máxima a 37 °C e pH 7,5, mas ela mantém mais de 40% de atividade quando submetida a 100 °C por 5 minutos em meio ideal e apenas de 14 a 30% quando submetida a tratamentos térmicos mais brandos, podendo causar problemas significativos nas condições normalmente utilizadas para o processamento e armazenamento de leite e produtos lácteos. Foi observada baixa expressão relativa da protease após 12 h de incubação a 25 °C, quando a bactéria encontra-se na fase logarítmica de crescimento, comparado com as temperaturas de refrigeração de 4 e 10 °C, quando a bactéria está ainda em fase lag. A produção da protease aumentou significativamente ($P < 0.05$) em 24 h a 25 °C e permaneceu constante por até 48 h, intervalo em que a bactéria permaneceu em fase

estacionária, indicando que a enzima pode ser produzida como uma estratégia adaptativa da bactéria. As frações de caseína de leite desnatado reconstituído foram completamente degradadas pela *P. fluorescens* 07A, pela protease purificada e pelo extrato da bactéria em até sete dias de incubação a 25 °C, e em menor extensão a 10 °C para a amostra incubada com a bactéria. O tratamento térmico de 90 °C por 5 minutos inativou a enzima purificada e inibiu sua atividade no extrato da bactéria. Este trabalho permitiu compreender as características bioquímicas e biológicas de uma protease extracelular da cepa *P. fluorescens* 07A, bem como as condições que influenciam a produção de proteases por esta bactéria em leite e sua atividade. Esses resultados podem auxiliar as indústrias de lácteos na busca por alternativas durante o processamento de produtos lácteos visando o controle de sua produção e atividade.

1. GENERAL INTRODUCTION

The storage and transport of raw milk under refrigerated conditions contributed to reducing deterioration of this material by the acidifying activity of mesophilic bacteria. However, the maintenance of this condition for a long period creates favorable conditions for multiplication of psychrotrophic bacteria.

Bacterial species from the genus *Pseudomonas* are predominant among the psychrotrophic contaminants of refrigerated raw milk and the species *P. fluorescens* stands out in the deterioration of foods, particularly dairy products. Many bacteria present in milk produce thermo resistant proteases which maintain activity even after heat treatments normally adopted by dairy industries. Specifically for cheese production, moderate concentrations of these enzymes may contribute to the development of flavor compounds and thus may have a beneficial effect. However, residual activity of proteolytic enzymes on casein micelles for many dairy products results in several technological problems and is associated with the formation of *off-flavor* in dairy products, UHT milk gelation and coagulation of milk proteins during storage.

The proteolytic strain *P. fluorescens* 07A was isolated from refrigerated raw milk and produces an extracellular protease with potential for deterioration of stored milk even at low temperatures (Pinto, 2004; Pinto et al., 2006). However, this protease still needs to be characterized, as well as the milk storage conditions that influence its activity and its production by the bacteria. Thus, in this work we've purified and characterized an extracellular protease produced by the proteolytic *P. fluorescens* 07A strain, as well as evaluated the differential expression of proteases by this strain under different incubation conditions in milk.

This document consists of articles describing the main aspects associated to proteolysis in milk by proteases of psychrotrophic microorganisms (Chapter 1); the purification of an extracellular protease produced by *P. fluorescens* 07A strain, its biochemical and biological properties (Chapter 2) and the evaluation of milk storage conditions that influences the production and activity of proteases by this strain (Chapter 3).

2. CHAPTER 1: LITERATURE REVIEW

PROTEOLITIC ACTIVITY OF PSYCHOTROPHIC BACTERIA IN MILK AND DAIRY PRODUCTS

Abstract

Proteolytic enzymes exhibit an important role in the food industry, being essential for the development of desirable characteristics of some dairy products. However, unwanted proteolytic activity may also affect the quality of different products. This occurs especially in dairy products made from raw milk contaminated with psychrotrophic bacteria. These bacteria multiply at refrigerated temperatures and produce thermo resistant proteases that maintain their activity even after the thermal treatments applied during the processing, presenting significant impact on the quality of milk and dairy products. This study reviews the main aspects associated with proteolysis of milk by proteases of psychrotrophic bacteria as well as the characteristics of these enzymes produced by different strains associated with milk deterioration.

Keywords: Psychrotrophic microorganisms, milk proteolysis, protease, *Pseudomonas fluorescens*.

System of refrigeration of raw milk

The 1990s represented a milestone in the modernization of milk production in Brazil. This modernization of milk and dairy products sector began with the first

technical regulations coming from MERCOSUL in 1992, which established patterns of identity and quality of dairy products of major commercial interest in this market (Oliveira et al., 2000).

In 1996, at the initiative of the Ministry of Agriculture, Livestock and Food Supply (MAPA), the "National Quality Improvement Program for Milk" (PNQL) was created. As a result of the diagnosis made in the PNQL, the federal legislation, dated 1952, was improved, providing the means to leverage, along with other initiatives of the public and private sectors, the development and modernization of the dairy sector in the country (Oliveira et al., 2000).

The regulation of storage of refrigerated raw milk at the source of production occurred then in 2002, through Normative Instruction 51 of the Ministry of Agriculture, Livestock and Food Supply (Brasil, 2002), later amended in 2011 by Normative Instruction 62 (Brasil, 2011).

According to the Normative Instruction of the Ministry of Agriculture, Livestock and Food Supply (Brasil, 2011), the raw milk collect system started to be done in bulk and by means of direct expansion refrigeration tanks that began to be sized in order to allow refrigeration of raw milk at a temperature of 4 °C or less, within a maximum of 3 h after the end of milking, regardless of their capacity. The immersion refrigerating tanks started to be sized to allow refrigeration of the milk at 7 °C or below, also within a maximum of 3 h after the end of milking. In addition, the use of community tanks is allowed if it is based on the operation principle of direct expansion, and the maximum temperature of conservation of milk in the rural property is up to 7 °C in community tanks, and 10 °C for reception in the processing plant.

The Normative Instruction of the Ministry of Agriculture, Livestock and Food Supply also established that the time between milking and receiving raw milk at the plant that will process it must be a maximum of 48 h, recommending as ideal a period not exceeding 24 h. And the collect and transport to the industry must be carried out in trucks equipped with isothermal tanks (Brasil, 2011).

This raw milk refrigerating system at the source of production represented a major advance for the milk production sector, leading to reduction of losses of this raw material due to the acidifying activity of mesophilic bacteria (Cempírková, 2002). However, the maintenance of raw milk at refrigeration temperatures for prolonged periods can cause deterioration problems associated with the multiplication and enzymatic activity of psychrotrophic bacteria (Sørhaug and Stepaniak, 1997).

Psychrotrophic microorganisms in milk

The psychrotrophic bacteria are distributed in a variety of environments and have mesophilic nature, however, they have the capacity to multiply in refrigerated temperatures, regardless of their optimum multiplication temperature (Cousin et al., 2001; Munsch-Alatossava and Alatossava, 2006).

Among the sources of contamination of raw milk by this group of bacteria are water, dust and animal feed. In addition, milk contamination may be associated with poor cleaning of surfaces and equipment during collect, transport and storage (McPhee and Griffiths, 2011). Under adequate sanitary conditions of milk production, these bacteria account for less than 10% of the microbiota compared to the percentage of up to 75% under unsatisfactory sanitary conditions (Cousin, 1982).

The group of psychrotrophic microorganisms of relevance in the dairy industry comprises both gram-negative and gram-positive bacteria. Among some genera isolated in different studies it can be mentioned: *Pseudomonas*, *Serratia*, *Aeromonas*, *Alcaligenes*, *Achromobacter* and *Acinetobacter* (gram-negative), *Bacillus*, *Streptococcus*, *Micrococcus* and *Corynebacterium* (gram-positive) (Munsch-Alatossava and Alatossava, 2006; Nörnberg et al., 2010; Raats et al., 2011; Tebaldi et al., 2008; Teh et al., 2011).

Different research groups have identified contaminating micro-organisms in refrigerated milk (Martins et al., 2006; Pinto et al., 2006; Tebaldi et al., 2008; Arcuri et al., 2008; Ercolini et al., 2009; Neubeck et al., 2015; Scatamburlo et al., 2015). Munsch-Alatossava and Alatossava (2006) observed that the majority of isolated bacteria from refrigerated raw milk samples in Finland belonged to the genera *Pseudomonas*. Samples of contaminated commercial UHT milk in Taiwan were also analyzed by Chen; Wei; Chen, (2011). Among the isolated gram-negative psychrotrophic bacteria, the authors observed that 22% were *Enterobacteriaceae* and 67% belonged to the genera *Pseudomonas* spp..

In Brazil, Arcuri et al. (2008) collected samples of refrigerated raw milk from 20 collective tanks and 23 individual tanks from properties in the Zona da Mata, region of Minas Gerais, and in the Southeast region of Rio de Janeiro. Psychrotrophic bacteria were found in the samples with counts raging from around 10^2 and 10^7 CFU·mL⁻¹. In this work, *Pseudomonas* was also the most isolated genera (43%), with *P. fluorescens* being the predominant species (37,6%).

It is evident, therefore, that the genera *Pseudomonas* covers the main contaminants of refrigerated raw milk (Ismail and Nielsen, 2010). They are gram-

negative, aerobic, catalase-positive microorganisms and the vast majority of species is oxidase-positive (McPhee and Griffiths, 2011). Among the bacteria that belong to this genera, the species *P. fluorescens* stands out in the deterioration of foods, especially dairy products (De Jonghe et al., 2011; Rasolofo et al., 2010).

Other species of the genera *Pseudomonas* spp. are also associated with milk deterioration, such as *P. fragi*, *P. lundensis*, *P. gessardii* (De Jonghe et al., 2011; Peix et al., 2009) and *P. putida* (Ercolini et al., 2009).

Adaptation of psychrotrophic microorganisms at low temperatures

Temperature influences the multiplication of microorganisms by affecting the conformation of cell macromolecules and other constituents, thus determining the rates of intracellular enzymatic reactions that are essential for cell viability (Russell, 2002; Beales, 2004). Thus, the ability of microorganisms to multiply at low temperatures depends on adaptive changes in their cellular proteins and lipids (Russell, 1990).

In low temperature conditions, the psychrotrophic microorganisms synthesize neutral lipids and phospholipids with a higher proportion of unsaturated fatty acids, which leads to a reduction in the melting point of the lipids. These changes occur to maintain fluidity of the cell membrane, thus allowing solutes transport and secretion of extracellular enzymes to continue (Beales, 2004).

Fonseca et al. (2011), by analysis of the transcriptome of *P. putida* KT2440 strain evaluated the changes that occur when cells are cultured at low temperature (10 °C) compared to a temperature higher (30 °C). The authors observed that

expression of at least 266 genes, about 5% of the genome, had been modified during cell growth at low temperature compared to cells grown at 30 °C. Several of these changes appeared to be directed toward neutralizing problems created by the low temperature, such as increased protein misfolding, increased stability of DNA/RNA secondary structures, reduced membrane fluidity and reduced growth rate.

In addition to cellular modifications that occur to maintain cell membrane fluidity and permeability, other mechanisms are known to be important for the multiplication of bacteria at low temperatures. These mechanisms include the synthesis of compatible solutes, changes in DNA folding, maintenance of the structural integrity of macromolecules and macromolecule groups, including ribosomes and other components that affect gene expression, in addition to the synthesis of cold shock proteins (Wouters et al., 2000; Cavicchioli et al., 2000; Chattopadhyay, 2006).

Proteolysis in the dairy industry

Enzymes that act on milk proteolysis may have both a beneficial and undesirable effect on the dairy industry. Many proteases are widely used in a controlled way during food processing, leading to improved digestibility, modifications in sensory quality, such as texture, flavor and aroma as well as promoting increased nutritional value (Tavano, 2013). For example, in cheese processing the amino acids released during the ripening process are used by microorganisms as substrate for a variety of catabolic reactions. These reactions generate important compounds for the development of the flavor characteristic of each type of cheese and depend on the

natural microbiota of the milk or the microorganisms added during the manufacturing process (Yvon and Rijnen, 2001).

However, although proteolysis has beneficial effects and is essential for the development of desirable characteristics of some dairy products, uncontrolled or unwanted proteolytic activity may adversely affect food quality. This occurs especially in dairy products made from milk contaminated with proteolytic psychrotrophic bacteria. These bacteria are able to multiply at low temperatures and can produce enzymes often associated with food spoilage (Baglinière et al., 2013; Quigley et al., 2013).

Much of these bacteria present in milk is inactivated by the heat treatment of pasteurization. However, many of its enzymes maintain activity even after pasteurization or ultra-high temperature treatments commonly adopted by the dairy industry (McPhee and Griffiths, 2011). Among the thermo resistant enzymes produced by this group of bacteria, proteases and lipases have a significant impact on the quality of milk and dairy products (Sørhaug and Stepaniak, 1997).

Proteolytic enzymes act on milk proteins causing deterioration in different dairy products during prolonged storage with reduced shelf life. Its activity modifies the physico-chemical properties of the final product, leading to changes in functionality and sensory characteristics (Chen et al., 2003). Consequently, the residual activity of proteases over the casein micelle results in several technological problems, and may be associated with loss of yield in cheeses production, off-flavor formation, UHT milk gelation and coagulation of milk proteins during storage (Datta and Deeth, 2001; MCPhee and Griffiths, 2011).

The proteolysis of UHT milk during storage at room temperature limits the shelf-life of this product due to changes in its sensory characteristics (Celestino et al., 1997; Datta and Deeth, 2003). Gaucher et al. (2011) studied the consequences of contamination of raw milk with a strain of *P. fluorescens* on the stability of UHT milk during storage. The authors have observed the destabilization of UHT milk by the formation of aggregates and reduction of stability to phosphate during storage as well as changes in the physico-chemical properties of casein micelles.

The study by Topçu et al. (2006) has also shown the effect of the microbiological quality of raw milk on proteolysis after UHT treatment. Sediment formation and gelation of UHT milk processed from raw milk with high somatic cell counts and psychrotrophic bacteria have been observed during storage at room temperature.

According to Cardoso (2006), one of the problems faced during cheese processing by producers is the reduction of yield due to the activity of bacterial proteases on the casein micelle. During the production of Minas Frescal cheese, this author has found a reduction of 6,78% in the yield in terms of liters of milk per kilogram of cheese and of 6,38% in grams of total solids in the cheese per liter of milk when the cheese had been manufactured with refrigerated milk stored for a period of four days.

Proteolytic enzymes in milk

Milk proteolysis may be caused by either natural milk proteases such as plasmin, or by thermostable proteases produced by psychrotrophic microorganisms

contaminating raw milk. These proteases differ in their specificity for milk proteins (Ismail and Nielsen, 2010).

Plasmin is an alkaline serine protease, thermostable and mainly active in the proteolysis of β -casein, which leads to the formation of γ -casein fractions. This enzyme also hydrolyzes α_{s2} -caseína, and to a lesser extent α_{s1} -caseína (Bars and Gripon, 1993).

Plasmin is present in milk both in its active form and in the form of its inactive precursor, plasminogen, and its activity in milk is controlled by a complex system formed of activating and inhibiting enzymes. The components of this system interact with each other and with other components of milk, such as whey proteins and casein, promoting or inhibiting proteolysis, depending on milk processing and storage conditions (Ismail and Nielsen, 2010).

Proteolytic enzymes of microbial origin can be produced by bacteria of different genera found in a variety of environments, including *Pseudomonas*, *Bacillus*, *Serratia*, among others (Cleto et al., 2012; Marchand et al., 2012). The production of these enzymes is a complex process, influenced by temperature, phase variation, ion level and quorum sensing (Liu et al., 2007; Woods et al., 2001; Nicodème et al., 2005; Broek et al., 2005) and usually occurs at the end of the exponential or stationary phase of microbial growth (Rajmohan et al., 2002). These enzymes may be located within the cell (intracellular), associated with the cell wall or excreted into the environment (extracellular).

Most of the proteases of psychrotrophs act on the casein micelle preferentially to the whey proteins, whereas the fractions most susceptible are κ -casein and β -casein (Chen et al., 2003). In milk, proteases produced by psychrotrophic

microorganisms preferentially hydrolyze κ -casein, then β -casein and, finally, α S1-casein, and still have low activity on whey proteins (Koka and Weimer, 2000; McPhee and Griffiths, 2011; Fairbairn and Law, 1986).

Proteolytic enzymes from psychrotrophic bacteria

Several proteolytic enzymes produced by psychrotrophic microorganisms maintain their activity even after the conventional heat treatments adopted by the dairy industry, which is one of the main current problems in the production of dairy products (Samaržija et al., 2012). Among the characteristics that allow the heat stability of these enzymes are additional salt bridges, additional hydrogen bonds, tighter Ca^{+2} -binding sites, maximized packing and expanded hydrophobic core (Sørhaug and Stepaniak, 1997).

Several studies have already been carried out to characterize proteases produced by different strains of *P. fluorescens* associated with milk deterioration, which differ in their physicochemical and biochemical properties. Most of them are metalloproteases rich in residues of alanine and glycine and poor in cysteine and methionine. Calcium is essential for its activity and stability (Mitchell and Ewings, 1986).

Mu et al. (2009) purified a heat-resistant extracellular protease (Ht13) produced by a proteolytic psychrotrophic bacteria, *P. fluorescens* Rm12. This protease has an estimated molar mass of 45 kDa and the presence of the Mn^{2+} ion showed a positive effect on the activity of this enzyme and may increase its resistance to heat. Based on its biochemical characteristics, Ht13 protease may be

included in the group of metalloproteases, which was inhibited by specific inhibitors of this group of enzymes, ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline. A protease purified by Dufour et al. (2008) also has a molar mass of approximately 45 kDa, with optimum activity at 45 °C and pH 8.5.

Liao and McCallus (1998) characterized an extracellular protease produced by *P. fluorescens* CY091 strain. This protease has an estimated molar mass of 50 kDa and its production was observed in medium containing CaCl₂ or SrCl₂. In addition, more than 20% of enzyme activity was maintained after heat treatment of the enzyme, indicating resistance to heat inactivation.

An extracellular protease produced by *P. fluorescens* BJ-10 strain showed optimum activity at 30 °C and pH 7.0. More than 94% of the activity of this enzyme was maintained after a heat treatment of 100 °C for 3 min, evidencing the characteristic of thermo resistance (Zhang and Lv, 2014).

The *aprX* gene encoding an extracellular protease from a proteolytic psychrotrophic bacterial strain, *P. fluorescens* 041, was cloned and expressed in *Escherichia coli*. The protease has a molar mass of approximately 50 kDa, activity in a wide range of pH and optimum temperature of 37 °C (Martins et al., 2015). In addition, the author observed proteolytic activity in the culture supernatant on TYEP medium from another strain, *P. fluorescens* 07A (Martins, 2007). This strain was isolated by Pinto (2004), in the Laboratory of Food Microbiology of the Universidade Federal de Viçosa, from refrigerated raw milk, and produces thermo resistant protease, presenting great potential for deterioration of stored milk at low temperatures. However, the protease produced by this strain still needs to be characterized as well as the milk storage conditions that favor the production and

activity of proteases by the bacteria. This knowledge can help to control the technological problems in dairy products associated with the activity of these enzymes.

References

- Arcuri, E.F., P.D.L. Silva, M.A.V.P. Brito, J.R.F. Brito, C.C. Lange, and M.M.A. Magalhães. 2008. Contagem, isolamento e caracterização de bactérias psicrófilas contaminantes de leite cru refrigerado. *Ciência Rural*. 38:2250–2255.
- Baglinière, F., A. Matéos, G. Tanguy, J. Jardin, V. Briard-Bion, F. Rousseau, B. Robert, E. Beaucher, J.L. Gaillard, C. Amiel, G. Humbert, A. Dary, and F. Gaucheron. 2013. Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilisation. *Int. Dairy J.* 31:55–61.
- Bars, D. Le, and J.C. Gripon. 1993. Hydrolysis of α s1-casein by bovine plasmin. *Lait*. 73:337–344.
- Beales, N. 2004. Adaptation of microorganisms to cold temperatures , weak acid preservatives , low ph , and osmotic stress : A Review. *Compr. Rev. Food Sci. Food Saf.* 3:1–20.
- Brasil. 2002. Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de Origem Animal. Instrução Normativa Nº 51, de 18 de Setembro de 2002. Diário Oficial da República Federativa do Brasil, p. 8-22, 20 set. 2002. Seção I.
- Brasil. 2011. Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de Origem Animal. Instrução Normativa Nº 62, de 29 de dezembro de 2011. Diário Oficial da República Federativa do Brasil, p. 13-20, 30

dez. 2011. Seção I.

- Broek, D.V.D., G.V. Bloemberg, and B. Lugtenberg. 2005. The role of phenotypic variation in rhizosphere *Pseudomonas* bacteria. *Environ. Microbiol.* 7:1686–1697.
- Cardoso, R. 2006. Influência da microbiota psicrotrófica no rendimento de queijo minas frescal elaborado com leite estocado sob refrigeração. Dissertação (Mestrado em Microbiologia Agrícola) - Universidade Federal de Viçosa. 57 pp.
- Cavicchioli, R., T. Thomas, and P.M.G. Curmi. 2000. Cold stress response in Archaea. *Extremophiles.* 4:321–331.
- Celestino, E.L., M. Iyer, and H. Roginski. 1997. Reconstituted UHT-treated milk : effects of raw milk , powder quality and storage conditions of uht milk on its physico-chemical attributes and flavour. *Int. Dairy J.* 7:129–140.
- Cempírková, R. 2002. Psychrotrophic vs . total bacterial counts in bulk milk samples. *Vet. Med.* 47:227–233.
- Chattopadhyay, M.K. 2006. Mechanism of bacterial adaptation to low temperature. *J. Biosci.* 31:157–165.
- Chen, L., R.M. Daniel, and T. Coolbear. 2003. Detection and impact of protease and lipase activities in milk and milk powders. *Int. Dairy J.* 13:255–275.
- Chen, T.R., Q.K. Wei, and Y.J. Chen. 2011. *Pseudomonas* spp. and *Hafnia alvei* growth in UHT milk at cold storage. *Food Control.* 22:697–701.
- Cleto, S., S. Matos, L. Kluskens, and M.J. Vieira. 2012. Characterization of contaminants from a sanitized milk processing plant. *PLoS One.* 7.

- Cousin, M.A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. *J. Food Prot.* 45:172–207.
- Cousin, M.A., J.M. Jay, and P.C. Vasavada. 2001. Psychrotrophic microorganisms. *In* Compendium of Methods for the Microbiological Examination of Foods - American Public Health Association. F.P. Downes and K. Ito, editors. 159–166.
- Datta, N., and H.C. Deeth. 2001. Age gelation of UHT milk - A Review. *Inst. Chem. Eng.* 79:197–210.
- Datta, N., and H.C. Deeth. 2003. Diagnosing the cause of proteolysis in UHT milk. *LWT - Food Sci. Technol.* 36:173–182.
- Dufour, D., M. Nicodème, C. Perrin, A. Driou, E. Brusseau, G. Humbert, J.-L. Gaillard, and A. Dary. 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. *Int. J. Food Microbiol.* 125:188–96.
- Ercolini, D., F. Russo, I. Ferrocino, and F. Villani. 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiol.* 26:228–31.
- Fairbairn, D.J., and B. Law. 1986. Proteinases of psychrotrophic bacteria: their production, properties, effects and control. *J. Dairy Res.* 53:139–177.
- Fonseca, P., R. Moreno, and F. Rojo. 2011. Growth of *Pseudomonas putida* at low temperature : global transcriptomic and proteomic analyses. *Environ. Microbiol. Rep.* 3:329–339.

- Gaucher, I., G. Tanguy, J. Fauquant, J. Jardin, F. Rousseau, B. Robert, M.-N. Madec, and F. Gaucheron. 2011. Proteolysis of casein micelles by *Pseudomonas fluorescens* CNRZ 798 contributes to the destabilisation of UHT milk during its storage. *Dairy Sci. Technol.* 91:413–429.
- Ismail, B., and S.S. Nielsen. 2010. Invited review: Plasmin protease in milk: current knowledge and relevance to dairy industry. *J. Dairy Sci.* 93:4999–5009.
- De Jonghe, V., A. Coorevits, K. Van Hoorde, W. Messens, A. Van Landschoot, P. De Vos, and M. Heyndrickx. 2011. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. *Appl. Environ. Microbiol.* 77:460–70.
- Koka, R., and B.C. Weimer. 2000. Isolation and characterization of a protease from *Pseudomonas fluorescens* RO98. *J. Appl. Microbiol.* 89:280–288.
- Liao, C.-H., and D.E. McCallus. 1998. Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. *Appl. Environ. Microbiol.* 64:914–921.
- Liu, M., H. Wang, and M.W. Griffiths. 2007. Regulation of alkaline metalloprotease promoter by N-acyl homoserine lactone quorum sensing in *Pseudomonas fluorescens*. *J. Appl. Microbiol.* 103:2174–84.
- Marchand, S., J. De Block, V. De Jonghe, A. Coorevits, M. Heyndrickx, and L. Herman. 2012. Biofilm formation in milk production and processing environments; influence on milk quality and safety. *Compr. Rev. Food Sci. Food Saf.* 11:133–147.

- Martins, M.L. 2007. Caracterização de protease e lipase de *Pseudomonas fluorescens* e Quorum sensing em bactérias psicotróficas isoladas de leite. Tese (Doutorado em Microbiologia Agrícola) - Universidade Federal de Viçosa. 184 pp.
- Martins, M.L., C.L.O. Pinto, R.B. Rocha, E.F. de Araújo, and M.C.D. Vanetti. 2006. Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. *Int. J. Food Microbiol.* 111:144–8.
- Martins, M.L., U.M. Pinto, K. Riedel, and M.C.D. Vanetti. 2015. Milk-deteriorating exoenzymes from *Pseudomonas fluorescens* 041 isolated from refrigerated raw milk. *Brazilian J. Microbiol.* 46:207–217.
- McPhee, J.D., and M.W. Griffiths. 2011. Psychrotrophic bacteria. *Pseudomonas* spp. In *Encyclopedia of Dairy Sciences*. J.W. Fuquay, P.F. Fox, and P.L.H. McSweeney, editors. Academic Press. 379–383.
- Mitchell, B.G.E., and K.N. Ewings. 1986. Physicochemical properties of proteinases from selected psychrotrophic bacteria. *J. Dairy Res.* 53:97–115.
- Mu, Z., M. Du, and Y. Bai. 2009. Purification and properties of a heat-stable enzyme of *Pseudomonas fluorescens* Rm12 from raw milk. *Eur. Food Res. Technol.* 228:725–734.
- Munsch-Alatossava, P., and T. Alatossava. 2006. Phenotypic characterization of raw milk-associated psychrotrophic bacteria. *Microbiol. Res.* 161:334–46.
- Neubeck, M. Von, C. Baur, M. Krewinkel, M. Stoeckel, S. Scherer, and M. Wenning. 2015. Biodiversity of refrigerated raw milk microbiota and their enzymatic

- spoilage potential. *Int. J. Food Microbiol.* 211:57–65.
- Nicodème, M., J.-P. Grill, G. Humbert, and J.-L. Gaillard. 2005. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. *J. Appl. Microbiol.* 99:641–8.
- Nörnberg, M.F.B.L., R.S.C. Friedrich, R.D.N. Weiss, E.C. Tondo, and A. Brandelli. 2010. Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. *Int. J. Dairy Technol.* 63:41–46.
- Oliveira, L.C., M.F. Gomes, and C.R.V. Velloso. 2000. Modernização da legislação sanitária federal sobre leite e derivados. In *Perspectivas e Avanços em Laticínios*. M.C.D. Castro and J.A.B. Portugal, editors. EPAMIG. Centro tecnológico da Zona da Mata, ILCT, Juiz de Fora. 278.
- Peix, A., M. Ramírez-Bahena, and E. Velázquez. 2009. Historical evolution and current status of the taxonomy of genus *Pseudomonas*. *Infect. Genet. Evol.* 9:1132–1147.
- Pinto, C.L.O. 2004. Bactérias psicrotróficas proteolíticas do leite cru refrigerado granelizado destinado à produção de leite UHT. Tese (Doutorado em Microbiologia Agrícola) - Universidade Federal de Viçosa. 97 pp.
- Pinto, C.L.O., M.L. Martins, and M.C.D. Vanetti. 2006. Qualidade microbiológica de leite cru refrigerado e isolamento de bactérias psicrotróficas proteolíticas. *Ciênc. Tecnol. Aliment.* 26: 645-651.
- Quigley, L., O.O. Sullivan, C. Stanton, T.P. Beresford, R.P. Ross, G.F. Fitzgerald, and P.D. Cotter. 2013. The complex microbiota of raw milk. *FEMS Microbiol.*

Rev. 37:664–698.

Raats, D., M. Offek, D. Minz, and M. Halpern. 2011. Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. *Food Microbiol.* 28:465–71.

Rajmohan, S., C.E.R. Dodd, and W.M. Waites. 2002. Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. *J. Appl. Microbiol.* 93:205–213.

Rasolofo, E.A., D. St-Gelais, G. LaPointe, and D. Roy. 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. *Int. J. Food Microbiol.* 138:108–18.

Russell, N.J. 2002. Bacterial membranes: the effects of chill storage and food processing . An overview. *Int. J. Food Microbiol.* 79:27–34.

Samaržija, D., Š. Zamberlin, and T. Pogačić. 2012. Psychrotrophic bacteria and milk and dairy products quality. *Mljekarstvo.* 62:77–95.

Scatamburlo, T.M., A.K. Yamazi, V.Q. Cavicchioli, F.A. Pieri, and L.A. Nero. 2015. Spoilage potential of *Pseudomonas* species isolated from goat milk. *J. Dairy Sci.* 98:759–764.

Sørhaug, T., and L. Stepaniak. 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. *Trends Food Sci. Technol.* 8:35–41.

Tavano, O.L. 2013. Protein hydrolysis using proteases: An important tool for food biotechnology. *J. Mol. Catal. B Enzym.* 90:1–11.

Tebaldi, V.M.R., T.L.C. de Oliveira, C.A. Boari, and R.H. Piccoli. 2008. Isolamento de

- coliformes , estafilococos e enterococos de leite cru provenientes de tanques de refrigeração por expansão comunitários: identificação , ação lipolítica e proteolítica. *Ciência e Tecnol. Aliment.* 28:753–760.
- Teh, K.H., S. Flint, J. Palmer, D. Lindsay, P. Andrewes, and P. Bremer. 2011. Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. *Int. Dairy J.* 21:742–747.
- Topçu, A., E. Numanoglu, and I. Saldamli. 2006. Proteolysis and storage stability of UHT milk produced in Turkey. *Int. Dairy J.* 21:633–638.
- Woods, R.G., M. Burger, C. Beven, and I.R. Beacham. 2001. The aprX – lipA operon of *Pseudomonas fluorescens* B52 : a molecular analysis of metalloprotease and lipase production. *Microbiology.* 147:345–354.
- Wouters, J.A., F.M. Rombouts, O.P. Kuipers, W.M. De Vos, and T. Abee. 2000. The role of cold-shock proteins in low-temperature adaptation of food-related bacteria. *Syst. Appl. Microbiol.* 23:165–173.
- Yvon, M., and L. Rijnen. 2001. Cheese flavour formation by amino acid catabolism. *Int. Dairy J.* 11:185–201.
- Zhang, S., and J. Lv. 2014. Purification and properties of heat-stable extracellular protease from *Pseudomonads fluorescens* BJ-10. *J. Food Sci. Technol.* 51:1185–90.

3. CHAPTER 2: CHARACTERIZATION OF THE PROTEASE FROM
***PSEUDOMONAS FLUORESCENS* 07A**

**CHARACTERIZATION OF A HEAT-RESISTANT EXTRACELLULAR PROTEASE
FROM *PSEUDOMONAS FLUORESCENS* 07A SHOWS THAT LOW
TEMPERATURE TREATMENTS ARE MORE EFFECTIVE IN DEACTIVATING ITS
PROTEOLYTIC ACTIVITY**

This article was published in:

Journal of Dairy Science, 2016. 99(10): p. 7842-7851.

Maura P. Alves, Rafael L. Salgado, Monique R. Eller, Pedro M. P. Vidigal, Antônio
Fernandes de Carvalho

**Interpretive Summary: Protease characterization and control of milk
degradation**

In this article, a protease from *Pseudomonas* was identified and biochemically characterized. Its conditions of highest activity were close to those of UHT milk storage, evidencing the potential of this enzyme in causing technological problems such as gelation of UHT milk during its storage. Moreover, pasteurization has decreased enzyme activity more than extreme heat treatments, highlighting new perspectives to control milk degradation by these enzymes.

Abstract

This work discusses the biological and biochemical characterization of an extracellular protease produced by *Pseudomonas fluorescens*. The enzyme has a molar mass of 49.486 kDa and hydrolyzes gelatin, casein and azocasein, but not BSA. Its maximum activity is found at 37 °C and pH 7.5, but it retained almost 70% activity in pH 10.0. It showed to be a metalloprotease inhibited by Cu^{2+} , Ni^{2+} , Zn^{2+} , Hg^{2+} , Fe^{2+} and Mg^{2+} , but induced by Mn^{2+} . After incubation at 100 °C for 5 min, the enzyme presented over 40% activity, but only 14-30% when submitted to milder heat treatments. This behavior may cause significant problems under conditions commonly used for the processing and storage of milk and dairy products, particularly UHT milk. A specific peptide sequenced by mass spectrometer analysis allowed the identification of gene that encodes this extracellular protease in the genome of *Pseudomonas fluorescens* 07A strain. The enzyme has 477 amino acids and highly conserved Ca^{2+} and Zn^{2+} -binding domains, indicating that Ca^{2+} , the main ion in milk, is also a cofactor. This work contributes to the understanding of the biochemical aspects of enzyme activity and associates them with its sequence and structure. These finding are essential for the full understanding and control of these enzymes and the technological problems they cause in the dairy industry.

Keywords: Milk proteolysis, protease, enzyme characterization, heat treatment

Introduction

The storage of raw milk under refrigeration conditions contributes to improved milk quality. However, a long-term storage allows the development of psychrotrophic bacteria (Sørhaug and Stepaniak, 1997), and predominantly the genera *Pseudomonas* (Ercolini et al., 2009; Decimo et al., 2014; Neubeck et al., 2015). The majority of these microorganisms produce thermo resistant proteases which maintains activity even after milk pasteurization or UHT treatment (McPhee and Griffiths, 2011). Residual activity of these enzymes on casein micelles can cause technological problems and is associated to loss of yield in cheese production, formation of *off-flavor* in dairy products, UHT milk gelation and coagulation of milk proteins during storage (Datta and Deeth, 2001; MCPhee and Griffiths, 2011).

A number of studies partially characterized proteases from psychrotrophic isolated from milk (Dufour et al., 2008; Liao and McCallus, 1998; Zhang and Lv, 2014). However, to fully understand protease activity in milk, is essential to associate this characteristic to others such as their biochemical properties, complete sequence and structure. These data will assist dairy industries in the development or adaptation of alternatives to control technological problems in dairy products.

Pseudomonas fluorescens 07A, a proteolytic strain, was isolated from raw bulk milk (Pinto, 2004; Pinto et al., 2006) and produces an extracellular protease responsible for milk deterioration, even at low temperatures. However, there are few information about this enzyme, the conditions in which it is produced and its activity in milk. Thus, this work describes the biological and biochemical properties of the

extracellular protease produced by *P. fluorescens* 07A and identifies its coding sequence in the genome of this bacterium.

Material and methods

Bacteria and Culture Conditions

The strain *P. fluorescens* 07A was isolated from raw milk collected in Minas Gerais, Brazil (Pinto, 2004; Pinto et al., 2006). The culture was cultivated in Luria-Bertani–LB Broth (Sigma Aldrich, St. Louis, MO) at 25 °C.

Proteolysis Assay

The proteolytic activity was determined using azocasein (Sigma-Aldrich, St. Louis, Missouri, USA) as substrate, as described by Ayora and Götz (1994), with modifications. An aliquot of 250 µL of azocasein solution at 2% (m/v) in diluted in Tris-HCl 40 mM, CaCl₂ 2 mM buffer, pH 7.5, was added to 150 µL of the test-solution. The mixture was agitated, incubated at 37 °C for 8 h and the reaction was interrupted with 1.2 mL of trichloroacetic acid at 10% (m/v). After incubation for 15 min at room temperature, the mixture was centrifuged at 12,000 x g for 15 min. A volume of 1.2 mL of the supernatant was neutralized with 1.0 mL of NaOH 1 M. The absorbance of this solution was measured at 440 nm using a SpectraMax M2 spectrometer (Molecular Devices, Sunnyvale, California, USA). One unit of enzymatic activity was defined as the quantity of enzyme sufficient to raise 0.01 of the solution absorbance at 440 nm per minute, at experimental conditions. All assays were performed as triplicates and the results were expressed as the mean \pm standard deviation (SD).

The protein concentration was estimated as Bradford (1976) using bovine serum albumin – BSA (Sigma-Aldrich, St. Louis, Missouri, USA) to build the calibration curve.

Protease Purification

A solution containing the bacteria *P. fluorescens* 07A in exponential phase was inoculated (1%) in LB, incubated for 18 h and centrifuged at 10,000 x g for 15 min. The enzyme was recuperate from crude supernatant by fractional precipitation using ammonium sulfate (40 to 60%) for 1 h and centrifugation at 12,000 x g for 30 min. Pellet was resuspended in Tris-HCl 10 mM, NaCl 50 mM buffer, pH 7.5 and dialyzed twice against this buffer in cellulose membrane of 14 kDa (Sigma-Aldrich, St. Louis, Missouri, USA), for 8 h. All steps were performed at 4 °C, and an aliquot was collected to monitor the enzyme activity.

Dialyzed solution containing the enzyme was applied in a HiPrep 26/60 Sephacryl™ S-200 HR column (GE Healthcare® Little Chalfont, Buckinghamshire, UK) equilibrated with Tris-HCl 10 mM, NaCl 50 mM buffer, pH 7.5, using an AKTA Purifier chromatographer (GE Healthcare® Little Chalfont, Buckinghamshire, UK). Proteins were recuperate at 1.6 mL/min and monitored at 280 nm. Fractions presenting proteolytic activity were subjected to ultrafiltration in Amicon® Ultra-15 (Millipore®, Cork, Ireland) and storage at 4 °C.

The aliquots collected from each purification step were subjected to a SDS-PAGE with 12% polyacrylamide (Laemmli, 1970) in a Mini-Protean® Tetra System (BioRad, California, USA). Proteins were stained with silver nitrate or 0.01% Coomassie Brilliant Blue R-250 solution.

The same aliquots were subjected to a zymogram using a 12% polyacrylamide gel and gelatin (3 mg/mL) as substrate, as described by Feitosa et al. (1998), with modifications. Gels were washed during 30 min with a Triton X-100 2.5% (v/v) solution and incubated in Tris-HCl 50 mM, NaCl 200 mM, CaCl₂ 5 mM buffer, pH 7.5, at 37 °C for 12 h. Proteolytic activity was visualized as clear zones in the gel stained with a 0.01% Coomassie Brilliant Blue R-250 solution.

Beyond gelatin, proteolytic activity was tested in zymograms using casein, azocasein and BSA as substrates.

Protease Characterization

Effect of Temperature and pH on Enzyme Activity

Proteolysis assay was performed with incubation temperatures of 4, 10, 20, 30, 50 and 60 °C. Enzyme activity was also evaluated at the pH range of 4.0 to 10.0. Enzyme solutions were adapted to each pH of the test using 1 M buffers with 50 mM NaCl and 2 mM CaCl₂. A sodium citrate/citric acid buffer was used for pH 4.0 and 5.0; a sodium phosphate buffer was used for pH 6.0 and a Tris-HCl for pH 7.5, 8.0, 9.0 and 10.0. A negative control with no enzyme was performed for all solutions. Results were expressed as relative unit, considering maximum activity as 100%.

Effect of Inhibitors and Metallic Ions on Enzyme Activity

The activity assay was performed with the addition of the following reagents: EDTA (1 and 5 mM), 1,10-phenantroline (1 and 5 mM), PMSF (1 and 5 mM), benzamidine (1 and 5 mM), DTT (1 and 5 mM), E-64 (1, 5 and 10 µM) and Pepstatin

A (1, 5 and 10 μM) (Sigma-Aldrich, St. Louis, Missouri, USA). The ions tested were Mg^{2+} , Cu^{2+} , Zn^{2+} , Ca^{2+} , Fe^{2+} , Mn^{2+} , Ni^{2+} and Hg^{2+} at concentrations of 2 and 5 mM. A negative control with no enzyme was performed for all solutions. Results were expressed as relative unit, considering the solution with no inhibitors and ions as 100%.

Enzyme Thermostability

Aliquots of 150 μL of the protease solution were incubated in microtubes at 80 $^{\circ}\text{C}$ during 5, 10, 15 and 20 min and at 90 and 100 $^{\circ}\text{C}$ during 1, 3 and 5 min. The protease was also incubated at 72 $^{\circ}\text{C}$ for 15 s and 65 $^{\circ}\text{C}$ for 30 min to test the heat-treatments of rapid and slow pasteurization, respectively, commonly applied to milk. The aliquots were immediately cooled in ice bath and subjected to the proteolysis assay. Results were expressed as relative unit, considering the no heat-treated solution as 100%. The effect of the ion Mn^{2+} (5 mM) on enzyme thermostability was also evaluated at 80 $^{\circ}\text{C}$ for 5, 10, 15 and 20 min.

Protease Sequencing Using MALDI TOF/TOF

Tryptic digestion of protease was based in the work of Wisniewski et al. (2009), with modifications. One milliliter of a solution with 90 μg of the enzyme was filtered using Amicon[®] Ultra 0.5 mL, with cut off molar mass of 10 kDa (Millipore[®], Cork, Ireland). The retained material was resuspended in ammonium bicarbonate - AMBIC 50 mM, agitated for 60 s, added of DTT 10 mM and incubated for 60 min at room temperature. The material was acetylated with Iodoacetamide – IAA 10 mM and incubated for 60 min at room temperature, protected from light. The solution was

centrifuged at 10,000 x g for 20 min and the retained material was washed twice with a AMBIC 50 mM and Urea 0.5 M solution, followed by centrifugation at 10,000 x g for 20 min. The material was then washed twice with a AMBIC 50 mM and centrifuged at 10,000 x g for 20 min. A trypsin solution was added to the retained material (Sigma-Aldrich, St. Louis, Missouri, USA), at a final concentration of 25 ng/μL, and the mixture was incubated at 37 °C, 400 rpm, for 20 h. The solution containing the peptides was centrifuged at 10,000 x g for 20 min and the filtrate was transferred to a new tube. Peptides were concentrated at a vacuum concentrator (Eppendorf®, USA) for approximately 12 h and subjected to a MALDI TOF/TOF analysis (Supplemental Material S1).

The ratios mass/charge of the theoretical tryptic peptides of proteins were analyzed using Mascot Server 2.4 (Matrix Science), Scaffold Q+ 3.0 Software (Proteome Software) and Peaks Online Server 7 (Bioinformatics Solutions) (Supplemental Material S1) and predicted by the *peptidemass* tool from ExPASy (http://web.expasy.org/peptide_mass). They were manually confronted with the lists of masses generated by the MS1 analysis from MALDI TOF/TOF (Altuntas et al., 2014).

Identification of the Gene Encoding the Extracellular Protease on the Bacteria P. fluorescens 07A Genome

The genome of the strain *P. fluorescens* 07A was partially sequenced in a previous study that isolated the UFV-P2 phage from this bacteria (Eller et al., 2014). The sequenced reads that assembled the UFV-P2 genome were excluded from data set and the remaining 45 million of paired reads with 51 nucleotides were selected for

P. fluorescens 07A genome. These reads were assembled in contigs using CLC Genomics Workbench version 6.5.1 (CLCbio), producing 603 sequences with an average size of 9.9 kb. Contigs sequences were analyzed using Prodigal software. A total of 5,446 genes were identified and the encoded proteins were functionally annotated using BLAST searches against reference proteomes of *P. fluorescens* available at UniProt (<http://www.uniprot.org/>). This protein data set was selected as reference on peptide sequencing. The sequenced peptides were submitted to BLAST searches against contigs of the *P. fluorescens* 07A genome and the predicted proteins.

Results

Protease Purification

The enzyme was partially purified from the *P. fluorescens* 07A supernatant. The fractions of 0 to 40% and 40 to 60% of ammonium sulfate presented proteolytic activity by the zymogram and proteolysis assay. The fraction 40 to 60% was chosen for the next steps since it contained less contaminant proteins (Figure 1, Line 2). This fraction was purified with a Sephacryl S-200 HR column and three peaks could be observed, one of which presented proteolytic activity. Fractions corresponding to this peak were concentrated with Amicon® Ultra-15 and subjected to electrophoresis. The apparent molar mass for this protease is 50 kDa (Figure 1, Line 3). Beyond gelatin, the protease hydrolyzed casein and azocasein, but not BSA.

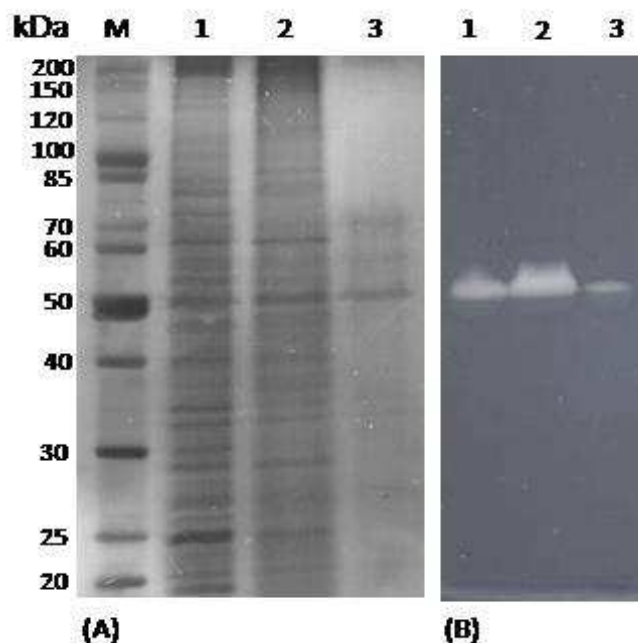


Figure 1. SDS-PAGE (A) and zymogram (B) of the proteolytic fractions in the purification steps. Line M: Molecular marker. Line 1: *Pseudomonas fluorescens* 07A crude extract; Line 2: fraction 40-60% of ammonium sulfate; Line 3: partially purified protease after chromatography.

Effect of Temperature and pH on Enzyme Activity

Protease activity was maintained in a wide range of conditions, of 4 to 60 °C (Figure 2a) and pH of 5.0 to 10.0 (Figure 2b). The enzyme presents maximum activity at 37 °C and pH 7.5. Over 95% activity was maintained at 50 °C, but above this temperature, enzyme activity was considerably reduced (Figure 2a). In pH 6.0, 90% of the maximum proteolytic activity was observed. This value is slightly below the pH of milk. In pH 8.0 the activity was reduced to 68% of the optimum and remained practically constant until pH 10.0 (Figure 2b).

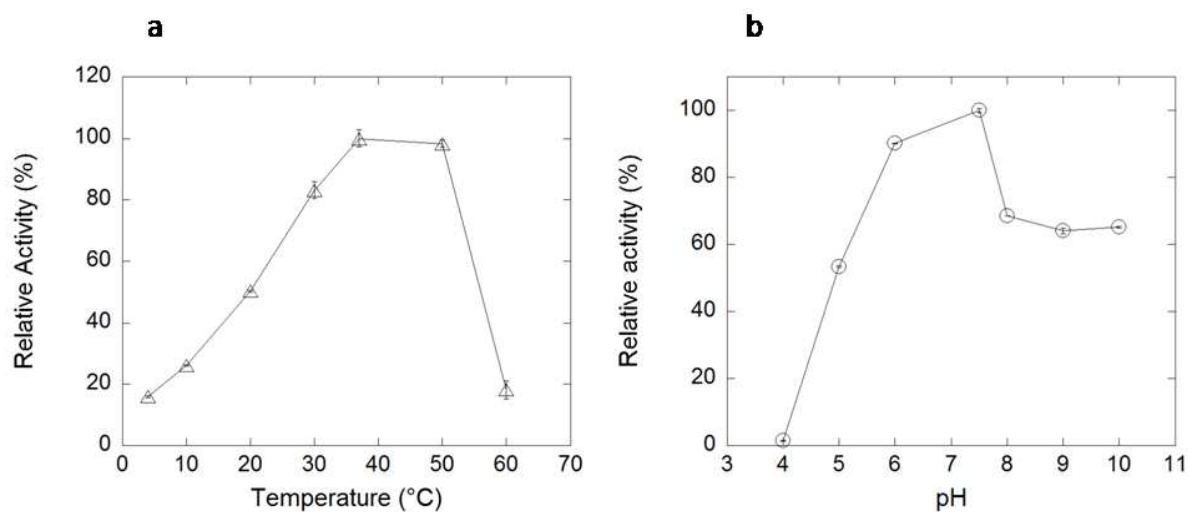


Figure 2. Effect of temperature (a) and pH (b) on the activity of the extracellular protease from *Pseudomonas fluorescens* 07A.

Effect of Inhibitors and Metallic Ions on Enzyme Activity

Inhibitors of aspartyl protease (pepstatin A) and serine protease (PMSF and benzamidine) had no effect on protease activity (Table 1), indicating that this enzyme doesn't belong to these catalytic groups. On the other hand, 5 mM of EDTA, an agent chelant of divalent ions, and 1,10-phenantroline, chelant of Zn^{2+} , have reduced enzyme activity in 87% and 97%, respectively. Protease activity was also reduced in the presence of DTT.

Table 1. Effect of inhibitors on protease activity

Inhibitor	Concentration (mM)	Relative activity (%)
Control	0	100.0±1.39
Pepstatin A	0.001	98.5±1.93
	0.005	97.1±1.83
	0.01	97.6±1.10
E-64	0.001	90.7±3.42
	0.005	88.5±3.13
	0.01	80.4±0.28
EDTA	1	91.3±1.71
	5	13.4±2.34
PMSF	1	92.2±2.00
	5	100.8±0.11
1,10-phenanthroline	1	44.8±1.71
	5	2.6±0.28
Benzamidine	1	94.0±0.21
	5	97.8±1.92
DTT	1	57.3±1.60
	5	47.7±0.32

Relative activity is the percentage of activity in the control treatment (without inhibitors).

E-64: trans-Epoxy succinyl-L-leucylamido(4-guanidino)butane, EDTA: ethylenediaminetetraacetic acid, PMSF: phenylmethylsulfonyl fluoride, DTT: Dithiothreitol.

The ion Ca^{2+} had no effect on protease activity, different from ions Cu^{2+} , Ni^{2+} , Zn^{2+} , Hg^{2+} , Fe^{2+} and Mg^{2+} , which had decreasing levels of inhibition, respectively, at 2 mM (Table 2). Moreover, protease activity was stimulated in the presence of Mn^{2+} ,

with a raise of 67.6% and 87.8% at manganese concentrations of 2 and 5 mM, respectively.

Table 2. Effect of metallic ions on protease activity

Ion	Concentration (mM)	
	2	5
Mg ²⁺	92.4±0.09	90.4±0.18
Cu ²⁺	39.7±0.18	11.7±0.46
Zn ²⁺	72.3±0.09	33.4±0.83
Ca ²⁺	99.1±2.57	100.2±3.21
Ni ²⁺	56.2±0.09	54.2±0.55
Mn ²⁺	167.6±1.10	187.8±3.67
Hg ²⁺	77.0±0.09	51.2±2.02
Fe ²⁺	78.0±2.11	75.3±1.30

Relative activity is the percentage of activity compared to a control treatment with no ions (100.00 ± 2.30%).

Protease Thermostability

The enzyme presented 31.7% of residual activity when subjected to the temperature of 72 °C for 15 s (HTST – high temperature and short time) and 14.3% when incubated at 65 °C for 30 min (LTLT – low temperature and long time), conditions similar to the heat treatments of milk pasteurization (Table 3). Moreover, the protease retained 44%, 44% and 40% of its initial activity, even after the treatments of 80, 90 and 100 °C respectively, for 5 min.

Table 3. Thermostability of the extracellular protease from *Pseudomonas fluorescens*

07A

Temperature (°C)	Time (min)	Relative activity (%)
Control	0	100.0±0.00
65	30	14.3±0.37
72	0.25	31.7 ± 0.37
80	5	44.1±1.10
	10	32.6±0.08
	15	33.0±0.44
	20	21.3±1.24
90	1	55.5±1.66
	3	44.7±0.83
	5	43.6±0.58
100	1	60.1±0.10
	3	43.9±0.84
	5	40.5±1.73

Since the protease activity was stimulated in the presence of Mn^{2+} , the thermostability of this enzyme was also assessed in the presence and absence of $MnCl_2$ at 80 °C. The presence of this ion had a positive effect on residual enzyme activity, but not in enzyme thermostability, since the rate of decrease of enzyme activity due to the heat treatment was greater in the presence of this ion (Figure 3).

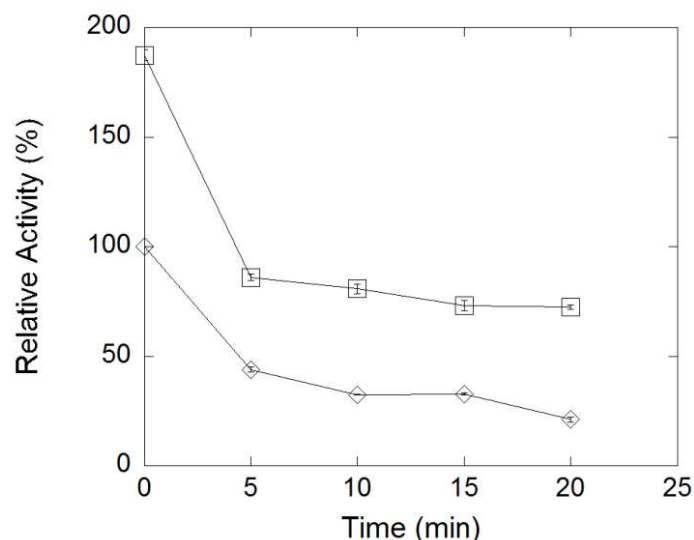


Figure 3. Effect of Mn^{+2} on enzyme thermostability. The protease was incubated at 80 °C for 5, 10, 15 and 20 min (◇) in the absence of manganese and (□) in the presence of 5 mM Mn^{+2} . Activity was expressed as percentage of the activity of a non heat-treated enzyme.

Identification of the Gene Encoding the Extracellular Protease on the Bacteria P. fluorescens 07A Genome

Tryptic peptides obtained from protease digestion were submitted to a MALDI TOF/TOF analysis and a peptide sequence could be associated to a specific protein encoded by a gene predicted in the *P. fluorescens* 07A genome (Figure 4). The contig sequence containing the extracellular protease has 14.63 kb and was sequenced with an average coverage of 400 fold (GenBank accession number KU170026). This contig contains 13 genes and the extracellular protease is encoded by gene g5. This sequence has 1,434 bp and encodes a protein with 477 amino acids, which was functionally annotated as extracellular metalloprotease (Supplemental Table S1). This protein has a predicted mass of 49.486 kDa and an

isoelectric point of 5.06. Similarity searches showed that this protein is highly conserved among *P. fluorescens* strains (Supplemental Table S1, Supplemental Figure S1). However, the sequence alignment showed that the extracellular protease of *P. fluorescens* 07A strain has few polymorphisms that differentiate this protein from other proteins identified in *P. fluorescens* strains (Supplemental Figure S1).

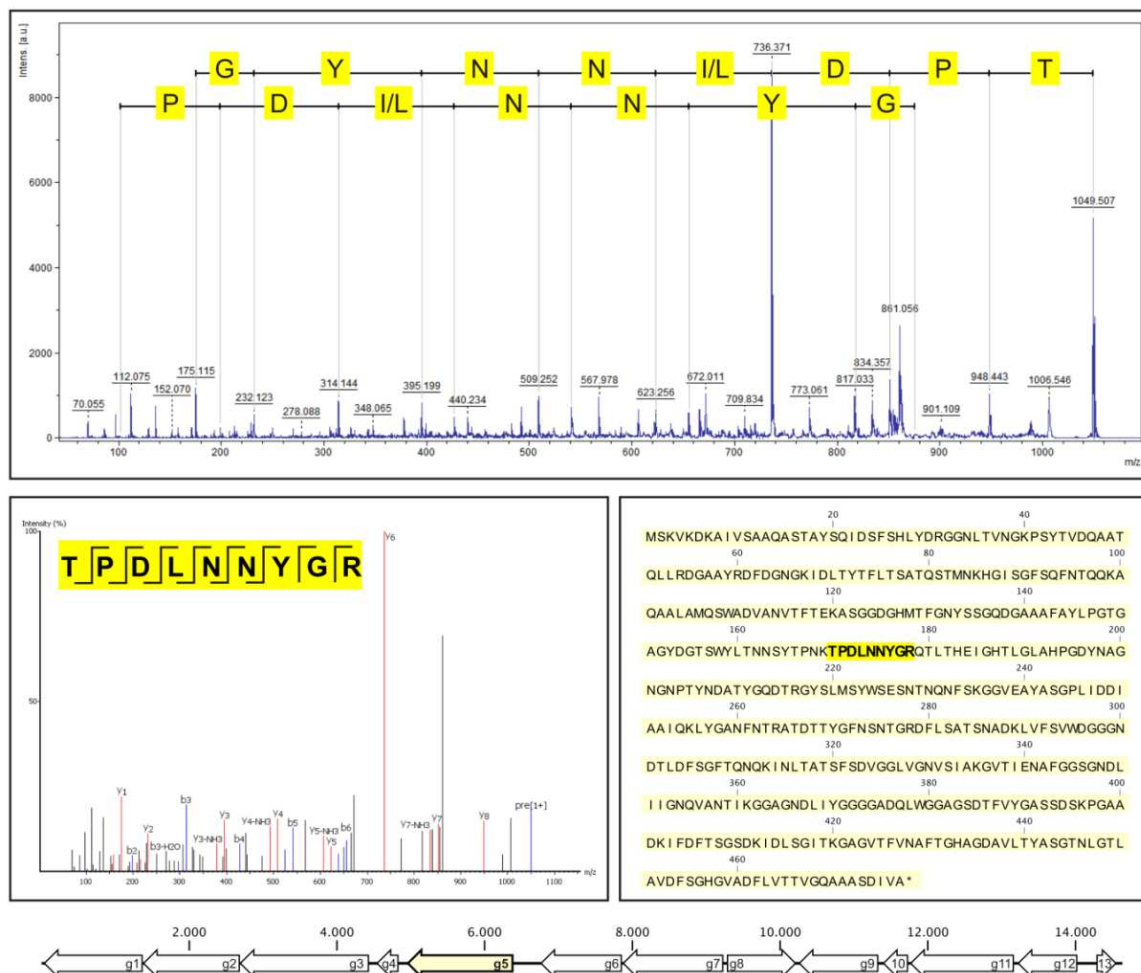


Figure 4. Sequencing and identification of the protease by a proteogenomics approach. The mass spectrums allowed the identification of a specific peptide sequence that was confirmed manually (upper) and using software (left). The differences between mass to charge ratios (x-axis) indicates the amino acids on sequenced peptide. The protease sequence (right) that contains the peptide was

predicted from a gene identified in the genomic sequence (bottom) of *Pseudomonas fluorescens* 07A.

Discussion

The presence of proteases produced by psychrotrophic microorganisms in milk results in degradation of casein micelles and hence in many technological problems in dairy industries (McPhee and Griffiths, 2011). The extracellular protease from *P. fluorescens* 07A presents activity in a wide range of conditions, as pH 5.0 to 10.0 and temperatures from 4 to 60 °C, which include many of the conditions for storage of milk and dairy products. For example, Prato Cheese is stored at approximately 12 °C during ripening and has a pH near to 5.5, conditions in which the enzyme presented over 26% and 54% activity, respectively. Similarly, pasteurized milk has a pH of 6.7 and is stored between 4 and 7 °C, conditions that also favor the hydrolytic activity. Considering UHT treatment, the milk can be stored for months at room temperature, which corresponds to the condition closer to the maximum protease activity and could explain why UHT milk is the product most affected by the technological problems caused by these proteases.

The high and almost constant activity presented by this protease in the range of pH over 7 was very particular. In the other hand, other proteases showed a continuous symmetrical reduction in their activities as the pH of the medium increased in relation to their pH of maximum activity (Zambare et al., 2011; Mu et al., 2009; Huston et al., 2004). The pH influences enzyme conformation and, consequently, the catalytic activity of their active site and their ability to recognize

and bind the substrate. This occurs through changes in the ionization degree of amino acid residues, which directly interferes with hydrophobic interactions maintaining the enzyme structure. Thus, in this study, deprotonation generated by increasing the pH allowed the stabilization of the enzyme activity at pH above 8.0. It means that deprotonating does not cause a disruptive change in the enzyme structure, contributing to the behavior observed in this work for pH from 8.0 to 10.0.

There are no patterns for stimulation or inhibition of protease activity by ions. As the enzyme characterized in this study, other proteases produced by different bacteria have their activity stimulated in the presence of Mn^{2+} (Salwan and Kasana, 2013; Ibrahim et al., 2011; Mu et al., 2009), or inhibited by Cu^{2+} , Zn^{2+} , Ni^{2+} , Hg^{2+} and Fe^{2+} (Zambare et al., 2011; Divakar et al., 2010; Sana et al., 2006). Stimulation of the enzyme activity by manganese ions suggests that it plays an important role in maintaining its active conformation (Kuddus and Ramteke, 2008), but how this stabilization occurs is still not known. However, this is again not a common pattern for *Pseudomonas* proteases, which usually require Ca^{2+} or Zn^{2+} as cofactors (Jankiewicz et al., 2010; Liao and McCallus, 1998; Sørhaug and Stepaniak, 1997).

Milk is a food rich in salts of phosphates, citrates, chlorides, sulfates and carbonates of sodium, potassium, calcium and magnesium (Fox and McSweeney, 1998). Thus, the activity of the protease from *P. fluorescens* 07A might not be stimulated in milk, since manganese salts are present only at low concentrations in this product (30 $\mu g/L$ or 0.55 μM) or even in concentrated milk products. An important finding is the fact that calcium, which is present in high concentrations in the milk (1200 mg/L), is probably a cofactor for this enzyme, since it has a calcium-binding domain in its structure, like other protease from psychrotrophic bacteria. This fact is

reinforced by the fact that this protease activity was inhibited by EDTA and 1,10-phenanthroline, confirming that this enzyme is a metalloprotease, which is a group of enzymes which require metallic ions in the active site to maintain their structure and stability.

The presence of zinc inhibited the protease activity in proportion to its concentration, although the analysis of its sequence revealed the presence of a Zinc-dependent metalloprotease, Serralysin-like subfamily and a Peptidase M10, a conserved protein domain family that suggest a zinc dependence for catalysis. An analysis of the sequence of a metalloprotease from a psychrophilic bacterium strain isolated from marine environment suggests that this enzyme needs Zn^{2+} , although its characterization also has shown complete inhibition of its activity in the presence of this ion at 0.1 mM, 1.0 mM and 10 mM (Huston et al., 2004). Furthermore, some Zn^{2+} -dependent metalloproteases (for example, aminopeptidase, thermolysin) could be inhibited by an excess of this ion due to the formation of a zincmonhydroxide bridge (Massaoud et al., 2011; Larsen and Auld, 1989).

Activity reduction by DTT would suggest that this protease is stabilized by disulfide bonds. Similar findings were reported for other proteases from *Pseudomonas* (Tang et al., 2010; Dufour et al., 2008; Gupta et al., 2005). However, the absence of cysteine residues in the protease sequence remits to alternative mechanisms of reduction of enzymes activity by DTT. For example, DTT could act as a metal ion chelator, what could explain the inhibition of cofactor-dependent enzymes (Cornell and Crivaro, 1972; Paoletti et al., 1997; Yang et al., 1996). It was also hypothesized that DTT could inhibit enzyme activity by competing with its substrates, or even by steric hindrance via hydrogen interactions with amino acid residues in the

catalytic domain (Alliegro, 2000). These findings demonstrate that the statement that enzymes inhibited by DTT would be stabilized by disulfide bonds, although true for many enzymes, must be confirmed by supplementary experiments.

Koka and Weimer (2000) affirmed that the competition for ions of these proteases with other milk proteins may be a factor regulating their activity. According to the authors, if the proteins present in milk have a higher binding affinity for metal ions than proteases, the hydrolysis of milk proteins by these proteases would be a strategy of these enzymes for obtaining ions in this product.

Heat treatments adopted by the dairy industry and the cooling conditions after processing may not be sufficient to completely inhibit the activity of proteolytic enzymes as the protease studied in this work (Figure 2). The thermostability pattern presented by this protease demonstrated the importance in determining the ideal combination of time and temperature for heat treatments in the industry, since a longer time for heat treatment (65 °C for 30 min) results in reduced enzymatic activity, although it may increase the destruction and inactivation of some milk constituents (Fellows, 2000). Alternatively, treatment HTST has been able to reduce activity to almost 30% within only 15 s. More importantly, the protease was more stable when subjected to the treatment at 100 °C for 5 min, compared to milder treatments.

Inactivation of some bacterial proteases deviates the first-order kinetics in certain temperature ranges (McKellar, 1989). In the literature, some reports describe proteases of psychrotrophic presenting this behavior, which is known as low-temperature inactivation - LTI. This could be mainly attributed to enzyme auto

digestion or its aggregation with molecules of casein (Stepaniak and Sørhaug, 1995; McKellar, 1989; Barach et al., 1978).

Many other proteases produced by strains of *P. fluorescens* are also heat-resistant and retain activity even after UHT treatment. This residual activity can cause changes in the physicochemical properties of the casein micelles and lead to destabilization and gelation of UHT milk during storage (Baglinière et al., 2013; McPhee and Griffiths, 2011). Thus, adoption of appropriate practices for obtaining milk is necessary in order to minimize contamination of the raw material by psychrotrophic microorganisms.

The gene that encodes the extracellular protease of *P. fluorescens* 07A was identified by a proteogenomics approach (Figure 4). A specific peptide sequenced by MALDI TOF/TOF allowed the identification of gene that encodes the extracellular protease in the *P. fluorescens* 07A genome and its complete amino acid sequence. The extracellular protease has 49.486 kDa and its sequence is highly conserved among *P. fluorescens* strains (Supplemental Figure S1), members of serralyisin-like proteins. Serralyisin are virulence factors in pathogenic bacteria and do not require signal sequences for transmembrane translocation. The proteases presenting similarity to the *P. fluorescens* 07A enzyme showed optimum activity in temperatures between 37 – 45 °C and pH 7.0 – 9.0 (Zhang et al., 2009; Anderson et al., 2004; Liao and McCallus, 1998). The characteristic of thermoresistance was also found for some of these proteases (Liao and McCallus, 1998).

The protease showing higher similarity with the protease sequenced in this study (C9WKP6) was isolated from the *P. fluorescens* strain TSS (Zhang et al., 2009), an aquaculture pathogen that can infect a number of fish species.

Surprisingly, this protease has its activity stimulated by 10 mM Ca^{2+} and Zn^{2+} , even presenting only two amino acids substitutions in its sequence. One of these substitutions is located in the calcium-binding domain (343-385), where a serine residue in position 356 in the *P. fluorescens* 07A sequence was substituted by an alanine residue. Miyajima et al. (1998) conducted an experiment with a *P. aeruginosa* protease wherein they replaced an aspartate residue at position 356 by an alanine and verified that the protease activity was completely inhibited, showing the importance of this residue in binding this ion and therefore in regulating the catalytic activity of the enzyme.

Conclusions

This work shows the importance of the knowledge about the characteristics of proteolytic enzymes in order to control their activity in raw milk and hence reduce technological problems in dairy products. The protease has maximum activity at 37 °C and pH 7.5, which is compounded by its high residual activity after the most intense heat treatments administered. On the other hand, the significant reduction on protease activity under LTLT and HTST treatments highlights new perspectives to the treatment of milk to control technological problems caused by these enzymes.

Acknowledgments

The authors acknowledge financial support from the following Brazilian agencies: Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors are grateful also to the Núcleo de Análise de Biomoléculas of the Universidade Federal de Viçosa for providing the facilities for the running and conduction of the experiments and to Edvaldo Barros and Humberto Josué de Oliveira Ramos, for assisting the experimental design for enzyme purification and sequencing.

References

- Alliegro, M.C. 2000. Effects of dithiothreitol on protein activity unrelated to thiol-disulfide exchange: for consideration in the analysis of protein function with cleland's reagent. *Anal. Biochem.* 282:102–106.
- Altuntas, E.G., K. Ayhan, S. Peker, B. Ayhan, and D.O. Demiralp. 2014. Purification and mass spectrometry based characterization of a pediocin produced by *Pediococcus acidilactici* 13. *Mol. Biol. Rep.* 41:6879–6885.
- Anderson, L.M., V.O. Stockwell, and J.E. Loper. 2004. An extracellular protease of *Pseudomonas fluorescens* inactivates antibiotics of *Pantoea agglomerans*. *Biol. Control.* 94:1228–1234.
- Ayora, S., and F. Götz. 1994. Genetic and biochemical properties of an extracellular neutral metalloprotease from *Staphylococcus hyicus* subsp. *hyicus*. *Mol. Gen. Genet.* 242:421–430.
- Baglinière, F., A. Matéos, G. Tanguy, J. Jardin, V. Briard-Bion, F. Rousseau, B. Robert, E. Beaucher, J.L. Gaillard, C. Amiel, G. Humbert, A. Dary, and F. Gaucheron. 2013. Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilisation. *Int. Dairy J.* 31:55–61.
- Barach, J.T., D.M. Adams, and M.L. Speck. 1978. Mechanism of low temperature inactivation of a heat-resistant bacterial protease in milk. *J. Dairy Sci.* 61:523–528.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*

72:248–254.

Cornell, N.W., and K.E. Crivaro. 1972. Stability constant for the zinc-dithiothreitol complex. *Anal. Biochem.* 47:203–208.

Datta, N., and H.C. Deeth. 2001. Age gelation of uht milk—A Review. *Inst. Chem. Eng.* 79:197–210.

Decimo, M., S. Morandi, T. Silvetti, and M. Brasca. 2014. Characterization of gram-negative psychrotrophic bacteria isolated from italian bulk tank milk. *J. Food Sci.* 79:2081–2090.

Divakar, K., J.D.A. Priya, and P. Gautam. 2010. Purification and characterization of thermostable organic solvent-stable protease from *Aeromonas veronii* PG01. *J. Mol. Catal. B Enzym.* 66:311–318.

Dufour, D., M. Nicodème, C. Perrin, A. Driou, E. Brusseaux, G. Humbert, J.-L. Gaillard, and A. Dary. 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. *Int. J. Food Microbiol.* 125:188–96.

Eller, M.R., P.M.P. Vidigal, R.L. Salgado, M.P. Alves, R.S. Dias, C.C. da Silva, A.F. de Carvalho, A. Kropinski, and S.O. De Paula. 2014. UFV-P2 as a member of the Luz24likevirus genus: a new overview on comparative functional genome analyses of the LUZ24-like phages. *BMC Genomics.* 15.

Ercolini, D., F. Russo, I. Ferrocino, and F. Villani. 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiol.* 26:228–31.

Feitosa, L., W. Gremski, S.S. Veiga, M.C.Q.. Elias, E. Graner, O.C. Mangili, and R.R.

- Brentani. 1998. Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in Brown spider (*Loxosceles intermedia*) venom. *Toxicon*. 36:1039–1051.
- Fellows, P. 2000. Food Processing Technology: Principles and Practice. Second Edition. CRC Press, Boca Raton. New York, Washington, DC. 591 pp.
- Fox, P.F., and P.L.H. McSweeney. 1998. Dairy Chemistry and Biochemistry. 1st ed. Kluwer Academic Publisher, London, UK. 478 pp.
- Gupta, A., I. Roy, S.K. Khare, and M.N. Gupta. 2005. Purification and characterization of a solvent stable protease from *Pseudomonas aeruginosa* PseA. *J. Chromatogr. A*. 1069:155–161.
- Huston, A.L., B. Methe, and J.W. Deming. 2004. Purification, characterization, and sequencing of an extracellular cold-active aminopeptidase produced by marine psychrophile *Colwellia psychrerythraea* Strain 34H. *Appl. Environ. Microbiol.* 70:3321–3328.
- Ibrahim, K.S., J. Muniyandi, and S.K. Pandian. 2011. Purification and characterization of manganese-dependent alkaline serine protease from *Bacillus pumilus* TMS55. *J. Microbiol. Biotechnol.* 21:20–27.
- Jankiewicz, U., U. Szawłowska, and M. Sobańska. 2010. Biochemical characterization of an alkaline metallopeptidase secreted by a *Pseudomonas fluorescens* isolated from soil. *J. Basic Microbiol.* 50:125–34.
- Koka, R., and B.C. Weimer. 2000. Isolation and characterization of a protease from *Pseudomonas fluorescens* RO98. *J. Appl. Microbiol.* 89:280–288.
- Kuddus, M., and P.W. Ramteke. 2008. A cold-active extracellular metalloprotease from *Curtobacterium luteum* (MTCC 7529): Enzyme production and

- characterization. *J. Gen. Appl. Microbiol.* 54:385–392.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.
- Larsen, K.S., and D.S. Auld. 1989. Carboxypeptidase A: Mechanism of zinc inhibition. *Biochemistry.* 28:9620–9625.
- Liao, C.-H., and D.E. McCallus. 1998. Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. *Appl. Environ. Microbiol.* 64:914–921.
- Massaoud, M.K., J. Marokházi, and I. Venekei. 2011. Enzymatic characterization of a serralyisin-like metalloprotease from the entomopathogen bacterium, *Xenorhabdus*. *Biochim. Biophys. Acta.* 1814:1333–9.
- McKellar, R.C. 1989. Enzymes of Psychrotrophs in Raw Food. CRC Press, Boca Raton.
- McPhee, J.D., and M.W. Griffiths. 2011. Psychrotrophic bacteria. *Pseudomonas* spp. In *Encyclopedia of Dairy Sciences*. J.W. Fuquay, P.F. Fox, and P.L.H. McSweeney, editors. Academic Press. 379–383.
- Miyajima, Y., Y. Hata, J. Fukushima, S. Kawamoto, K. Okuda, Y. Shibano, and K. Morihara. 1998. Long-range effect of mutation of calcium binding asparates on the catalytic activity of alkaline protease from *Pseudomonas aeruginosa*. *J. Biochem.* 123: 24–27.
- Mu, Z., M. Du, and Y. Bai. 2009. Purification and properties of a heat-stable enzyme of *Pseudomonas fluorescens* Rm12 from raw milk. *Eur. Food Res. Technol.* 228:725–734.
- Neubeck, M. Von, C. Baur, M. Krewinkel, M. Stoeckel, S. Scherer, and M. Wenning.

2015. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *Int. J. Food Microbiol.* 211:57–65.
- Paoletti, P., P. Ascher, and J. Neyton. 1997. High-affinity zinc inhibition of NMDA NR1–NR2A Receptors. *J. Neurosci.* 17:5711–5725.
- Pinto, C.L.O. 2004. Bactérias psicrotróficas proteolíticas do leite cru refrigerado granelizado destinado à produção de leite UHT. Tese (Doutorado em Microbiologia Agrícola) - Universidade Federal de Viçosa. 97 pp.
- Pinto, C.L.O., M.L. Martins, and M.C.D. Vanetti. 2006. Qualidade microbiológica de leite cru refrigerado e isolamento de bactérias psicrotróficas proteolíticas. *Ciênc. Tecnol. Aliment.* 26: 645-651.
- Salwan, R., and R.C. Kasana. 2013. Purification and characterization of an extracellular low temperature-active and alkaline stable peptidase from psychrotrophic *Acinetobacter* sp. MN 12 MTCC (10786). *Indian J. Microbiol.* 53:63–9.
- Sana, B., D. Ghosh, M. Saha, and J. Mukherjee. 2006. Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma-Proteobacterium isolated from the marine environment of the Sundarbans. *Process Biochem.* 41:208–215.
- Sørhaug, T., and L. Stepaniak. 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. *Trends Food Sci. Technol.* 8:35–41.
- Stepaniak, L., and T. Sørhaug. 1995. Thermal Denaturation of Bacterial Enzymes in Milk. *In* Heat Induced Changes in Milk. P.F. Fox, editor. International Dairy Federation, Brussels, Belgium. 349–363.
- Tang, X.-Y., B. Wu, H.-J. Ying, and B.-F. He. 2010. Biochemical properties and

- potential applications of a solvent-stable protease from the high-yield protease producer *Pseudomonas aeruginosa* PT121. *Appl. Biochem. Biotechnol.* 160:1017–31.
- Wiśniewski, J.R., A. Zougman, N. Nagaraj, and M. Mann. 2009. Universal sample preparation method for proteome analysis. *Nat. Methods.* 6:359–362.
- Yang, Y., H.R. Wang, and H.M. Zhuo. 1996. Kinetics of inhibition of aminoacylase activity by dithiothreitol or 2-mercaptoethanol. *Int. J. Pept. Protein Res.* 48:532–538.
- Zambare, V., S. Nilegaonkar, and P. Kanekar. 2011. A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. *N. Biotechnol.* 28:173–81.
- Zhang, S., and J. Lv. 2014. Purification and properties of heat-stable extracellular protease from *Pseudomonads fluorescens* BJ-10. *J. Food Sci. Technol.* 51:1185–90.
- Zhang, W., Y. Hu, H. Wang, and L. Sun. 2009. Identification and characterization of a virulence-associated protease from a pathogenic *Pseudomonas fluorescens* strain. *Vet. Microbiol.* 139:183–188.

Supplemental Material

Supplemental material S1

Methodology of Peptide Sequencing by Mass Spectrometry

Tryptic peptides were resuspended in 10 L of 50% acetonitrile acidified with 0.1% trifluoroacetic acid. The matrix used was α -cyano-4-hydroxycinnamic acid - HCCA (BrukerDaltonics, Germany) solubilized in the same solution used to resuspend the tryptic peptides, to a final concentration of 10 mg/mL, following manufacturer's instructions. For calibration of the MS1 analysis method, standard peptides were used (Peptide Calibration Standard II) (BrukerDaltonics, Germany). The samples were applied to a plate appropriate to MALDI-TOF/TOF, Ultraflex III (BrukerDaltonics, Germany), performed manually and in duplicate by adding 1 μ L sample and 1 μ L matrix for each spot.

The MS1 data was obtained by using the positive and reflective mode, with a detection range of 500-3500 Da. To the MS2, we used the LIFT method in positive mode. For this, ions with intensity greater than 3,000 were selected and ratio charge-mass (m/z) greater than 700 Da.

All data were analyzed by the software Flexcontrol, version 3.3 (BrukerDaltonics, Germany), and the spectra resulting from the MS1 and MS2 analysis were processed with the flexanalysis application, version 3.3 (BrukerDaltonics, Germany). The lists of MS1 peaks were generated in extensible markup language (XML) format by flexanalysis software. The lists of MS2 peaks

were generated in mascot generic format (mgf) by Biotoools software, version 3.2 (BrukerDaltonics, Germany).

The lists with the mass values were compared with the proteins of the bacterium *P. fluorescens* 07A using the MASCOT software, version 2.4.0 (Matrix Science, London, UK). The parameters used for the research were: enzymatic digestion by trypsin with a missed cleavage; an error tolerance for the parental ion of 0.2 Da and 0.5 Da for fragments; cysteine carbamidomethylation as fixed modification and oxidation of methionine as a variable modification.

The result obtained by MASCOT was validated by SCAFFOLD application, version 4.4.1.1 (Proteome Software Inc., Portland, OR). To validate the peptides identified by MASCOT, the algorithm Peptide Prophet was applied (Keller et al., 2002) and for proteins, the Protein Prophet algorithm (Nesvizhskii et al., 2003), with acceptance criteria for those with the probability of identification greater than 90 % in both cases. Proteins that had similar peptides and could not be differentiated based on the analysis of MS/MS data were grouped, satisfying the principles of parsimony. The lists of MS/MS were also subjected to PEAKS software, version 7.0 (BioinformaticsSolutions Inc., Canada) in order to achieve greater coverage of protein identification using an FDR rate ≤ 1 .

References

Keller, A., A.I. Nesvizhskii, E. Kolker, and R. Aebersold. 2002. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and data base search. *Anal Chem.* 74: 5383–5392.

Nesvizhskii, A.I., A. Keller, E. Kolker, and R. Aebersold. 2003. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem.* 75: 4646–4658.

Table S1. Functional annotation of genes predicted from a contig of the *Pseudomonas fluorescens* 07A genome. This contig sequence contains the gene (g5) that encodes the protease identified in this work. The contig sequence was registered in GenBank under accession number KU170026.

Query	Annotation	Sequence aa length	E- value	Identity(%)	Similarity(%)	UniProt ID	UniProt Description	Organism
g1	Outer membrane protein	446	0	99.55	100.00	I4KBX6	Outer membrane protein	<i>Pseudomonas fluorescens</i> SS101
g2	Membrane fusion protein	437	0	98.86	99.54	I4K616	Membrane fusion protein	<i>Pseudomonas fluorescens</i> SS101
g3	ABC transporter	583	0	99.14	99.66	I2BSQ5	ABC transporter	<i>Pseudomonas fluorescens</i> A506
g4	Protease inhibitor Inh	99	1.81E- 66	97.98	97.98	I2BMS8	Protease inhibitor Inh	<i>Pseudomonas fluorescens</i> A506
g5	Extracellular metalloprotease	477	0	99.58	100.00	C9WKP6	Extracellular metalloprotease	<i>Pseudomonas fluorescens</i>
g6	Peptidase, T4 family	367	0	97.55	99.18	I4KB76	Peptidase, T4 family	<i>Pseudomonas fluorescens</i> SS101
g7	Amino acid permease, APC family	454	0	99.56	99.78	I4KFW5	Amino acid permease, APC family	<i>Pseudomonas fluorescens</i> SS101
g8	Transcriptional regulator, LuxR family	312	0	98.47	99.23	I2BMY9	Transcriptional regulator, LuxR family	<i>Pseudomonas fluorescens</i> A506
g9	Periplasmic putrescine- binding protein, PotF family	359	0	95.82	99.44	I2BKK3	Periplasmic putrescine- binding protein, PotF family	<i>Pseudomonas fluorescens</i> A506
g10	Uncharacterized protein	113	2.75E- 77	96.46	96.46	W2F7P2	Cupin	<i>Pseudomonas fluorescens</i> FH5
g11	FAD dependent oxidoreductase	468	0	96.80	98.08	I4K7P7	FAD dependent oxidoreductase	<i>Pseudomonas fluorescens</i> SS101

g12	Transcriptional regulator, AraC family	269	0	96.28	98.14	I2BMU2	Transcriptional regulator, AraC family	<i>Pseudomonas fluorescens</i> A506
g13	Uncharacterized protein	90	5.68E- 62	100.00	100.00	I2BWN5	Uncharacterized protein	<i>Pseudomonas fluorescens</i> A506

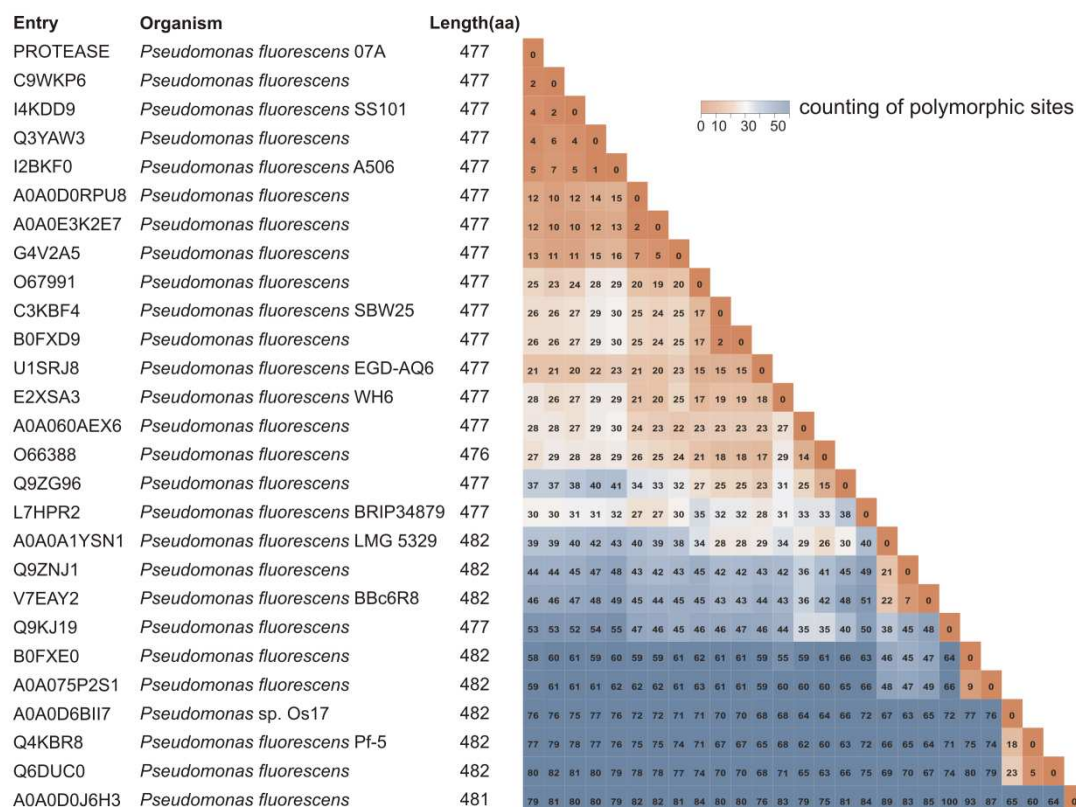


Figure S1. Pairwise sequence comparisons between the proteases of *Pseudomonas fluorescens* strains. A pairwise matrix containing the number of differences (polymorphic sites) among the amino acid sequences was calculated from the sequences that present high similarity with the protease characterized in this work (upper).

**4. CHAPTER 3: PRODUCTION AND ACTIVITY OF PROTEASES FROM
PSEUDOMONAS FLUORESCENS 07A IN MILK**

TEMPERATURE MODULATES THE PRODUCTION AND ACTIVITY OF PROTEOLYTIC ENZYMES FROM *PSEUDOMONAS* IN DAIRY

Abstract

This work evaluated the expression and activity of proteases by *Pseudomonas fluorescens* 07A strain in milk at different temperatures. Low relative expression of proteases by the bacteria was observed after incubation for 12 h at 25 °C, when the bacteria is found in the logarithmic growth phase, compared to refrigeration temperatures of 4 and 10 °C, when the bacteria is in lag phase. After 24 h, protease production significantly increased ($P < 0.05$) at 25 °C and remained constant up to 48 h, range in which the bacteria remained in stationary phase. These data indicate that this protease could be produced as an adaptive strategy to the environment under the conditions used. An additional extracellular protease of approximately 30 kDa was produced after 36 h of incubation at 25 °C in Luria Bertani, but not in milk, and it completely disappeared at 48 h. Casein fractions were massively degraded by *P. fluorescens* 07A, the purified protease and the bacterial extract on the seventh day of incubation at 25 °C and to a lesser extent at 10 °C for the sample incubated with the bacteria. Heat treatment at 90 °C for 5 min completely inactivated the proteolytic activity of the purified protease and the bacterial extract. This work contributes to the understanding of the milk storage conditions that influence the production and activity of these enzymes. These results show the need to search for alternative strategies

by dairy industries to control the synthesis and activity of these enzymes in order to ensure the quality of processed products.

Keywords: Milk storage, control of milk proteolysis, protease;

Introduction

Refrigeration of raw milk at farms and dairy processing plants maintains milk quality by reducing the growth of mesophilic bacteria (Samaržija et al., 2012). However, it favours multiplication of psychrotrophic bacteria, especially the genus *Pseudomonas* (Neubeck et al., 2015; Rasolofo et al., 2010; De Jonghe et al., 2011). Among them, the species *P. fluorescens* has considerable importance in milk deterioration (De Jonghe et al., 2011; Rasolofo et al., 2010).

Although psychrotrophic microorganisms are inactivated by heat treatments, their extracellular proteases are usually thermoresistant, keeping their activities even after milk pasteurization or ultra high temperature (UHT) treatments (McPhee and Griffiths, 2011). Extracellular proteases are able to cause degradation of casein micelles, which results in modifications to the physico-chemical properties and sensory characteristics of dairy products (Chen et al., 2003; MCPhee and Griffiths, 2011), leading to important economic losses.

Thus, the knowledge of storage conditions that favor expression of these enzymes by this group of bacteria is fundamental in order to avoid their presence in milk to be subjected to heat treatment.

Production of proteases by psychrotrophic bacteria is a complex process influenced by medium composition, especially iron availability (McKellar et al., 1987), culture conditions and growth phase (Nicodème et al., 2005; Rajmohan et al., 2002) and quorum sensing (Liu et al., 2007). Temperature is also a major factor associated with regulation of protease production by psychrotrophic microorganisms (Woods et al., 2001; McKellar and Cholette, 1987). However, most works has evaluated

protease production in culture media, and the results are not reproducible when extended for milk matrix.

The *P. fluorescens* 07A strain produces at least one protease of 49.5 kDa, which has maximum activity at 37 °C and pH 7.5. We have shown that this enzyme has high residual activity when subjected to intense heat treatment, while milder heat treatments lead to protease hydrolysis and massive loss of activity (Alves et al., 2016). In this work, we evaluated the expression and activity of this protease in milk, refrigerated or not, as well as the degradation of casein micelles caused by this enzyme. For this, we simulated different situations for the presence of this enzyme in milk before and after heat treatment, searching for information that may assist in the development of more effective systems for better quality and control of dairy products.

Material and Methods

The strain *P. fluorescens* 07A was isolated from raw milk collected from a dairy industry in Minas Gerais, Brazil (Pinto, 2004; Pinto et al., 2006). It was cultivated in Luria-Bertani broth (Sigma Aldrich, St. Louis, MO) at 25 °C.

Protease purification

A solution containing the bacteria in exponential phase was inoculated (1%) in Luria-Bertani broth, incubated for 18 h and centrifuged at 10,000 x g for 15 min at 4 °C. The enzyme was isolated from crude supernatant and partially purified by fractional precipitation using ammonium sulfate (40 - 60%) for 1 h followed by

centrifugation at 12,000 x g for 30 min at 4 °C. The pellet was resuspended in 10 mM Tris-HCl, 50 mM NaCl buffer, pH 7.5, and dialyzed twice against this buffer using a cellulose membrane of 14 kDa (Sigma-Aldrich, St. Louis, Missouri, USA) for 8 h. All steps were performed at 4 °C, and an aliquot was collected to monitor enzyme activity.

The dialyzed solution containing the enzyme was applied in a HiPrep 26/60 Sephacryl™ S-200 HR column (GE Healthcare® Little Chalfont, Buckinghamshire, UK) equilibrated with 10 mM Tris-HCl, 50 mM NaCl buffer, pH 7.5, using an AKTA Purifier chromatographer (GE Healthcare® Little Chalfont, Buckinghamshire, UK). Samples were collected at 1.6 mL/min and monitored by absorbance at 280 nm. Fractions containing proteolytic activity were concentrated by ultrafiltration in Amicon® Ultra-15 (Millipore®, Cork, Ireland) and stored at 4 °C.

Purification steps were monitored by SDS-PAGE and zymogram.

SDS-PAGE and zymogram

SDS-PAGE was performed with a 12% polyacrylamide gel according to the method of Laemmli (Laemmli, 1970). Proteins were stained with silver nitrate or 0.01% Coomassie Brilliant Blue R-250 solution.

The zymogram was performed using a 12% polyacrylamide gel and gelatin (3 mg/mL) as substrate, as described by Feitosa (Feitosa et al., 1998), with modifications. After electrophoresis, gels were washed for 30 min with a Triton X-100 2.5% (v/v) solution and incubated in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂ buffer, pH 7.5, at 37 °C for 12 h. Proteolytic activity was visualized as clear zones in the gel stained with a 0.01% Coomassie Brilliant Blue R-250 solution.

Activity Assay

The proteolytic activity was determined using azocasein (Sigma-Aldrich, St. Louis, Missouri, USA) as a substrate, as described by Ayora and Gotz (1994), with modifications. An aliquot of 250 μ L of azocasein solution at 2% (m/v) in 40 mM Tris-HCl, 2 mM CaCl₂, pH 7.5, was added to 150 μ L of the test solution. The mixture was agitated and incubated at 37 °C for 8 h, and the reaction was stopped with the addition of 1.2 mL of 10% (m/v) trichloroacetic acid. After incubation for 15 min at room temperature, the mixture was centrifuged at 12,000 x g for 15 min at 4 °C. The supernatant was neutralized with the addition of 1.0 mL of 1 M NaOH. The absorbance of the resulting solution was measured at 440 nm using a SpectraMax M2 spectrometer (Molecular Devices, Sunnyvale, California, USA). One unit of enzymatic activity was defined as the amount of enzyme sufficient to raise 0.01 of the solution absorbance at 440 nm per minute under experimental conditions. All assays were performed as triplicates and the results were expressed as the mean \pm standard deviation.

Protein concentration was estimated by the Bradford assay (Bradford, 1976) using bovine serum albumin – BSA (Sigma-Aldrich, St. Louis, Missouri, USA) as pattern to the calibration curve.

Protease expression in milk

To assess the differential expression of proteases by the proteolytic *P. fluorescens* 07A, three flasks containing 10% (w/v) reconstituted skimmed milk were inoculated with approximately 10³ CFU/mL of this bacteria and incubated at 4, 10 and

25 °C. Aliquots of each sample were collected at 0, 12, 24, 36 and 48 h to monitor enzyme activity, microbial growth and enzyme production by RT-qPCR.

Total RNA extraction and cDNA synthesis

Aliquots of 4 mL were centrifuged at 3,000 x g for 5 min at 4 °C. The pellet was lysed for total RNA extraction using Purezol™ RNA Isolation Reagent (Bio-Rad, California, USA) and cDNA was synthesized using an iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA) according to the manufacturer's recommendations. The efficiency of RNA extraction and cDNA synthesis were evaluated by quantification using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware USA).

Real-time PCR

The primers used to monitor protease production were synthesized based on the protease sequence (Alves et al., 2016) and are described as follows: Apr07 F - 5' TCT CAC GGT CAA TGG CAA AC 3' and Apr07 R – 5' TTG CCG TTG CCA TCA AAG TC 3'. The cDNAs were used as templates for Real-time PCR using a Ssofast™ EvaGreen Supermix kit (Bio-Rad, California, USA). Reactions were made in triplicate, and the level of basal expression was compared to that of 16S gene expression, using the primers 530F and 110R (Silva et al., 2016).

Statistical analysis

Relative expression of the protease by *P. fluorescens* 07A at different temperatures, within the same time, were compared using one-way analysis of

variance (ANOVA) and Tukey's test. Data from three-independent replicates were shown as the mean \pm SD. Analyses were performed using GraphPad Prism software, version 7.00 (GraphPad, San Diego, CA, USA). $P < 0.05$ were considered statistically significant.

Protease production in LB

P. fluorescens 07A strain was inoculated in Luria-Bertani (10^3 CFU/mL) and incubated at 4, 10 and 25 °C, and enzyme activity in this medium was evaluated by zymogram at the times 0, 12, 24, 36 and 48 h of incubation.

Proteolysis assay

Milk proteolysis by the purified protease was compared to that caused by the bacteria, under different conditions. For this, flasks containing 100 mL of sterile 10% (m/v) reconstituted skimmed milk were inoculated as follows:

Treatment 1. Control

Treatment 2. *P. fluorescens* 07A

Treatment 3. Purified protease (2.5% v/v)

Treatment 4. 10 mM Tris-HCl, 50 mM NaCl, pH 7.5 buffer (2.5% v/v)

Treatment 5. Heat-treated purified protease (2.5% v/v)

Treatment 6. Heat-treated *P. fluorescens* 07A extract (2.5% v/v)

Treatment 7. *P. fluorescens* 07A extract (2.5% v/v)

Each treatment was inoculated in three flasks and incubated at 4, 10 and 25 °C, as biological replicates. The initial count of *P. fluorescens* 07A (treatment 2, 6 and

7) was approximately 10^3 CFU/mL and bacterial extracts (treatments 6 and 7) were resuspended in 10 mM Tris-HCl, 50 mM NaCl buffer, pH 7.5. After addition of the purified protease and the *P. fluorescens* extract in milk, treatments 5 and 6, respectively, the samples were subjected to heat-treatment in a bath at 90 °C for 5 min. Temperatures of reconstituted skimmed milk inside the flasks were monitored with a test flask.

Aliquots of 10 mL of each treatment were collected at 0, 1, 2, 3, 5 and 7 days after inoculation. Sample preparation for proteolysis monitoring was adapted from Adams (Adams et al., 1976). Samples were acidified to pH 4.0 by using 3 M chloridric acid and centrifuged at 12,000 x g for 10 minute at 4 °C to precipitate milk caseins. The supernatant (milk serum) was discarded and the casein-containing pellet was stored at -20 °C. On the last day of assay, samples were thawed, resuspended in 10 mL of Tris-HCl 0.5 M buffer, pH 9.0, and subjected to SDS-PAGE with a 12% polyacrylamide gel (Laemmli, 1970). Proteins were stained with 0.01% Coomassie Brilliant Blue R-250 solution.

Gels were digitalized, and milk caseins concentrations were estimated by densitometry analysis using Image J® software (National Institute of Health, Bethesda, Maryland, USA).

Results

Protease expression and activity in milk

The protease expression by *P. fluorescens* 07A was proportional to the phase of microbial growth in milk. At 12 h at 25 °C (Figure 1a), when the culture was in

middle of exponential phase of growth (Supplemental Figure S2), protease production was low. However, after 24 h of incubation at this same temperature, the relative expression was significantly increased ($P < 0.05$) and remained constant up to 48 h. Associated with this result, only 0.006 U of enzyme activity was detected at 25 °C after 12 h in milk (Figure 1b), and this activity significantly increased and accumulated during incubation into stationary phase and reached 0.15 U after 48 h.

Conversely, the relative expression of proteases at 10 °C at 12 h of incubation was significantly higher ($P < 0.05$) than at 25 °C (Figure 1a), followed by a reduction in the last 24 h of incubation at refrigeration temperatures. The enzymatic activity in these treatments remained low throughout the incubation period compared to activity at 25 °C.

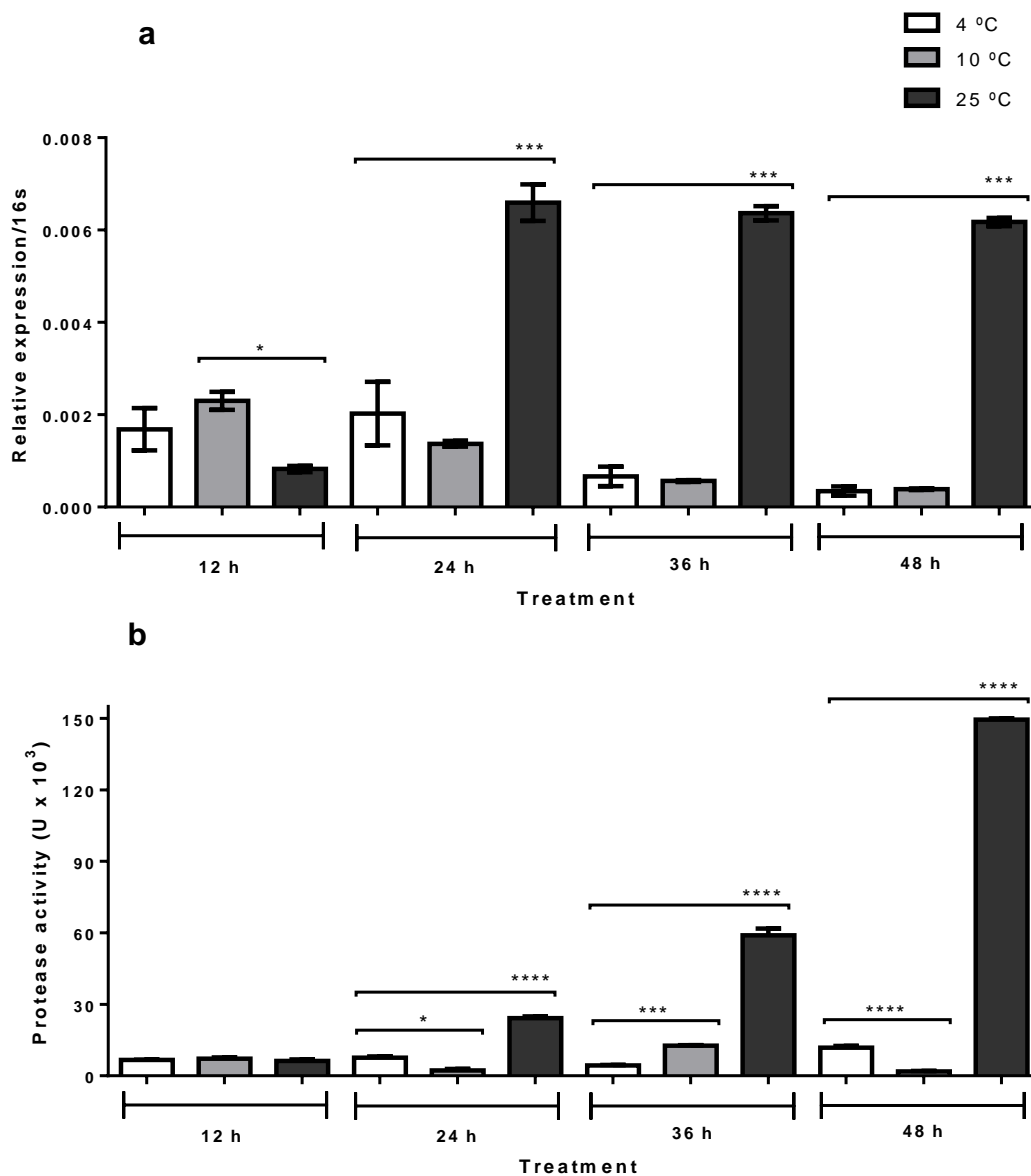


Figure 1. Relative expression of protease (a) and enzymatic activity (b) by *P. fluorescens* 07A at 4, 10 and 25 °C. Values are mean \pm SD (n = 3).

Protease production in LB

In general, when cultivated in LB medium, *P. fluorescens* 07A produces an extracellular protease of approximately 50 kDa. At 25 °C, this protease activity is

detectable within first 12 h of incubation, while at 10 °C, a discrete band can be observed at 24 h. Only after 36 h can protease activity be detected at 4 °C. However, when incubated at 25 °C, a second protease was produced after 36 h, with an estimated size of 30 kDa (Figure 2).

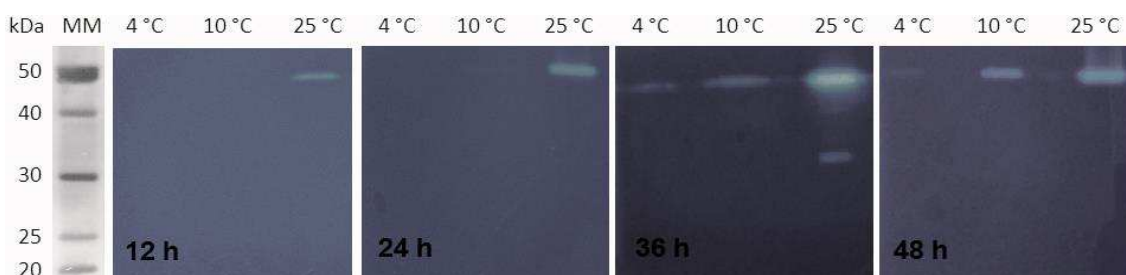


Figure 2. Zymogram of protease production by *P. fluorescens* 07A in Luria-bertani at 12, 24, 36 and 48 h of incubation at 4, 10 and 25 °C.

Proteolysis assay

The inoculation of *P. fluorescens* 07A (treatment 2), the purified protease (treatment 3) or bacterial extract (treatment 7) in LDR at 25 °C led to near complete hydrolysis of caseins (Figure 3) and visually modified the appearance of the milk in these treatments (Supplemental Figure S1). To a lesser extent, the same was observed in treatment 2, incubated with the bacteria, at 10 °C.

The degradation of the casein fractions on the seventh day after inoculation of *P. fluorescens* 07A in LDR relative to day zero was 42.98% and 99.47% for treatments at 10 °C and 25 °C, respectively (Supplemental Table S1). Similarly, samples incubated with the purified protease and bacterial extract at 25 °C also showed a high degree of casein degradation since the second day after inoculation in milk, of 98.47% and 99.41%, respectively, on the seventh day of incubation.

Heat treatment at 90 °C for 5 min was effective in inactivating this protease since no proteolysis was observed for the purified protease and the bacterial extract subjected to this condition.

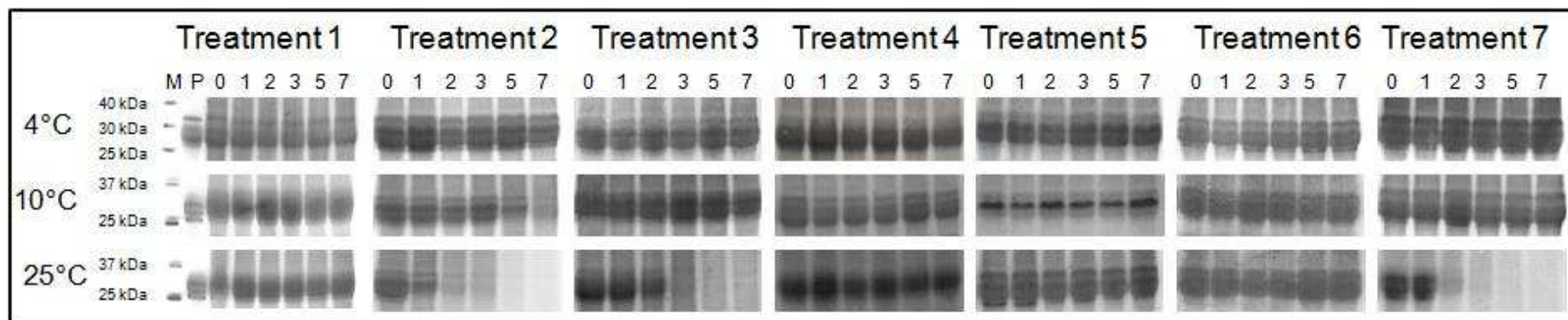


Figure 3. Biological assay – Profile of casein fractions in reconstituted skim milk at days 0, 1, 2, 3, 5 and 7. M: Commercial Molecular Marker; P: Casein pattern. Treatment 1: Control. Treatment 2: *P. fluorescens* 07A (10^3 CFU/mL). Treatment 3: Purified protease (2.5% v/v). Treatment 4: Tris-HCl 10 mM, NaCl 50 mM, pH 7.5 Buffer (2.5% v/v). Treatment 5: Heat-treated purified protease (2.5% v/v). Treatment 6: Heat-treated *P. fluorescens* 07A extract (2.5% v/v). Treatment 7: *P. fluorescens* 07A extract (2.5% v/v).

Discussion

To understand the conditions that influence the production and activity of proteases by psychrotrophic bacteria in milk, we have evaluated the expression of proteases by *P. fluorescens* 07A at different temperatures and compared proteolysis of caseins by this bacteria and the purified protease under different conditions.

The temperature of 4 °C is recommended for milk storage and the maximum temperature accepted for milk reception for processing by industries is 10 °C. However, the production of the protease by the bacteria within the first 24 h of incubation (Figure 1a) shows that refrigeration is not sufficient to prevent the production of these enzymes in milk if the initial count of psychrotrophic bacteria is above 10^3 CFU/mL. However, the temperature of 4 °C minimized enzyme production by the bacteria in the first 12 h of incubation in comparison to 10 °C. After 24 h, protease production is significantly reduced (Figure 1a), but the protease accumulates in milk. The high levels of expression of this protease in the lag growth phase indicate that it could be produced as an adaptive strategy to the environment under the conditions used, which is reinforced by the increased production of this enzyme in stationary phase at 25 °C (Figure 1a).

After 36 and 48 h, enzyme production at 25 °C was approximately ten and sixteen times higher, respectively, than those at refrigeration temperatures. In addition, enzyme activity significantly increased with time (Figure 1b), showing the retention of the enzyme in the extracellular medium during stationary phase.

The proteolytic activity of *P. fluorescens* strains is normally detected in the late logarithmic phase of growth (Matselis and Roussis, 1998). The regulation of

production of these enzymes may be associated with high cell density, which may partly explain the high and constant expression of proteases by *P. fluorescens* 07A strain at 25 °C after 24 h. Extracellular proteases, including those produced by *Pseudomonas* strains, can act as virulence factors released in response to the signal molecules of quorum sensing (Tan et al., 2013). Although the proteolytic activity of *P. fluorescens* 07A is not regulated by signal molecules (AHL) (Pinto et al., 2010), the activity of the extracellular protease produced by this strain is only detected when cell concentration is higher than 10^8 CFU/mL, confirming that its proteolytic activity could be related to cell density.

Protease IV of *P. aeruginosa* is produced as a precursor, which is intracellularly processed in order to be activated (Traidej et al., 2003). It is possible to infer that there is a system for transcriptional and post-translational control of enzyme expression and secretion and that it is highly regulated by extrinsic factors such as temperature and cell concentration. This pattern of response to external stimuli could explain the lower expression of this protease during the logarithmic phase of growth of the bacteria *P. fluorescens* 07A, followed by an increase and a constant expression at the end of this phase, without an instantaneous corresponding increase in the activity (Figure 1a and 1b).

Protease production by *P. fluorescens* has been investigated by several groups and has varied depending on temperature, stage of growth and medium composition (Hellio et al., 1993; Peterson and Gunderson, 1960). However, these studies did not assess the differential expression and activity of these enzymes in milk, but only in culture media. Peterson and Gunderson (1960) observed that the proteolytic activity in the supernatant of a *P. fluorescens* culture in tryptone-glucose-

meat extract broth was greater at refrigeration temperatures and decreased with increasing temperature, contrary to what was found in this study in milk. According to the authors, the tendency to increase production of proteases at low temperatures may be related to the fact that under these conditions, enzyme activity is lower. As temperature increases, higher enzyme activity limits the need for extensive production.

Hellio et al. (1993) reported that the *P. fluorescens* MF0 strain optimal proteolytic activity at 17.5 °C. They demonstrated that this activity gain was due to an increase in the amount of protease secreted, excluding the hypothesis that differences in activities at different growth temperatures might correspond to a difference in the enzymatic activity.

Differential expression of proteases in different substrates and stages of bacterial growth has already been described for other bacteria. It was reported that the production of this type of enzyme depends upon the availability of nutrients in the medium (Rajmohan et al., 2002). In this work, protease activity in LB medium at 10 °C after 48 h was fairly intense as at 25 °C (Figure 2). The activity of an extracellular protease with an approximate molecular mass of 30 kDa after 36 h of incubation at 25 °C in LB medium (Figure 2), when the bacteria is at the end of the stationary phase, could be due to the limitation of a specific protein substrate and then the attempt of that enzyme to use another substrate.

P. fluorescens 07A was able to hydrolyse casein fractions even when stored at 10 °C for seven days (Figure 3), indicating that milk storage at that temperature may be of concern because it allows the multiplication of psychotropic bacteria, in addition to protease production and activity. This fact reinforces the importance of maintaining

hygienic conditions to obtain quality milk since in Brazil, 10 °C is the maximum allowed temperature for reception of milk for processing in dairy industries (Brasil, 2011). Raw milk contains approximately between 10^3 and 10^5 CFU/mL psychrotrophic bacteria (Ercolini et al., 2009; Pinto et al., 2006), which indicates that the production of the protease may be even greater in routine situations, since the results obtained in this study are based on an initial count of 10^3 CFU/mL. At 25 °C the intensity of protein degradation was even greater, and on the second day, it has already been possible to observe degradation of all caseinic fractions.

Pinto et al. (2014) evaluated the degradation of casein fractions of inoculated milk samples with 10^6 CFU /mL of the *P. fluorescens* 07A and 041 strains stored at 2, 4, 7 and 10 °C. The authors verified degradation of the κ -casein fractions of the samples even when incubated at 2 °C, after 6 days. The temperature increase to 4 °C accelerated degradation, with complete hydrolysis of all the casein fractions being observed on the fourth day of storage in milk inoculated with *P. fluorescens* 041, and on the sixth day, in milk inoculated with *P. fluorescens* 07A.

The intense proteolysis caused by the purified protease at 25 °C (Figure 3) shows the importance of controlling milk contamination by psychrotrophic bacteria since this temperature is close to those used for storage of UHT milk. Again, obtaining quality milk is essential, otherwise, psychrotrophic contaminants can produce those heat resistant enzymes, resulting in loss of dairy product quality (Sørhaug and Stepaniak, 1997). Interesting results were that storage of the milk with the purified protease at 4 °C or 10 °C inhibited its activity, suggesting that refrigeration temperatures could reduce or minimize UHT gelation caused by these

enzymes during storage. However, UHT milk is stored at room temperature because it is considered a commercially sterile product.

The heat treatment at 90 °C for 5 min was sufficient to inactivate the purified enzyme and the bacteria. We have previously show that this protease retains approximately 44% of its residual activity when subjected to 90 °C for 5 min in L B medium, while only approximately 14% of this residual activity is observed when subjected to 65 °C for 30 min (Alves et al., 2016) . Thus, the observed inactivation may be due to the longer time taken to achieve the temperature in milk and differences associated with medium composition.

The intense degradation of casein fractions in the milk incubated with *P. fluorescens* 07A extract at 25 °C (Figure 3) for two days suggests that the proteases continued to be produced by the bacteria even after precipitation of the cells and their inoculation into milk. This could then lead to accumulation of this enzyme in the medium and potent activity in degrading milk proteins.

Conclusions

We have shown that refrigeration temperatures are not sufficient to inhibit protease production but do inhibit protease activity, which could lead to misinterpretations and mask the presence of these enzymes in milk. This protease activity leads to a massive loss of casein fractions during storage at room temperature in UHT milk due to residual activity of these enzymes after heat treatments. Since production of these enzymes occurs mainly within the first 12 h of incubation at 4 or 10 °C, the control of psychrotrophics in raw milk is essential to

avoid the presence of these enzymes in the subsequent steps. Thus, this work affirmed the need for strict microbiological control when obtaining raw milk for processing in the dairy industry.

Acknowledgements

The authors acknowledge financial support from the following Brazilian agencies: Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

- Adams, D.M., J.T. Barach, and M.L. Speck. 1976. Effect of psychrotrophic bacteria from raw milk on milk proteins and stability of milk proteins to ultrahigh temperature treatment. *J. Dairy Sci.* 59:823–7.
- Alves, M., R. Salgado, M. Eller, P. Vidigal, and A. Carvalho. 2016. Characterization of a heat-resistant extracellular protease from *Pseudomonas fluorescens* 07A shows that low temperature treatments are more effective in deactivating its proteolytic activity. *J. Dairy Sci.* 99:7842–7851.
- Ayora, S., and F. Götz. 1994. Genetic and biochemical properties of an extracellular neutral metalloprotease from *Staphylococcus hyicus* subsp. *hyicus*. *Mol. Gen. Genet.* 242:421–430.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Brasil. 2011. Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de Origem Animal. Instrução Normativa Nº 62, de 29 de dezembro de 2011. Diário Oficial da República Federativa do Brasil, p. 13-20, 30 dez. 2011. Seção I.
- Chen, L., R.M. Daniel, and T. Coolbear. 2003. Detection and impact of protease and lipase activities in milk and milk powders. *Int. Dairy J.* 13:255–275.
- Ercolini, D., F. Russo, I. Ferrocino, and F. Villani. 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiol.* 26:228–31.

- Feitosa, L., W. Gremski, S.S. Veiga, M.C.Q.. Elias, E. Graner, O.C. Mangili, and R.R. Brentani. 1998. Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in Brown spider (*Loxosceles intermedia*) venom. *Toxicon*. 36:1039–1051.
- Hellio, F.C., N. Orange, and J.F. Guespin-Michel. 1993. Growth temperature controls the production of a single extracellular protease by *Pseudomonas fluorescens* MF0, in the presence of various inducers. *Res. Microbiol.* 144:617–25.
- De Jonghe, V., A. Coorevits, K. Van Hoorde, W. Messens, A. Van Landschoot, P. De Vos, and M. Heyndrickx. 2011. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. *Appl. Environ. Microbiol.* 77:460–70.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227:680–685.
- Liu, M., H. Wang, and M.W. Griffiths. 2007. Regulation of alkaline metalloprotease promoter by N-acyl homoserine lactone quorum sensing in *Pseudomonas fluorescens*. *J. Appl. Microbiol.* 103:2174–84.
- Matselis, E., and I. Roussis. 1998. Proteinase and lipase production by *Pseudomonas fluorescens*. Proteolysis and lipolysis in thermized ewe's milk. *Food Control*. 9:251–259.
- McKellar, R.C., and H. Cholette. 1987. Effect of temperature shifts on extracellular proteinase-specific mRNA pools in *Pseudomonas fluorescens* B52. *Appl. Environ. Microbiol.* 53:1973–1976.
- Mckellar, R.C., K. Shamsuzzamano, C.S. Jose, and H. Cholette. 1987. Influence of iron (III) and pyoverdine on extracellular proteinase and lipase production by

- Pseudomonas fluorescens* B52. *Arch. Microbiol.* 147:225–230.
- McPhee, J.D., and M.W. Griffiths. 2011. Psychrotrophic bacteria. *Pseudomonas* spp. In J.W. Fuquay, P.F. Fox, and P.L.H. McSweeney. (Eds.), *Encyclopedia of dairy sciences*. Academic Press. 379–383.
- Neubeck, M. Von, C. Baur, M. Krewinkel, M. Stoeckel, S. Scherer, and M. Wenning. 2015. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *Int. J. Food Microbiol.* 211:57–65.
- Nicodème, M., J.-P. Grill, G. Humbert, and J.-L. Gaillard. 2005. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. *J. Appl. Microbiol.* 99:641–8.
- Peterson, A.C., and M.F. Gunderson. 1960. Some characteristics of proteolytic enzymes from *Pseudomonas fluorescens*. *Appl. Microbiol.* 8:98–104.
- Pinto, C.L.O. 2004. Bactérias psicrotróficas proteolíticas do leite cru refrigerado granelizado destinado à produção de leite UHT. Tese (Doutorado em Microbiologia Agrícola) - Universidade Federal de Viçosa. 97 pp.
- Pinto, C.L.O., M.L. Martins, and M.C.D. Vanetti. 2006. Qualidade microbiológica de leite cru refrigerado e isolamento de bactérias psicrotróficas proteolíticas. *Ciênc. Tecnol. Aliment.* 26: 645-651.
- Pinto, U.M., Costa, E.D., Mantovani, H.C., Vanetti, M.C.D. 2010. The proteolytic activity of *Pseudomonas fluorescens* 07A isolated from milk is not regulated by quorum sensing signals. *Brazilian J Microbiol.* 41:91–96.
- Pinto, C.L.O.; Machado, S.G.; Cardoso, R.R.; Alves, R.M.; Vanetti, M.C.D. 2014. Proteolytic potential of *Pseudomonas fluorescens* isolated from refrigerated raw milk. *Revista Brasileira de Agropecuária Sustentável.* 4:16-25.

- Rajmohan, S., C.E.R. Dodd, and W.M. Waites. 2002. Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. *J. Appl. Microbiol.* 93:205–213.
- Rasolofo, E.A., D. St-Gelais, G. LaPointe, and D. Roy. 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. *Int. J. Food Microbiol.* 138:108–18.
- Samaržija, D., Š. Zamberlin, and T. Pogačić. 2012. Psychrotrophic bacteria and milk and dairy products quality. *Mljekarstvo.* 62:77–95.
- Silva, M.C.S., I.R. Mendes, T.A. Paula, R.S. Dias, S.O. Paula, C.C. Silva, D.M.S. Bazzolli, and M.C.M. Kasuya. 2016. Expression of the *nifH* gene in diazotrophic bacteria in *Eucalyptus urograndis* plantations. *Can. J. For. Res.* 46:190–199.
- Sørhaug, T., and L. Stepaniak. 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. *Trends Food Sci. Technol.* 8:35–41.
- Tan, S.Y.Y., S.L. Chua, Y. Chen, S.A. Rice, S. Kjelleberg, T.E. Nielsen, L. Yang, and M. Givskov. 2013. Identification of five structurally unrelated quorum-sensing inhibitors of *Pseudomonas aeruginosa* from a natural-derivative database. *Antimicrob. Agents Chemother.* 57:5629–5641.
- Traidej, M., A.R. Caballero, Mary E. Marquart, B.A. Thibodeaux, and R.J. O’Callaghan. 2003. Molecular analysis of *Pseudomonas aeruginosa* Protease IV expressed in *Pseudomonas putida*. *Invest. Ophthalmol. Vis. Sci.* 44:190–196.
- Woods, R.G., M. Burger, C. Beven, and I.R. Beacham. 2001. The *aprX* – *lipA* operon of *Pseudomonas fluorescens* B52 : a molecular analysis of metalloprotease and lipase production. *Microbiology.* 147:345–354.

Supplemental Material

Table S1. Biological assay - Percentage of degradation of casein fractions in reconstituted skim milk at day 7. Treatment 1: Control. Treatment 2: *P. fluorescens* 07A (10^3 CFU/mL). Treatment 3: Purified protease (2.5% v/v). Treatment 4: 10 mM Tris-HCl, 50 mM NaCl, pH 7.5 Buffer (2.5% v/v). Treatment 5: Heat-treated purified protease (2.5% v/v). Treatment 6: Heat-treated *P. fluorescens* 07A extract (2.5% v/v). Treatment 7: *P. fluorescens* 07A extract (2.5% v/v).

Temperature of incubation	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7
4 °C	2,39	1,27	1,09	0,14	0,47	1,46	2,04
10 °C	2,22	42,98	1,24	0,95	2,36	1,67	1,38
25 °C	0,81	99,15	98,47	1,55	1,11	1,54	99,41

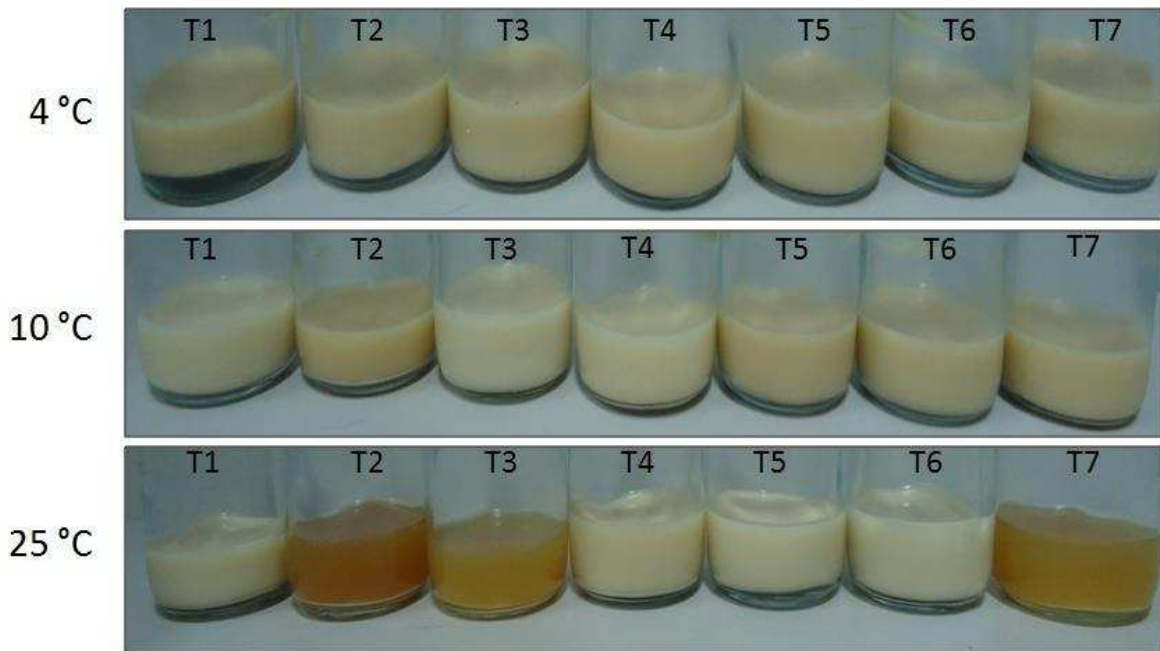


Figure S1. Biological assay – visual effects of treatments on reconstituted skim milk at day 7. Treatment 1: Control. Treatment 2: *P. fluorescens* 07A (10^3 CFU/mL). Treatment 3: Purified protease (2.5% v/v). Treatment 4: 10 mM Tris-HCl, 50 mM NaCl, pH 7.5 Buffer (2.5% v/v). Treatment 5: Heat-treated purified protease (2.5% v/v). Treatment 6: Heat-treated *P. fluorescens* 07A extract (2.5% v/v). Treatment 7: *P. fluorescens* 07A extract (2.5% v/v).

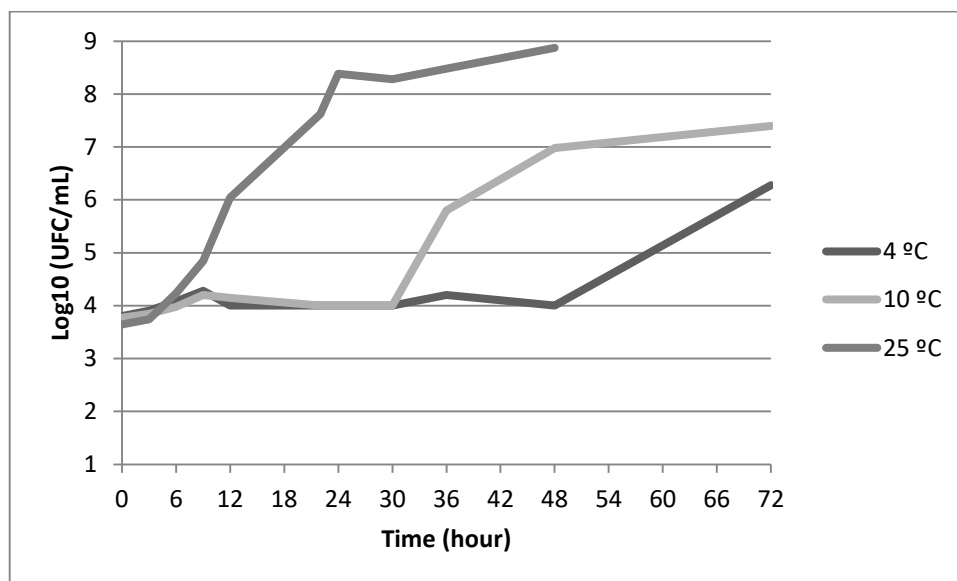


Figure S2. Growth curve of *P. fluorescens* 07A in reconstituted skimmed milk at 4, 10 and 25 °C.

5. GENERAL CONCLUSION AND PERSPECTIVES

In this work an extracellular protease from *P. fluorescens* 07A strain was purified and characterized in relation to its biochemical and biological properties. The enzyme is a metalloprotease with a molar mass of 49,486 kDa and maximal activity at 37 °C and pH 7.5. Low temperature treatments, such as milk pasteurization, were more effective in reducing protease activity when compared to more intense heat treatments. This behavior can cause significant problems during processing and storage of milk and dairy products, especially UHT milk. The gene encoding the protease was identified in the *P. fluorescens* 07A strain genome. The enzyme has 477 amino acids and highly conserved Ca²⁺ and Zn²⁺-binding domains.

The expression of proteases by *P. fluorescens* 07A presented variations at different temperatures according to the growth phases of this bacteria, indicating that this protease could be produced as an adaptive strategy to the environment. Low relative expression was observed after 12 h of incubation at 25 °C compared to refrigerated temperatures of 4 and 10 °C, but the production of this enzyme significantly increased after 24 h at 25 °C and remained constant for up to 48 h. Casein fractions were highly degraded by the *P. fluorescens* 07A, the purified protease and the bacterial extract until the seventh day of incubation at 25 °C, and at a lower intensity also the sample incubated with the bacteria at 10 °C.

These results reinforce the importance to adopt adequate practices in obtaining raw milk in order to minimize its contamination by psychrotrophic microorganisms. In addition, the knowledge about the characteristics of these enzymes and the conditions that favor their production and activity may help dairy industries in the search for strategies and technologies to control the technological

problems associated to the activity of these enzymes. As example, we can mention the development of rapid tests to quantify these enzymes in order to assist industries to direct milk for processing.

This work raise the perspective for the prediction of the structure and functions of this protease, as well as evaluation of the physico-chemical modifications caused by it in milk aiming to better understand its activity. Finally, proteolytic enzymes may have beneficial effects in food products when used at controlled concentrations and conditions. Thus, additional matters that remains to be answered would be if this enzyme could be used, for example, in cheese production and to the development of aroma compounds in this product.