Characterization of a heat-resistant extracellular protease from *Pseudomonas fluorescens* 07A shows that low temperature treatments are more effective in deactivating its proteolytic activity

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ABSTRACT

This work discusses the biological and biochemical characterization of an extracellular protease produced by *Pseudomonas fluorescens*. The enzyme has a molecular weight 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 at pH 10.0. It was shown to be a metalloprotease inhibited by Cu²⁺, Ni²⁺, Zn²⁺, Hg²⁺, Fe²⁺, and Mg²⁺, but induced by Mn²⁺. After incubation at 100°C for 5 min, the enzyme presented over 40% activity, but only 14 to 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 AA and highly conserved Ca²⁺- and Zn²⁺-binding domains, indicating that Ca²⁺, 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 findings are essential for the full understanding and control of these enzymes and the technological problems they cause in the dairy industry.

Key words: 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; von Neubeck et al., 2015). The majority of these microorganisms produce thermo-resistant proteases, which maintain 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 with 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).

Several studies partially characterized proteases from psychrotrophic bacteria isolated from milk (Liao and McCallus, 1998; Dufour et al., 2008; Zhang and Lv, 2014). However, to fully understand protease activity in milk, it is essential to associate this characteristic with 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; Martins et al., 2005) and produces an extracellular protease responsible for milk deterioration, even at low temperatures. However, little information is known 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.

MATERIALS AND METHODS

Bacteria and Culture Conditions

The strain *P. fluorescens* 07A was isolated from raw milk collected in Minas Gerais, Brazil (Pinto, 2004;

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Martins et al., 2005). The culture was cultivated in Luria-Bertani broth (Sigma-Aldrich, St. Louis, MO) at 25°C.

Proteolysis Assay

The proteolytic activity was determined using azocasein (Sigma-Aldrich) as substrate, as described by Ayora and Götz (1994), with modifications. An aliquot of 250 μ L of azocasein solution at 2% (mass/vol) in diluted in 40 mM Tris-HCl, 2 mM CaCl_2 buffer, pH 7.5, was added to 150 μ L 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% (mass/vol). After incubation for 15 min at room temperature, the mixture was centrifuged at $12,000 \times g$ for 15 min at 4°C. A volume of 1.2 mL of the supernatant was neutralized with 1.0 mL of 1 M NaOH. The absorbance of this solution was measured at 440 nm using a SpectraMax M2 spectrometer (Molecular Devices, Sunnyvale, CA). 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.

The protein concentration was estimated as Bradford (1976) using BSA (Sigma-Aldrich) to build the calibration curve.

Protease Purification

A solution containing the bacteria *P. fluorescens* 07A in exponential phase was inoculated (1%) in Luria-Bertani, incubated for 18 h, and centrifuged at $10,000 \times g$ for 15 min at 4°C. The enzyme was recuperated from crude supernatant by fractional precipitation using ammonium sulfate (40 to 60%) for 1 h and centrifugation at $12,000 \times g$ for 30 min at 4°C. Pellet was resuspended in 10 mM Tris-HCl, 50 mM NaCl buffer, pH 7.5, and dialyzed twice against this buffer in cellulose membrane of 14 kDa (Sigma-Aldrich), 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, UK) equilibrated with 10 mM Tris-HCl, 50 mM NaCl buffer, pH 7.5, using an AKTA Purifier chromatographer (GE Healthcare). Proteins were recuperated 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 (Bio-Rad, Hercules, CA). 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% (vol/vol) solution and incubated in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl_2 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 CaCl_2 . 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 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 units, 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), dithiothreitol (DTT; 1 and 5 mM), E-64 (1, 5, and 10 μ M), and Pepstatin A (1, 5, and 10 μ M; Sigma-Aldrich). 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 units, considering the solution with no inhibitors and ions as 100%.

Enzyme Thermostability. Aliquots of 150 μ L of the protease solution were incubated in microtubes at 80°C during 5, 10, 15, and 20 min and at 90 and 100°C during 1, 3, and 5 min. The protease was also incubated at 72°C for 15 s and 65°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 units, considering the non-heat-treated solution as 100%. The effect of the ion Mn^{2+} (5 mM) on enzyme thermostability was also evaluated at 80°C for 5, 10, 15, and 20 min.

Protease Sequencing Using MALDI TOF/TOF

Tryptic digestion of protease was based in the work of Wiśniewski et al. (2009), with modifications. One milliliter of a solution with 90 µg of the enzyme was filtered using 0.5 mL of Amicon Ultra, with cut off molar mass of 10 kDa (Millipore). The retained material was resuspended in 50 mM ammonium bicarbonate (AMBIC), agitated for 60 s, added with 10 mM DTT, and incubated for 60 min at room temperature. The material was acetylated with 10 mM iodoacetamide and incubated for 60 min at room temperature, protected from light. The solution was centrifuged at $10,000 \times g$ for 20 min at 4°C and the retained material was washed twice with a 50 mM AMBIC and 0.5 M urea solution, followed by centrifugation at $10,000 \times g$ for 20 min at 4°C. The material was then washed twice with a AMBIC 50 mM and centrifuged at $10,000 \times g$ for 20 min at 4°C. A trypsin solution was added to the retained material (Sigma-Aldrich), 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 $10,000 \times g$ for 20 min at 4°C and the filtrate was transferred to a new tube. Peptides were concentrated at a vacuum concentrator (Eppendorf, Hauppauge, NY) for approximately 12 h and subjected to a matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF) analysis (Supplemental Material; <http://dx.doi.org/10.3168/jds.2016-11236>).

The mass/charge ratios of the theoretical tryptic peptides of proteins were analyzed using Mascot Server 2.4 (Matrix Science, London, UK), Scaffold Q+ 3.0 Software (Proteome Software, Portland, OR), and Peaks Online Server 7 (Bioinformatics Solutions Waterloo, ON, Canada; Supplemental Material S1; <http://dx.doi.org/10.3168/jds.2016-11236>) and predicted by the *peptidemass* tool from Expasy (http://web.expasy.org/peptide_mass). They were manually compared 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 *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 the data set and the remaining 45 million of paired reads with 51 nucleotides were selected for the *P. fluorescens* 07A genome. These reads were assembled in contigs using CLC Genomics Workbench version 6.5.1 (CLC bio, Aarhus, Denmark), producing 603 sequences with an average size of 9.9 kb. Contigs sequences were analyzed using Prodigal software (<http://prodigal.ornl.gov/>). A total of 5,446 genes were identified and the encoded proteins were functionally annotated using Basic Local Alignment Search Tool (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 because it contained less contaminant proteins (Figure 1, line 2). This fraction was purified with a Sephacryl S-200 HR column and 3 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 molecular weight for this protease is 50 kDa (Figure 1, line 3). Beyond gelatin, the protease hydrolyzed casein and azocasein, but not BSA.

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 presented 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).

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

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

protease activity (Table 1), indicating that this enzyme does not belong to these catalytic groups. On the other hand, 5 mM of EDTA, an agent chelant of divalent ions, and 1,10-phenanthroline, chelant of Zn^{2+} , have reduced

enzyme activity in 87 and 97%, respectively. Protease activity was also reduced in the presence of DTT.

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 an increase of 67.6 and 87.8% at manganese concentrations of 2 and 5 mM, respectively.

Figure 2. Effect of temperature (a) and pH (b) on the activity of the extracellular protease from *Pseudomonas fluorescens* 07A. Error bar represents the mean of triplicate $\pm$ SD.

Table 1. Effect of inhibitors on protease activity¹

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

¹Relative activity is the percentage of activity in the control treatment (without inhibitors). E-64 = *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, PMSF = phenylmethylsulfonyl fluoride, DTT = dithiothreitol. Values of relative activity are expressed as mean of triplicate $\pm$ SD.

Table 2. Effect of metallic ions on protease activity¹

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

¹Relative activity is the percentage of activity compared with a control treatment with no ions (100.00 ± 2.30%). Values of relative activity are expressed as mean of triplicate ± SD.

Protease Thermostability

The enzyme presented 31.7% of residual activity when subjected to the temperature of 72°C for 15 s (HTST) and 14.3% when incubated at 65°C for 30 min (low temperature and long time; **LTLT**), 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.

Because the protease activity was stimulated in the presence of Mn²⁺, the thermostability of this enzyme was also assessed in the presence and absence of MnCl₂ at 80°C. The presence of this ion had a positive effect on residual enzyme activity, but not in enzyme thermostability, because the rate of decrease of enzyme activity due to the heat treatment was greater in the presence of this ion (Figure 3).

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 with 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 AA, which was functionally annotated as extracellular metalloprotease (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2016-11236>). This protein has a predicted mass of 49,486 Da and an isoelectric point of 5.06. Similarity searches showed that this protein is highly conserved among *P. fluorescens* strains (Supplemental Table S1

Table 3. Thermostability of the extracellular protease from *Pseudomonas fluorescens* 07A¹

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

¹Values of relative activity are expressed as mean of triplicate ± SD.

and Figure S1; <http://dx.doi.org/10.3168/jds.2016-11236>). 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).

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,

Figure 3. Effect of Mn²⁺ 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 Mn²⁺ (□). Activity was expressed as a percentage of the activity of a non-heat-treated enzyme. Error bar represents the mean of triplicate ± SD.

Figure 4. Sequencing and identification of the protease by a proteogenomics approach. The mass spectra 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 AA on sequenced peptide. The protease sequence (right) that contains the peptide was predicted from a gene identified in the genomic sequence (g5; bottom) of *Pseudomonas fluorescens* 07A. Color version available online.

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 (Huston et al., 2004; Mu et al., 2009; Zambare et al., 2011). 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 AA 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.

No patterns are present 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+} (Mu et al., 2009; Ibrahim et al., 2011; Salwan and Kasana, 2013), or inhibited by Cu^{2+} , Zn^{2+} , Ni^{2+} , Hg^{2+} , and Fe^{2+} (Sana et al., 2006; Divakar et al., 2010; Zambare et al., 2011). 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 (Sørhaug and Stepaniak, 1997; Liao and McCallus, 1998; Jankiewicz et al., 2010).

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 because manganese salts are present only at low concentrations in this product (30 µg/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 (1,200 mg/L), is probably a cofactor for this enzyme, since it has a calcium-binding domain in its structure, like other proteases 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 that 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, Serralyisin-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, 1.0, 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 zinc monhydroxide bridge (Larsen and Auld, 1989; Massaoud et al., 2011).

Activity reduction by DTT would suggest that this protease is stabilized by disulfide bonds. Similar findings were reported for other proteases from *Pseudomonas* (Gupta et al., 2005; Dufour et al., 2008; Tang et al., 2010). 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; Yang et al., 1996; Paoletti et al., 1997). 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, HTST treatment 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 with 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 bacteria presenting this behavior, which is known as low-temperature inactivation. This could be mainly attributed to enzyme auto digestion or its aggregation with molecules of casein (Barach et al., 1978; McKellar, 1989; Stepaniak and Sørhaug, 1995).

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 (McPhee and Griffiths, 2011; Baglinière et al., 2013). Thus, adoption of appropriate practices for obtaining milk is necessary 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 the gene that encodes the extracellular protease in the *P. fluorescens* 07A genome and its complete AA sequence. The extracellular protease has 49.486 kDa and its sequence is highly conserved among *P. fluorescens* strains (Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2016-11236>), 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 to 45°C and pH 7.0 to 9.0 (Liao and McCallus, 1998; Anderson et al., 2004; Zhang et al., 2009). 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 several fish species. Surprisingly, this protease has its activity stimulated by 10 mM Ca^{2+} and Zn^{2+} , even presenting only 2 AA 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 *Pseudomonas 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 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.

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