Temperature modulates the production and activity of a metalloprotease from *Pseudomonas fluorescens* 07A in milk

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ABSTRACT

This work evaluated the expression and activity of a metalloprotease released by *Pseudomonas fluorescens* 07A in milk. Low relative expression of the protease by the strain was observed after incubation for 12 h at 25°C while the strain was in the logarithmic growth phase. After 24 h, protease production significantly increased and remained constant for up to 48 h, a time range during which the strain remained in the stationary phase. Conversely, at refrigeration temperatures, at 12 h the strain was still in the lag phase and expressed the protease at higher levels than when the logarithmic phase was reached. Casein fractions were highly degraded by *P. fluorescens* 07A, the purified protease, and the bacterial pellet on d 7 of incubation at 25°C and to a lesser extent at 10°C for the sample incubated with the bacterium. Heat treatment at 90°C for 5 min completely inactivated the proteolytic activity of the purified protease and the bacterial pellet. This work contributes to the knowledge about the conditions of milk storage that influence the production and activity of this extracellular metalloprotease. The results demonstrate the need to find alternative strategies to control the synthesis and activity of proteolytic enzymes in the dairy industry to ensure the quality of processed products.

Key words: milk storage, psychrotrophic bacteria, control of milk proteolysis, real-time polymerase chain reaction

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, refrigeration favors the multiplication of psychrotrophic bacteria, particularly the genus *Pseudomonas*, mainly when cold storage conditions are inadequate (Rasolofo et al., 2010; De Jonghe et al., 2011; von Neubeck et al., 2015). Among the genus *Pseudomonas*, the species *Pseudomonas fluorescens* has shown considerable importance in milk spoilage (Rasolofo et al., 2010; De Jonghe et al., 2011).

Although psychrotrophic microorganisms are inactivated by heat treatments commonly used in the dairy industry, their extracellular proteases are usually thermoresistant and retain their activities even after milk pasteurization or UHT treatments (McPhee and Griffiths, 2011). Extracellular proteases can cause degradation of casein micelles, which results in modifications in physicochemical properties and sensory characteristics of dairy products (Chen et al., 2003; MCPhee and Griffiths, 2011). These issues lead to economic losses due to product recalls associated with UHT milk gelation or changes in the sensorial characteristics and, more indirectly, damage the brand image associated with the product (Machado et al., 2017). Thus, an understanding of storage conditions that favor the bacterial expression of these enzymes is fundamental to avoid the enzymes' activity in milk subjected to heat treatment.

Production of proteases by psychrotrophic bacteria is a complex process influenced by medium composition, particularly iron availability (McKellar et al., 1987), culture conditions and growth phase (Rajmohan et al., 2002; Nicodème et al., 2005), and quorum sensing (Liu et al., 2007). Temperature is also a major factor associated with protease production regulation by psychrotrophic microorganisms (McKellar and Cholette, 1987; Woods et al., 2001). However, these and other previous studies have evaluated protease production in a culture medium, meaning that the results cannot be reproduced when extended to a milk matrix.

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The *P. fluorescens* 07A strain isolated from raw milk produces at least 1 protease of 49.5 kDa, which demonstrates maximum activity at 37°C and pH 7.5. This enzyme shows high residual activity when subjected to intense heat treatment, whereas milder heat treatments lead to protease hydrolysis and massive loss of activity (Alves et al., 2016). This study evaluated the expression and activity of this protease in refrigerated and unrefrigerated milk as well as the degradation of casein micelles caused by the enzyme. For this purpose, different milk incubation conditions were simulated before and after heat treatment to acquire information that may help develop more effective systems to improve milk management at the dairy industry level.

MATERIALS AND METHODS

Bacteria and Culture Conditions

The strain *P. fluorescens* 07A was isolated from raw milk collected from a dairy producer in Minas Gerais, Brazil (Pinto, 2004). It was cultivated in Luria-Bertani (LB) broth (Sigma-Aldrich, St. Louis, MO) at 25°C for 24 h.

Protease Purification

Protease purification was performed as previously defined and standardized by Alves et al. (2016). A cell suspension containing the bacteria in the exponential phase was inoculated (1%) in LB broth, incubated for 18 h, and centrifuged at $10,000 \times g$ 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 $12,000 \times g$ 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) 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, Buckinghamshire, UK) equilibrated with 10 mM Tris-HCl, 50 mM NaCl buffer (pH 7.5) using an AKTA Purifier chromatographer (GE Healthcare). 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 protease activity was monitored by zymogram.

SDS-PAGE and Zymogram of the Fractions Obtained in the Purification Steps

Sodium dodecyl sulfate-PAGE was performed with a 12% polyacrylamide gel according to the method of 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 et al. (1998) with modifications. After electrophoresis, gels were washed for 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. All products sourced from Bio-Rad (Hercules, CA).

Activity Assay

The proteolytic activity was determined using azocasein (Sigma-Aldrich) as a substrate as described by Ayora and Götz (1994) with modifications. An aliquot of 250 μL of azocasein solution at 2% (wt/vol) in 40 mM Tris-HCl, 2 mM CaCl_2 (pH 7.5) was added to 150 μL 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% (wt/vol) trichloroacetic acid. After incubation for 15 min at room temperature, the mixture was centrifuged at $12,000 \times g$ 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, CA). One unit of enzymatic activity was defined as the amount of enzyme sufficient to raise 0.01 of the solution absorbance at 440 nm/min under experimental conditions. All assays were performed in triplicate, and the results were expressed as the mean $\pm$ standard deviation.

Protease Expression in Milk

To assess the differential expression of proteases by the proteolytic *P. fluorescens* 07A, 3 flasks containing 100 mL of 10% (wt/vol) sterile reconstituted skim milk were inoculated with approximately 10^3 cfu/mL of the 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 real-time quantitative PCR. One aliquot was also collected at 72 h to monitor microbial growth at 4 and 10°C. Microbial growth was monitored

in plate count agar (Becton Dickinson, Franklin Lakes, NJ) at $32 \pm 1^\circ\text{C}$ for 48 h (Wehr and Frank, 2004).

Total RNA Extraction and cDNA Synthesis. Aliquots of 4 mL were centrifuged at $3,000 \times g$ for 5 min at 4°C . The pellet was lysed for total RNA extraction using Purezol RNA isolation reagent (Bio-Rad, Hercules, CA), and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's recommendations. The efficiency of RNA extraction and cDNA synthesis was evaluated by quantification using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA).

Real-Time PCR. The synthesized primers were made based on the AprX sequence found in the *P. fluorescens* 07A genome (KU170026.1; Alves et al., 2016) and are described as follows: Apr07 R-5'-TCTCACGGTCAATGGCAAAC-3' and Apr07 F-5'-TTGCCGTTGCCATCAAAGTC-3'. The cDNA were used as templates for real-time PCR using a Ssofast EvaGreen Supermix kit (Bio-Rad). Reactions were made in triplicate, and the level of basal expression was compared with that of 16S gene expression using 530F and 110R primers (Silva et al., 2016).

Statistical Analysis. Relative expressions of the protease by *P. fluorescens* 07A at different temperatures within the same time were compared using 1-way ANOVA and Tukey's test. Data from 3 independent replicates were shown as the mean $\pm$ standard deviation. Analyses were performed using GraphPad Prism software (version 7.00; GraphPad, San Diego, CA). A *P*-value of <0.05 was considered statistically significant.

Protease Production in LB

The *P. fluorescens* 07A strain was inoculated in LB broth (10^3 cfu/mL) and incubated at 4, 10, and 25°C . Enzyme activity in this medium was evaluated by zymogram at the following times: 0, 12, 24, 36, and 48 h of incubation.

Proteolysis Assay

Milk proteolysis by the purified protease was compared with that caused by the bacteria under 7 different conditions. For this, flasks containing 100 mL of 10% (wt/vol) sterile reconstituted skim milk were inoculated as follows: treatment 1 = control; treatment 2 = *P. fluorescens* 07A; treatment 3 = purified protease (2.5% vol/vol); treatment 4 = 10 mM Tris-HCl, 50 mM NaCl, pH 7.5 buffer (2.5% vol/vol; control); treatment 5 = heat-treated purified protease (2.5% vol/vol); treatment 6 = heat-treated *P. fluorescens* 07A pellet (2.5% vol/vol); and treatment 7 = *P. fluorescens* 07A pellet (2.5% vol/vol).

Three flasks were inoculated according to the composition of each treatment and incubated at 4, 10, and 25°C as biological replicates. The initial count of *P. fluorescens* 07A in treatment 2 was approximately 10^3 cfu/mL, and the volume of 2.5% (vol/vol) of the purified protease added in treatments 3 and 5 corresponded to 0.82 U of enzymatic activity. Bacterial pellets (treatments 6 and 7) were produced from a cell suspension containing 10^3 cfu/mL of *P. fluorescens* 07A, which had been centrifuged at $10,000 \times g$ for 10 min and resuspended in 10 mM Tris-HCl, 50 mM NaCl buffer (pH 7.5). After addition of the purified protease and the *P. fluorescens* pellet to the milk (treatments 5 and 6, respectively), the samples were subjected to heat treatment in a 90°C water bath for 5 min. Temperatures of reconstituted skim 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 d after inoculation. Sample preparation for proteolysis monitoring was adapted from Adams et al. (1976). Samples were acidified to pH 4.0 using 3 M hydrochloric acid and centrifuged at $12,000 \times g$ for 10 min at 4°C to precipitate milk caseins. The supernatant (whey) was discarded, and the casein-containing pellet was stored at -20°C . On the last day of the 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 casein concentrations were estimated by densitometry analysis using Image J software (National Institutes of Health, Bethesda, MD). Densitometry was performed according to each band corresponding to time 0 of each treatment to provide a cumulative estimation among all casein fractions.

RESULTS

Protease Expression and Activity in Milk

The protease expression by *P. fluorescens* 07A varied according to the phase of microbial growth in milk. At 12 h at 25°C (Figure 1a), when the culture was in the middle of the exponential growth phase (Figure 1c), protease production was low. However, after 24 h of incubation at the same temperature, the relative expression had significantly increased ($P < 0.05$) and remained constant up to 48 h. In addition, only 0.006 U of enzyme activity was detected at 25°C after 12 h in milk (Figure 1b). This activity significantly increased ($P < 0.05$) and accumulated during incubation and then into the stationary phase, and then reached 0.15 U after 48 h.

Figure 1. Relative expression of (a) protease, (b) enzymatic activity, and (c) growth curve of *Pseudomonas fluorescens* 07A in milk at 4, 10, and 25°C. Values are mean $\pm$ SD ($n = 3$). Statistics were performed with one-way ANOVA, Tukey's test; * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 2. Zymogram of protease production by *Pseudomonas fluorescens* 07A in Luria-Bertani broth at 12, 24, 36, and 48 h of incubation at 4, 10, and 25°C. MM = commercial molecular marker.

Conversely, the relative expression of protease at 10°C at 12 h of incubation was significantly higher ($P < 0.05$) than at 25°C (Figure 1a). This was then followed by a reduction over the last 24 h of incubation at refrigeration temperatures. Similarly, the relative expression of protease at 4°C at 12 h of incubation was also higher than at 25°C. The enzymatic activity in these treatments remained low throughout the incubation period when compared with activity at 25°C.

Protease Production in LB

When cultivated in LB broth, *P. fluorescens* 07A generally produces an extracellular protease of approximately 50 kDa (Figure 2). At 25°C, this protease production is detectable within the first 12 h of incubation, whereas at 10°C a discrete band can be observed at 24 h. At 4°C, the band relative to protease was detected only after 36 h. Moreover, after 36 h of incubation, zymograms revealed an unexpected band with an estimated size of 30 kDa for cells incubated at 25°C (Figure 2).

Proteolysis Assay

The inoculation of *P. fluorescens* 07A (treatment 2), the purified protease (treatment 3), and the bacterial

pellet (treatment 7) in reconstituted skim milk at 25°C led to complete casein hydrolysis after 7 d of incubation (Figure 3) and a visible modification of the milk tested in these treatments (see Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-13238>). To a lesser extent, the same result was observed in the treatment incubated with bacterium (treatment 2) at 10°C with 7 d of incubation.

The casein fraction degradation on d 7 following inoculation of *P. fluorescens* 07A (treatment 2) in reconstituted skim milk compared with d 0 was 42.98 and 99.15% for samples incubated at 10 and 25°C, respectively (see Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13238>). Similarly, samples incubated with the purified protease and bacterial pellet at 25°C showed a high degree of casein degradation since d 2 following milk inoculation. A 90°C heat treatment for 5 min effectively inactivated this protease because no proteolysis was observed for the purified protease and the bacterial pellet subjected to this condition.

DISCUSSION

In Brazil, 4°C is the recommended temperature for milk storage in direct expansion refrigeration tanks on farms, and the maximum temperature for milk destined for processing in dairy industries is 10°C (Ministério da

Figure 3. Biological assay—profile of casein fractions in reconstituted skim milk at d 0, 1, 2, 3, 5, and 7. M: commercial molecular marker; P: casein pattern; treatment 1: control; treatment 2: *Pseudomonas fluorescens* 07A (10^3 cfu/mL); treatment 3: purified protease (2.5% vol/vol); treatment 4: Tris-HCl 10 mM, NaCl 50 mM, pH 7.5 buffer (2.5% vol/vol); treatment 5: heat-treated purified protease (2.5% vol/vol); treatment 6: heat-treated *P. fluorescens* 07A pellet (2.5% vol/vol); treatment 7: *P. fluorescens* 07A pellet (2.5% vol/vol).

Agricultura, Pecuária e Abastecimento, 2011). However, the production of the protease by the strain *P. fluorescens* 07A at these temperatures within the first 24 h of incubation (Figure 1a) shows that refrigeration is not sufficient to prevent the enzyme production in milk if the initial psychrotrophic bacteria count is above 10^3 cfu/mL. After 24 h, protease production is significantly reduced ($P < 0.05$) under refrigeration (Figure 1a). The higher levels of protease expression were found in the lag and stationary phases under refrigeration temperatures and at 25°C, respectively (Figure 1a).

After 36 and 48 h, enzyme production at 25°C was approximately 10 and 16 times higher, respectively, than that observed at refrigeration temperatures. In addition, enzyme activity significantly increased ($P < 0.05$) over time (Figure 1b), demonstrating enzyme maintenance in the extracellular medium during the stationary phase.

The proteolytic activity of *P. fluorescens* strains is usually detected in the late logarithmic phase of growth (Matselis and Roussis, 1998). The regulation on the production of these enzymes may be associated with high cell density, which could partly explain the high and constant expression of proteases by the *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 (Pinto et al., 2010), the extracellular protease activity produced by this strain is detected only when the cell concentration is higher than 10^8 cfu/mL, confirming that its proteolytic activity could be related to cell density. However, this would not be the only form of regulation of this enzyme by the bacteria considering the greater expression observed also at the lag phases under refrigeration temperatures (Figure 1a).

Protease IV of *Pseudomonas aeruginosa* is produced as a precursor, which is processed intracellularly to be activated (Traidej et al., 2003). It is possible that there is a system for transcriptional and posttranslational control of enzyme expression and secretion that is highly regulated by extrinsic factors such as temperature and cell concentration. This pattern of response to external stimuli could explain the lower protease expression during the logarithmic phase by *P. fluorescens* 07A, followed by an increase and then constant expression at the end of this phase, with a concomitant gradual increase of the enzymatic activity (Figure 1a and 1b).

Protease production by *P. fluorescens* has been investigated and has been shown to vary depending on temperature, stage of growth, and medium composition (Peterson and Gunderson, 1960; Hellio et al., 1993).

However, the studies assessed the differential expression and activity of these enzymes in culture medium only and not in milk. 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 of this study, under low temperatures the enzymatic activity is usually lower, probably because the bacterium compensated the decrease of the activity with greater protease production in these conditions. As the temperature increases, higher enzyme activity limits the need for extensive production.

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

Differential protease expression in different substrates and bacterial growth stages has already been described for other bacteria. It was reported that the production of this type of enzyme depends on nutrient availability in the medium (Rajmohan et al., 2002). In this work, protease activity in LB broth at 10°C after 48 h was fairly intense as at 25°C (Figure 2). The motive for the presence of an extracellular protease activity with an approximate molecular mass of 30 kDa after 36 h of incubation at 25°C in LB broth (Figure 2), when the strain of *Pseudomonas* is at the end of the stationary phase (data not shown), is still unclear. The differential expression of extracellular enzymes by microorganisms occurs due to different factors, such as the limitation of a specific substrate, which in turn would lead to an attempt by bacteria to use alternative substrates in the medium by altering the structure and, probably, the specificity of the enzyme (Meijer and Hugenholtz, 1997; Nicodème et al., 2005). A second hypothesis is the possibility of occurring posttranslational processing of the enzyme with maintenance of its active site to confer stability, change its specificity, or provide nutrients for bacterium maintenance (Traidej et al., 2003; Gupta et al., 2007). The reason why the band at 48 h of incubation was not observed is not clear. This could be due to the limited sensitivity of the method or to the inability of the novel protease to confer an adaptive advantage to *P. fluorescens* 07A.

Pseudomonas fluorescens 07A was able to hydrolyze casein fractions even when stored at 10°C for 7 d (Figure 3). This indicates that milk storage at that temperature may be of concern because it allows protease production and activity. The fact reinforces the importance of

maintaining hygienic conditions to obtain quality milk, as the maximum acceptable temperature for milk destined for processing in dairy industries in Brazil is 10°C (Ministério da Agricultura, Pecuária e Abastecimento, 2011). Raw milk contains approximately between 10^3 and 10^5 cfu/mL of psychrotrophic bacteria (Pinto et al., 2006; Ercolini et al., 2009), which indicates that the production of the protease may be even greater in routine situations because the results obtained in this study are based on an initial count of 10^3 cfu/mL. At 25°C, the protein degradation intensity was even greater, and on d 2 it was already possible to observe degradation of all casein fractions.

The intense proteolysis caused by the purified protease at 25°C (Figure 3) shows the importance of controlling milk contamination by psychrotrophic bacteria because this temperature is close to the temperature range used for UHT milk storage. Again, obtaining quality milk is essential; otherwise, psychrotrophic contaminants can produce heat-resistant enzymes, resulting in the loss of dairy product quality (Sørhaug and Stepaniak, 1997). Storage of the milk under refrigeration inhibited protease activity, suggesting that refrigeration of the UHT milk could reduce or minimize gelation during storage. However, UHT milk is stored at room temperature because it is considered a commercially sterile product. Considering that refrigerated milk storage will not prevent protease production by this bacteria (Figure 1a), new control options are required.

The 90°C heat treatment for 5 min was sufficient to inactivate the purified enzyme and the strain *P. fluorescens* 07A. Previously, it was shown that this protease retains approximately 44% of its residual activity when subjected to 90°C for 5 min in LB broth, whereas only approximately 14% of this residual activity is observed when subjected to 65°C for 30 min (Alves et al., 2016). Thus, the inactivation observed may be attributable to the longer time taken to achieve the temperature of 90°C in milk or even to the differences associated with composition of LB medium and milk.

Considering the hypothesis that the protease could be produced by the bacterium and remain in the cell membrane, besides the supernatant, the maintenance of the proteolytic activity was verified for bacterial pellets that were and were not subjected to thermal treatment (treatments 6 and 7). It was believed that the bacterium, when inactivated by temperature, would no longer produce the enzyme; therefore, any activity detected in treatment 6 would confirm the presence of proteases in the membrane. However, the heat treatment inactivated the purified enzyme (treatment 5), and it was therefore concluded that it would probably inactivate proteases that may be found in the membrane. The intense casein fraction degradation in the

milk incubated with *P. fluorescens* 07A pellet at 25°C (Figure 3) corroborates the theory that the protease continued to be produced by the strain even after the precipitation of the cells and inoculation into milk.

CONCLUSIONS

It has been shown that refrigeration temperatures are not sufficient to inhibit protease production, although they do significantly inhibit protease activity, which may lead to misinterpretations and mask the presence of these enzymes in milk. Protease activity leads to a massive loss of casein fractions in UHT milk while stored at room temperature due to residual activity of these enzymes after heat treatment. Because production of these enzymes within the first 12 h occurs mainly at 4 or 10°C, the control of psychrotrophics in raw milk is essential to avoid the presence of these enzymes in subsequent production steps.

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