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Extracellular thermostable proteolytic activity of the milk spoilage bacterium *Pseudomonas fluorescens* PS19 on bovine caseins

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ABSTRACT

We studied the thermostable proteolytic activity of *Pseudomonas fluorescens* PS19 isolated from raw bovine milk. The heat-treated cell-free supernatant (HT-CFS) contained a thermostable protease of approximately 45 kDa, as revealed by casein zymography. We assigned this enzyme to *P. fluorescens* AprX metalloprotease (UniProtKB Acc. No. C9WKP6). After concentration by ultrafiltration at 10 kDa, the HT-CFS showed 2 other thermostable proteolytic bands on zymogram, with molecular masses of approximately 15 and 25 kDa. The former resulted a fragment of the AprX protease, whereas the 25-kDa protease was not homologous to any known protein of *Pseudomonas* spp. Subsequently, we assessed the proteolytic activity of the HT-CFS on bovine α_s-, β-, and κ-casein during in vitro incubation at 7 or 22°C. By means of ultra-performance liquid chromatography-tandem mass spectrometry we identified the released peptides (n = 591). Some of them resisted proteolysis during the whole incubation period at both incubation temperatures and, therefore, they could be assumed as indicators of the proteolytic action of *P. fluorescens* PS19 on bovine caseins.
Key words: AprX protease, *Pseudomonas fluorescens*, casein, liquid chromatography-mass spectrometry

INTRODUCTION

The microbial contaminants of bovine raw milk include gram-negative psychrotrophic bacteria that are able to grow at 7°C or below (Champagne et al., 1994; Sørhaug and Stepaniak, 1997). These bacteria are ubiquitous in nature, primarily in water and soil, because of their remarkable degree of physiological and genetic adaptability (Spiers et al., 2000). Typically, most of the psychrotrophs isolated from bovine raw milk are the gram-negative aerobic *Pseudomonas* spp. strains (Griffiths et al., 1987). Compared with other psychrotrophic bacteria, *Pseudomonas* spp. are characterized by a short generation time (<4 h) at 0 to 7°C and, for this reason, storage of raw milk for a long time at refrigeration temperature creates conditions for the selective growth of *Pseudomonas* spp. (Chen et al., 2003; Lafarge et al., 2004; Ercolini et al., 2009).

Among members of the genus, *Pseudomonas fluorescens* is the most commonly isolated bacterium in raw milk at the time of its spoilage, which occurs as change of flavor, curdling, and lipolysis (Fairbairn and Law, 1986; Boor and Murphy, 2002; Datta and Deeth, 2003). Spoilage of milk by *Pseudomonas* spp. often depends on the activity of thermostable extracellular proteases that resist the pasteurization and UHT treatments (Fairbairn and Law, 1986; Chen et al., 2003; Datta and Deeth, 2003). In particular, *P. fluorescens* produces heat-resistant extracellular AprX proteases, which can degrade milk casein and have been often associated with spoilage of milk (Sørhaug and Stepaniak, 1997; Dufour et al., 2008). Most extracellular thermostable proteases of *P. fluorescens* are 40- to 50-kDa metalloenzymes, called AprX, belonging to the serralysin family (Nicodème et al., 2005; Marchand et al., 2009; Martins et al., 2015). Calcium or zinc are essential for the activity and stability of these enzymes that present an optimum pH value of 7 to 9 and an optimal temperature range between 30 and 45°C (Martins et al., 2015).

Great need currently exists for more knowledge on the effect of the activities of enzymes from the genus *Pseudomonas* to better support the quality control of milk before and after processing. Thus, the molecular and biochemical characterization of these spoilage enzymes would address the identification of pertinent indicators of their activities, as well as the development of tools for their detection in milk. In recent years, only a few studies have provided detailed information

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on the proteolytic activity of *Pseudomonas* spp. Moreover, information about specific effects of their enzymes on milk proteins is lacking. Based on this, the present study was carried out to attain further knowledge on the proteolytic traits of the *P. fluorescens* PS19 strain isolated from bulk bovine raw milk (Decimo et al., 2014). In particular, we aimed to characterize the extracellular thermostable proteolytic activities of the heat-treated cell-free supernatant (**HT-CFS**) of *P. fluorescens* PS19. Likewise, we aimed to evaluate the caseinolytic activity of this HT-CFS and to characterize the peptides released from single casein fractions of bovine milk.

MATERIALS AND METHODS

Bacterial Strain

The *P. fluorescens* PS19 strain was isolated from bulk bovine raw milk from 28 different farms located in the Lombardy region of the northern Italy. This strain showed a high proteolytic activity by diffusion method assay on skim milk agar at 7 and 22°C (Decimo et al., 2014).

Preparation of the HT-CFS of P. fluorescens PS19

The production of thermostable extracellular proteases by *P. fluorescens* PS19 was studied in minimal salt medium (**MSM**) containing 0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.02% $MgSO_4 \times 7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.4% glycerol, and 1 m*M* $CaCl_2$ (Liao and McCallus, 1998).

The *P. fluorescens* PS19 was first recovered in brain heart infusion broth (Biolife Italiana S.r.l., Milan, Italy) and incubated at 22°C for 6 h. Subsequently, 10 mL of MSM was inoculated and incubated overnight at 22°C. This procedure was repeated to favor the adaptation of the *P. fluorescens* PS19 to the MSM. Afterward, 1 L of MSM was inoculated in an Erlenmeyer flask. After 24 h of incubation at 22°C in aerobic conditions, cells were harvested by centrifugation (6,000 × *g* for 20 min at 4°C). The supernatant was filter-sterilized (0.22 μm) and heated at 95°C for 8.45 min to select heat-resistant proteases. The obtained HT-CFS was used as a crude enzyme extract for casein zymography. Specific proteolytic activity of HT-CFS was determined by azocasein assay according Nicodème et al. (2005).

Casein Zymography

Casein zymography was performed as described by Rajmohan et al. (2002) and Marchand et al. (2009). Briefly, the HT-CFS was resuspended in 5× zymogram sample buffer containing 156.25 m*M* Tris-HCl (pH 6.8), 10% SDS, 62.5% glycerol, and 0.025% bromophenol blue. The mix was loaded on a 12% SDS-polyacrylamide gel polymerized with 0.1% of sodium caseinate. The sample was run initially at 60 V for 30 min and then at 100 V for 100 min on the Hoefer SE 250 apparatus (Hoefer, Holliston, MA). The PageRuler Plus Prestained protein ladder (10–250 kDa; Thermo Scientific, Pierce, Rockford, IL) was used as a molecular weight marker. After the run, the gel was renatured in 2.5% (vol/vol) Triton X-100**[AU1: Please add name and location for the manufacturer for TritonX.]** for 30 min and then incubated overnight at 37°C in development buffer [50 m*M* Tris-HCl (pH 7.5), 200 m*M* NaCl, 5 m*M* $CaCl_2 \times 2H_2O$, and 0.02% Brij 35]. After incubation, the gel was stained with Coomassie Brilliant Blue R-250. All reagents (if not stated otherwise) for casein zymography were from Sigma-Aldrich (St. Louis, MO).

The HT-CFS from *P. fluorescens* PS19 was further concentrated in a stirred cell unit for UF (Amicon-8010; Merck Millipore, Darmstadt, Germany) using a 10-kDa regenerated cellulose membrane. The retentate was subsequently tested on zymogram gel with 0.1% of either κ-, β-, α_{s1}-CN (Sigma-Aldrich) or sodium caseinate and loaded in parallel on 12% SDS-polyacrylamide gel polymerized without casein for further proteomic characterization.

Proteomic Identification of P. fluorescens PS19 Extracellular Proteases

The regions of SDS-polyacrylamide gel, corresponding to the proteolytic bands of *P. fluorescens* PS19 on the zymogram were excised, destained, and subsequently digested in gel with trypsin (Roche Diagnostics, Monza, Italy) according Wilm et al. (1996). For in-solution digestion, the concentrated HT-CSF of *P. fluorescens* PS19 was mixed with urea buffer containing 8 *M* urea in 0.1 *M* Tris-HCl (pH 8.5). The digestion was performed according the filter-aided sample preparation protocol described by Wiśniewski et al. (2009). The enzymes LysC and trypsin (Roche Diagnostics) were used.

The peptides obtained either by the in-gel or in-solution digestions were desalted and concentrated using reverse-phase chromatography using C18 StageTips (Thermo Scientific, San Jose, CA) as described by manufacturer (Rappsilber et al., 2007). After the washing steps, peptides were eluted with 0.1% formic acid in 80% acetonitrile. Peptides were lyophilized and

resuspended in 0.1% formic acid for nano liquid chromatography (**nLC**)-MS/MS analysis.

Five microliters of desalted peptides was injected on a C18 HALO PicoFrit column (75 μm × 10 cm, 2.7 μm, 100 Å; New Objective, Woburn, MA) by an Ultimate3000 RSLCnano system (Thermo Scientific). Peptide separation was performed by a linear gradient starting from 1% acetonitrile/0.1% formic acid to 40% acetonitrile/0.1% formic acid over 60 min, at a constant flow rate of 300 nL/min. Eluting peptides were sprayed into a LTQ Orbitrap XL mass spectrometer through a nanoESI source (Thermo Scientific). The instrument operated in data-dependent mode to automatically switch between full MS and MS/MS acquisitions. Full MS spectra were acquired in profile mode by the Fourier transform analyzer in an m/z window 300 to 1500, with the automatic gain control (**AGC**) scan 5×10^5, and full width at half maximum resolution 100,000 at m/z 400. Tandem mass spectra were acquired in centroid mode by the linear ion trap for the 2 the most intense ions exceeding 1×10^4 counts. The MS/MS spectra acquisition was set as follows: precursor ion isolation width 2.5 m/z, AGC target 1×10^4, and normalized collision energy of 30 eV. Dynamic exclusion was enabled to reduce redundant spectra acquisition for 45 s after 3 repeat counts in 30 s. The mass spectrometer and spectra analysis were fully automated and controlled by the Xcalibur software (version 2.0.7, Thermo Scientific).

Raw data obtained by nLC-MS/MS analysis were analyzed by the software Proteome Discoverer 1.4 (Thermo Scientific), which was used to extract peaks from MS and MS/MS spectra and to match them against the database of *Pseudomonas* spp. strains (taxon ID 286, 1,353,566 entries, downloaded from UniProt on 08.05.2015). The selected protease was trypsin or LysC; up to 2 missed cleavages were allowed. Cysteine carbamidomethylation was set as a fixed modification, whereas Asn deamidation and Met oxidation were set as variable modifications. The precursor mass tolerance was set to 5 ppm and the fragment mass tolerance was 0.5 Da.

Characterization of Hydrolytic Action on Bovine Casein Fractions

The hydrolytic activity on bovine α_s-, β-, and κ-CN was tested by incubating a single protein fraction in 20 m*M* Tris-HCl (pH 6.7) with concentrated HT-CFS of *P. fluorescens* PS19. The reaction mixtures were incubated for 1, 2, 4, 5, and 6 d at 7°C and for 4, 8, 24, 48, and 96 h at 22°C. Aliquots taken at different time points were submitted to UPLC coupled to high-resolution tandem mass spectrometry (**UPLC-MS/MS**) analysis for peptide identification. Samples, consisting of single caseins or UF HT-CFS were used as controls.

Separation and Identification of Peptides from Hydrolysis of Casein Fractions

Separation of peptides in casein hydrolysates was performed on an Alliance 2695 HPLC system (Waters, Milford, MA) equipped with a Waters 2996 diode array detector. Samples were loaded on a PLRP-S column (2.1 × 250 mm, 5 μm, 300 Å; Polymer Laboratories Ltd., Church Stretton, UK) kept at 40°C. The eluents used for the separation were solvent A, 0.1% (vol/vol) trifluoroacetic acid (**TFA**) in MilliQ-treated water (Merck Millipore), and solvent B, 0.1% (vol/vol) TFA in acetonitrile. The elution gradient, expressed as the solvent B proportion, was as follows: 0 to 5 min, 5%; 5 to 65 min, 55%; 65 to 70 min, 95%; 70 to 72 min, 95%, and 72 to 76 min, 5%. The flow rate was 0.2 mL/min, and run-to-run time was 90 min. Absorbance was recorded at 210 nm, and data were processed using the Empower software package (Waters).

Identification of peptides in hydrolysates of casein fractions was carried out by UPLC-MS/MS on an Acquity separation module (Waters), interfaced with a Q Exactive hybrid quadrupole-Orbitrap MS through an HESI-II probe for electrospray ionization (Thermo Scientific). Peptides from 5 μL of each sample were separated on an Aeris Widepore XB-C18 column (2.1 × 150 mm, 3.6 μm, 200 Å; Phenomenex; Torrance, CA) kept at 40°C. The eluents were: 5% acetonitrile, 0.08% formic acid, 0.01% TFA in MilliQ-treated water (solvent A) and 5% water, 0.08% formic acid, 0.01% TFA in acetonitrile (solvent B). The UPLC separation was performed by using a linear elution gradient (0–80% of solvent B in 32 min) at a flow rate of 0.3 mL/min. The ion source and MS interface conditions were: spray voltage 3.5 kV, sheath gas flow rate 35, auxiliary gas flow rate 15**[AU2: Is there a unit for this?]**, temperature 300°C, and ion transfer tube temperature 350°C. The UPLC eluate was analyzed by MS using a full scan (400–2,000 m/z) and data-dependent MS/MS analysis of 10 the most intense ions [**ddMS^2** (Top10**[AU3: Clarify what Top10 means here.]**)]. The resolution was set 70,000 and 17,500 full width at half maximum at m/z 200 for full MS and ddMS^2 scan types, respectively. The AGC target was 5×10^5 for full MS and 1×10^5 for ddMS^2 scans. The maximum ion injection times were 50 and 100 ms for full MS and ddMS^2 scans, respectively. The MS data were automatically processed using the Xcalibur software (version 3.0, Thermo Scientific). Identification of peptides was performed using the software Proteome Discoverer 1.4 (Thermo Scientific).

RESULTS AND DISCUSSION

Evaluation of the HT-CFS Caseinolytic Activity

In a previous work, we identified 19 strains of *P. fluorescens* among 63 psychrotrophic bacteria isolated from bulk bovine raw milk (Decimo et al., 2014). The *P. fluorescens* strain PS19 presented the highest proteolytic activity in vitro at both 7 and 22°C and, for this reason, we further characterized it on bovine casein in the current study. We prepared the CFS after bacterial growth in an MSM supplemented with 1 m*M* $CaCl_2$, without milk addition. We adopted this procedure to characterize the proteolytic activity on single casein fractions. In this regard, the use of the MSM permitted us to obtain a crude enzyme extract depleted of interfering milk caseins and derived peptides, as well as of any pre-existing endogenous or exogenous proteolytic activities potentially present in milk. Subsequently, we heated the CFS to select for thermostable proteases (Marchand et al., 2008, 2009). Preliminary azocasein assay did not show any difference between the proteolytic activity of HT-CFS deriving from bacteria grown in MSM or in reconstituted skim milk powder (10% wt/vol; data not shown).

We further characterized the HT-CFS from the strain PS19 cultured in MSM by casein zymography. We associated the caseinolytic activity of the HT-CFS with the presence of a protease of approximately 45 kDa (data not shown). This result confirmed previous findings, which reported the proteolytic activity of different *P. fluorescens* strains to derive mainly from thermostable proteases with a molecular weight of 40 to 50 kDa (Koka and Weimer, 2000; Nicodème et al., 2005; Dufour et al., 2008; Marchand et al., 2009; Martins et al., 2015; Mateos et al., 2015). After concentration by UF at 10 kDa, we used the HT-CFS of the strain PS19 for further analysis of the proteolytic extracellular activity. We carried out a zymography with either sodium caseinate or single casein fractions (α_s, β, and κ; Figure 1). Two proteolytic bands with molecular masses of approximately 15 and 25 kDa appeared on zymography gels together with the 45-kDa protease (Figure 1). Previously, Rajmohan et al. (2002) reported the presence of some low-molecular weight proteases on zymogram, when they had concentrated these enzymes by UF of supernatants of *P. fluorescens* isolated from milk. Also Marchand et al. (2009) reported some *P. fluorescens* strains displayed 2 or 3 clearance zones of low (<45–50 kDa) molecular weight on casein zymography. Those authors assumed the appearance of low-molecular weight extracellular proteases was likely attributable to substrate depletion, by which the protease of 45 to 50 kDa was degraded into smaller active fragments, being the only protein source left. Similarly, Nicodème et al. (2005) and Martins et al. (2015) described the presence of different low-molecular weight protease bands for some strains of *Pseudomonas* spp., as revealed by zymography.

Proteomic Characterization of the Extracellular Proteases

Although the production of both 45- to 50-kDa and lower-molecular mass proteases by a single strain of *P.*

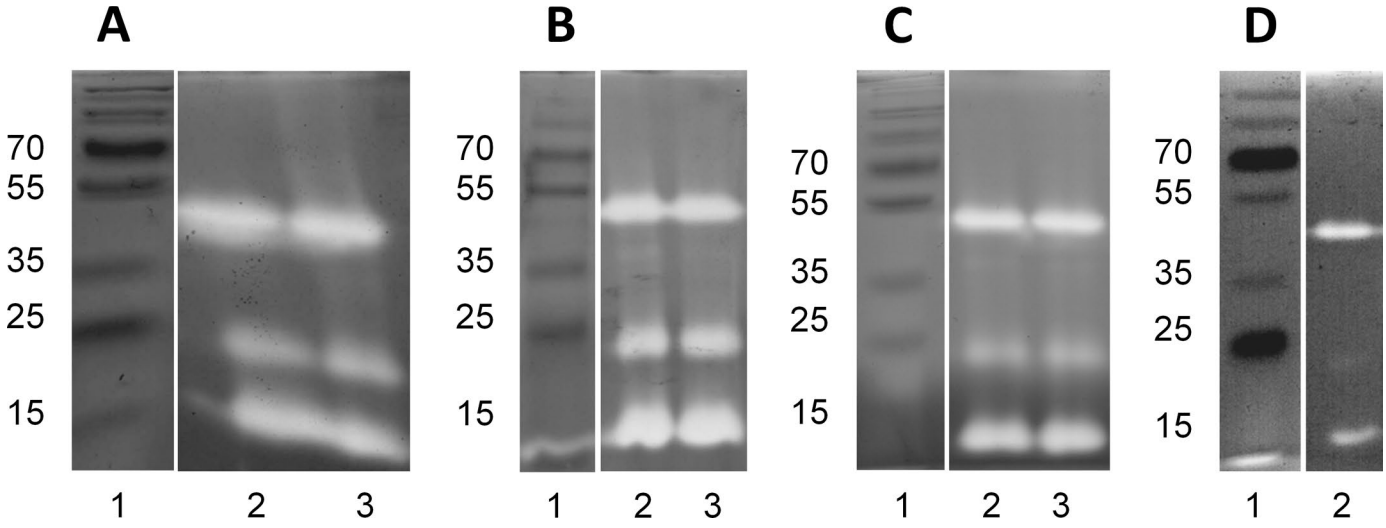

Figure 1. Caseinolytic activity of the concentrated heat-treated cell-free supernatant (HT-CFS) of *Pseudomonas fluorescens* PS19 on sodium caseinate (A), α_s-CN (B), β-CN (C), and κ-CN (D). Lanes: 1 = protein molecular weight ladder, 10–250 kDa; 2 and 3 = concentrated HT-CFS[AU6: Verify D has no lane 3.] or version available online.

fluorescens has been already reported, the molecular identification of these proteases has been studied to a lesser extent. Based on this, we further subjected the 15-, 25-, and 45-kDa proteases present in the HT-CFS of *P. fluorescens* PS19 to proteomic analysis by nLC-MS/MS after both in-gel and in-solution digestions to obtain higher identification coverage. As a result, we attributed the band of 45 kDa on casein zymogram to the AprX metalloprotease of *P. fluorescens* (UniProtKB Acc. No. C9WKP6) with sequence coverage of 55.14% (data not shown). The AprX proteases are believed to be responsible for strong proteolytic spoilage of milk, leading to gelification or coagulation of (heat-treated) milk during storage (Datta and Deeth, 2003; Dufour et al., 2008). These proteases are encoded by a gene *aprX*, which has been characterized in several strains of *Pseudomonas* spp. isolated from raw milk, in particular in *P. fluorescens* (Marchand et al., 2009; Martins et al., 2015). The PS19 strain also possessed the *aprX* gene, as noted via screening with an AprX-PCR test (Decimo et al., 2014). Recently, Baglinière et al. (2013) revealed a similar molecular weight AprX protease from *P. fluorescens* F and, using a proteomic approach, identified this protease as the extracellular alkaline protease (UniProtKB Acc. No. Q7X4S5). Seven AA substitutions differentiated the protease of this study, UniProtKB Acc. No. C9WKP6, and the AprX, UniProtKB Acc. No. Q7X4S5 (data not shown). According Mateos et al. (2015), AA sequences of serralysin proteases are highly conserved within *Pseudomonas* spp.; a 76 to 99% similarity for AprX proteases was observed for the *P. fluorescens* group. Our findings confirm this high similarity for AprX proteases from *P. fluorescens* PS19 and *P. fluorescens* F (data not shown).

The results obtained from the digestions of the band at 15 kDa matched different fragments present in the AA sequence of both the AprX metalloprotease, UniProtKB Acc. No. C9WKP6, and the AprX metalloprotease, UniProtKB Acc. No. E6Z7L2, of *P. fluorescens* R-37987 (data not shown). This last enzyme is a 275 AA-long protease with a molecular weight of 29.6 kDa. In our study, the 15 kDa band can be likely assumed to be a fragment of the 45-kDa AprX metalloprotease, UniProtKB Acc. No. C9WKP6. Findings of Kumura et al. (1999) support this hypothesis, as those authors showed the alkaline protease (48.9 kDa) of *P. fluorescens* 33 (UniProtKB Acc. No. Q9ZNJ1) to auto-digest leading to formation of different autolytic fragments. In addition, Martins et al. (2015) recently assigned the low-molecular weight protease revealed by casein zymography to degradation products of the 50-kDa AprX protease produced by *P. fluorescens* 041 isolated from raw milk. The proteomic analysis of the band at 25 kDa did not yield any peptides present in the sequence of any known protein possessing proteolytic activity.

Identification of the Peptides Released from Bovine Casein Fractions

We studied the peptidic profiles generated in vitro by the action of the concentrated HT-CFS from *P. fluorescens* PS19 on α_s-, β-, and κ-CN after HPLC separation and UV detection of casein hydrolysates. In detail, we incubated the single casein fractions with or without the HT-CFS for 1, 2, 4, 5, and 6 d at 7°C, and for 4, 8, 24, 48, and 96 h at 22°C, as described in Materials and Methods. As expected, HPLC patterns showed the highest degradation of casein fractions to occur when incubation was carried out at 22°C (Figure 2), that is closer to the optimal temperature range (30–45°C) of AprX activity (Dufour et al., 2008). Nonetheless, the HPLC patterns confirmed that thermostable proteases from *P. fluorescens* PS19 remained strongly active at 7°C (Mitchell and Marshall, 1989). Indeed, we did not reveal the intact β- and κ-CN in the HPLC patterns after 2 d of incubation at this temperature. In contrast, α_s-CN partly resisted proteolysis, and it was still detectable in the HPLC chromatogram at the end of incubation (Figure 2). At 22°C, we observed again the strongest proteolysis for κ- and β-CN, which were no longer present in the HPLC patterns at 24 h of sampling (Figure 2). We observed almost complete degradation of α_s-CN after 96 h of incubation at the same temperature. Overall, extracellular proteases present in the HT-CFS of *P. fluorescens* PS19 cleaved β-CN = κ-CN > α_s-CN preferentially. These findings agree with the results of previous studies on the activity of extracellular proteases from different *Pseudomonas* spp. strains. For instance, Koka and Weimer (2000) found that the protease isolated from *P. fluorescens* RO98 preferentially hydrolyzed κ-CN. Similarly, Costa et al. (2002) demonstrated that the extract of *P. fluorescens* RV10 culture mainly proteolyzed κ- and β-CN. In addition, Nicodème (2006) showed *Pseudomonas* spp. to hydrolyze casein in the following order: κ-CN > β-CN > α_s-CN. Finally, studying the destabilization of UHT milk caused by the AprX protease from *P. fluorescens* F, Baglinière et al. (2013) found the enzyme to hydrolyze all casein with a preference for β-CN.

By UPLC-MS/MS, we identified peptides released in vitro from casein fractions. After different times of incubation, the identified peptides varied in quantity according to temperature and digested casein fraction. In total, about 200 peptides were released from each casein fraction during incubation with the concentrated HT-CFS of *P. fluorescens* PS19 at 7 and 22°C (Supple-

mental Table S1; http://dx.doi.org/10.3168/jds.2016-10894). We did not reveal any of these peptides in the control samples constituted by either the HT-CFS or the single caseins alone.

Among the identified peptides, we paid the particular attention to those that promptly formed and resisted further proteolysis until the end of the incubation. For instance, we identified 14 peptides as fragments of α_{s1}-CN at the first sampling (24 h) at 7°C. Most of them were released from the N-terminal part of the protein chain, in particular from the sequences 1–10 and 9–24. Gaucher et al. (2011) found several peptides arising from the sequence 8–24 of α_{s1}-CN, which are specifically released during storage of an UHT milk prepared from raw milk inoculated with *P. fluorescens* CNRZ 798. In the present study, after 24 h of incubation at 7°C, only 4 peptides were released from α_{s2}-CN, being represented by the fragment f(117–125). Three peptides derived from the α_{s2}-CN sequence 190–207; these findings overlap the results obtained by Baglinière et al. (2013), who identified similar peptide sequences in an UHT milk stored at 20°C and produced from raw milk contaminated with purified AprX protease from *P. fluorescens* F. During our in vitro experiments, as the incubation time increased, several other peptides formed. However, most of them still derived from the previously mentioned sequences of α_{s1}- and α_{s2}-CN. Interestingly, the peptides f(4–7), f(1–7), and f(101–105) of α_{s1}-CN resisted further proteolysis, and they were detected at every sampling time until 6 d of refrigerated incubation. On the contrary, at the end of the experiment at the same temperature we did not recover any of the peptides that were promptly formed after 2 h of incubation at 22°C.

Forty-six peptides originated from κ-CN after 24 h of incubation at 7°C. Most of the peptides, which survived until the end of incubation (6 d), arose from the sequences 62–69 (n = 4) and 105–114 (n = 7) of this casein fraction (see Supplemental Table S1; http://dx.doi.org/10.3168/jds.2016-10894). According to Gaucher et al. (2011), the κ-CN sequence 105–123 was mainly hydrolyzed in UHT milk prepared from raw milk

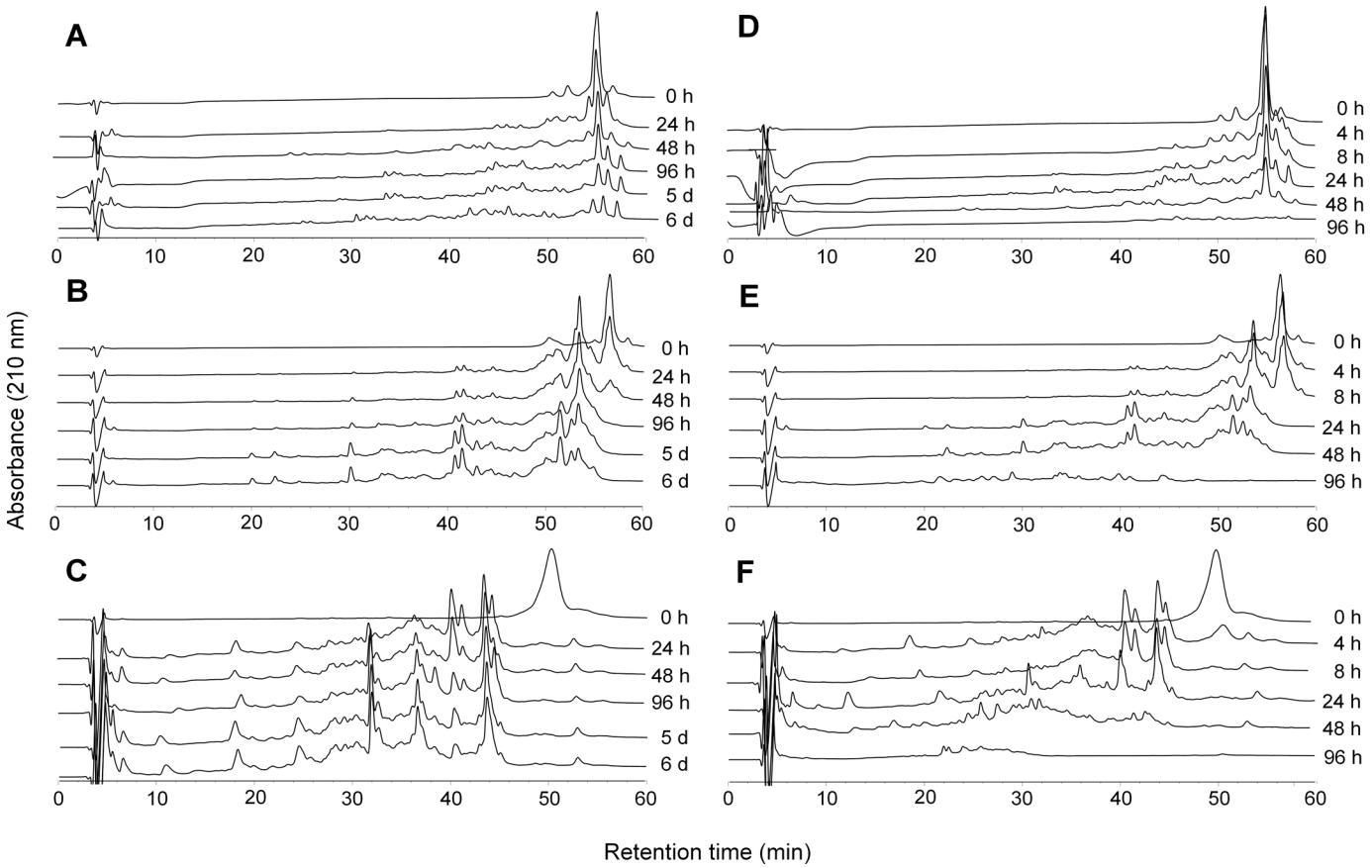

Figure 2. The HPLC patterns of peptides generated from hydrolysis of single casein fractions by the concentrated heat-treated cell-free supernatant of *Pseudomonas fluorescens* PS19 after different hydrolysis times at 7°C (A = α_s-CN; B = β-CN; C = κ-CN) and 22°C (D = α_s-CN; E = β-CN; F = κ-CN).

inoculated with *P. fluorescens* CNRZ 798. Baglinière et al. (2013) also revealed some peptides released from this last sequence of κ-CN in stored UHT milk upon the action of the AprX protease from *P. fluorescens* F. The activity of the HT-CFS of *P. fluorescens* P19 released 19 peptides from κ-CN after 4 h of incubation at 22°C, and 2 of them, f(69–72) and f(108–111), were revealed at every sampling time. Interestingly, at both temperatures, none of the identified κ-CN peptides occurred upon cleavage of the 105–106 bond, which is the specific peptide site cleaved by chymosin. Nonetheless, cleavage of κ-CN chain at positions 105 was observed in UHT milk (Recio et al., 1996), in UHT milk prepared from raw milk contaminated with *P. fluorescens* CNRZ 798 (Nicodème, 2006), and in in vitro experiments with proteases from *P. fluorescens* B52 (Recio et al., 2000).

Strong proteolysis characterized the β-CN at 7 and 22°C, and several peptides arose from the C-terminal sequence 190–209 (Supplemental Table S1; http://dx.doi.org/10.3168/jds.2016-10894) known to contain peptide sequences conferring bitterness to dairy products (Visser et al., 1983). Only 6 peptides that readily generated at 7°C were still present at the end (6 d) of the experiment. They comprised the fragments f(190–202) and f(192–202), which were also identified in the UHT milk studied by Gaucher et al. (2011).

In summary, 26 peptides were promptly released from caseins, and they were detected until the end of incubation at 7°C (Table 1). On the contrary, only the fragments f(69–72) and f(108–111) from κ-CN behaved similarly at 22°C, and they were still recovered at the end of the experiment (Table 1). Among the peptides released at 7°C incubation, 17, 6, and 3 were derived from κ-, β-, and α_s-CN, respectively. Despite about 200 peptides being released from each CN fraction, most of those resisted further proteolysis and arose from the sequence 105–113 of κ-CN (Table 1). In a recent work, Mateos et al. (2015) identified the peptides released in vitro from single casein fractions by the AprX protease of *Pseudomonas* sp. LBSA1, a strain isolated from bulk bovine raw milk. Those authors found several peptides deriving from the hydrolysis of peptide bonds 103–104, 104–105, 105–106, and 112–113 of κ-CN. These findings partially agree with our findings and indicate the proteolytically resistant peptides from the sequence 105–113 of κ-CN as interesting markers of the action of HT-CFS from *P. fluorescens* P19. In the work of Mateos et al. (2015), some of the peptides generated from α_{s1}-CN also overlapped the proteolytically resistant sequences [f(4–7) and f(1–7)] of α_s-CN, which we found in the present work (Table 1). Mateos et al. (2015) also discovered the AprX protease to release several peptides from the N-terminal region of β-CN upon the cleavage of several peptidic bonds, including the 189–190 and the 191–192 ones. In our work, these sites were cleaved upon the formation of the peptides f(190–202) and f(192–202; Table 1). Overall, we did not identify any specific cleavage sites upon the action of the proteases contained in the HT-CFS of *P. fluorescens* PS19. Nonetheless, in the case of κ- and α_s-CN, the presence of aliphatic (I, P) and basic (K, H) AA in the sequence of peptides likely hindered their further hydrolysis during incubation at refrigerated conditions with the HT-CFS of *P. fluorescens* PS19.

Table 1. Peptides released from α_s-, β-, and κ-CN upon the action of proteases present in the heat-treated cell-free supernatant of *Pseudomonas fluorescens* PS19 and revealed at every sampling time of incubation at 7 or 22°C

Item	7°C	22°C
α_s-CN	f(4–7) HPIK	
	f(1–7) RPKHPIK	
	f(101–105) LKKYK	
β-CN	f(162–169) VLSLSQSK	
	f(165–169) LSQSK	
	f(167–176) QSKVLPVPQK	
	f(186–189) PIQA	
	f(190–202) FLLYQEPVLGPVR	
	f(192–202) LYQEPVLGPVR	
κ-CN	f(17–24) FFSDKIAK	f (69–72) SPAQ
	f(31–37) VLSRYPS	f (108–111) IPP
	f(31–41) VLSRYPSYGLN	
	f(40–43) LNYY	
	f(62–66) AKPAA	
	f(65–68) AAVR	
	f(69–72) SPAQ	
	f(69–74) SPAQIL	
	f(98–104) HPHPHLS	
	f(98–105) HPHPHLSF	
	f(105–111) FMAIPPK	
	f(107–112) AIPPKK	
	f(108–111) IPPK	
	f(108–112) IPPKK	
	f(108–113) IPPKKN	
	f(108–114) IPPKKNQ	
	f(109–112) PPKK	

CONCLUSIONS

The results of this work highlight the strong in vitro activity on bovine caseins of the HT-CFS of *P. fluorescens* PS19 at both refrigerated and room temperature conditions. Many of the released peptides were previously discovered in UHT milk, generated by the action of thermostable proteases of *P. fluorescens* strains, especially by the activity of the AprX enzymes. Moreover, some of the identified peptides resisted further in vitro proteolysis and demonstrated to be stable hydrolytic end products of casein breakdown at refrigerated conditions. For this reason, these peptides could be regarded as potential indicators of the action of metalloproteases thus providing a better understanding of the enzymatic activities of *P. fluorescens* in milk.

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