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

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ABSTRACT

The thermostable proteolytic activity of *Pseudomonas fluorescens* PS19, isolated from raw bovine milk, was studied. The heat-treated cell-free supernatant (HT-CFS) contained a thermostable protease of approximately 45 kDa as revealed by casein zymography. This enzyme was assigned to *P. fluorescens* AprX metalloprotease (UniProtKB Acc. No. C9WKP6). After concentration by UF at 10 kDa, the HT-CFS showed two 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. The proteolytic activity of the HT-CFS was subsequently assessed on α_s, β and κ bovine caseins (CNs) during *in vitro* incubation at 7 °C or 22 °C. The released peptides (n=591) were identified by means of UPLC-MS/MS. Some of these peptides resisted

proteolysis during the whole incubation period and, therefore, they could be assumed as indicators of the proteolytic action of *P. fluorescens* PS19 on bovine CNs.

Keywords: AprX protease; *Pseudomonas fluorescens*; casein; proteomics; liquid chromatography

Highlights

- The heat-treated cell-free supernatant (HT-CFS) of *P. fluorescens* PS19 was studied
- After HT-CFS concentration, proteases of 15, 25 and 45 kDa were revealed
- The 45 kDa protease was assigned to AprX metalloprotease (UniProtKB Acc. No. C9WKP6)
- The 15 kDa enzyme resulted a fragment of the AprX protease
- The HT-CFS released 591 peptides from β-=κ->α_s-CNs, as revealed by UPLC-MS/MS

1. Introduction

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

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 flavour, curdling and lypolysis (Boor & Murphy, 2002; Datta & Deeth, 2003; Fairbairn & Law, 1986). Spoilage of milk by *Pseudomonas* spp. often depends on the activity of thermostable extracellular proteases that survive the pasteurization and UHT

treatments (Chen et al., 2003; Datta & Deeth, 2003; Fairbairn & Law, 1986). In particular, *P. fluorescens* produces heat-resistant extracellular AprX proteases, which can degrade milk casein (CN) and have been often associated with spoilage of milk (Dufour et al., 2008; Sørhaug & Stepaniak, 1997). Most of the extracellular thermostable proteases of *P. fluorescens*, isolated from raw milk kept refrigerated, are 40–50 kDa metalloenzymes called AprX, with molecular mass, which belongs to the serralysin family (Marchand et al., 2009, Martins, de Araujo, Mantovani, Moraes & Vanetti, 2005; Martins, Pinto, Riedel, & Vanetti, 2015; Nicodème et al., 2005). Calcium or zinc are essential for the activity and stability of these enzymes that present an optimum pH value of 7–9, and an optimal temperature range between 30 and 45 °C (Martins et al., 2005).

Nowadays, there is a great need for more knowledge on the impact of the activities of enzymes from the genus *Pseudomonas* in order to better support the quality control of milk before and after processing. Thus, the molecular and biochemical characterisation of these spoilage enzymes would address the identification of pertinent indicators of their activities as well as for the development of tools for their detection in milk. Over the past years, only some studies have provided detailed information 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 firstly addressed 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, the research aimed to characterise the extracellular thermostable proteolytic activities of the heat-treated cell-free supernatant (HT-CFS) of *P. fluorescens* PS19. Secondly, this study was addressed to evaluate the caseinolytic activity of this HT-CFS and to characterise the peptides released from single CN fractions of bovine milk.

2. Materials and methods

2.1. Bacterial strain

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

2.2. 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 mM $CaCl_2$.

The *P. fluorescens* PS19 was firstly recovered in Brain Heart Infusion (BHI) broth (Biolife, Milan, Italy) and incubated at 22 °C for 6 h. Subsequently, 10 mL of MSM were inoculated (3% of inoculum) and incubated overnight at 22 °C. This procedure was repeated to favour the adaptation of the *P. fluorescens* PS19 to the MSM. Afterwards, 1 L of MSM was inoculated (3% of inoculum) in an Erlenmeyer flask. After 24 h of incubation at 22 °C in aerobic conditions, cells were harvested by centrifugation (6000 *g* for 20 min at 4 °C). The supernatant was filter-sterilised (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 CN zymography. Specific proteolytic activity of HT-CFS was determined by azocasein assay according Nicodème, Grill, Humbert & Gaillard (2005).

2.3. Casein zymography

Casein zymography was performed as described by Marchand et al. (2009) and Rajmohan, Dood, & Waites (2002). Briefly, the HT-CFS was resuspended in 5× zymogram sample buffer containing 156.25 mM 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 polymerised 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. The PageRuler Plus Prestained protein ladder (10–250 kDa) (Thermo Scientific, Pierce, Rockford, IL, USA) was used as a molecular weight marker. After the run, the gel was renatured in 2.5% (v/v) Triton X-100 for 30 min and then incubated overnight at 37 °C in

development buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2 \times 2H_2O$ and 0.02% Brij 35). After incubation, the gel was stained with Coomassie Brilliant Blue R-250.

The HT-CFS from *P. fluorescens* PS19 was further concentrated in a stirred cell unit for ultrafiltration (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}-CNs (Sigma-Aldrich, St. Louis, MO, USA) or sodium caseinate and loaded in parallel on 12% SDS-polyacrylamide gel polymerized without casein for further proteomic characterization.

2.4. 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 (FASP) protocol described by Wiśniewski, Zougman, Nagaraj & Mann (2009). The enzymes LysC and trypsin (Roche Diagnostics, Monza, Italy) 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, USA) 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 lyophilised and re-suspended in 0.1% formic acid for nLC–MS/MS analysis.

Five µL of desalted peptides were injected on a C18 HALO PicoFrit column (75 µm × 10 cm, 2.7 µm, 100 Å; New Objective, Woburn, MA, USA) 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^{-1}. Eluting peptides were on-line sprayed into a LTQ Orbitrap XL mass spectrometer through a nanoESI source (Thermo Scientific). The instrument operated in data-dependent mode (DDA) to automatically switch

between full MS and MS/MS acquisitions. Full MS spectra were acquired in profile mode by the FT analyzer in a *m/z* window 300–1500 with the AGC scan 5×10^5 and resolution 100000 FWHM at *m/z* 400. Tandem mass spectra were acquired in centroid mode by the linear ion trap for the 2 most intense ions exceeding 1×10^4 counts. 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 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 analysed 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, 1353566 entries, downloaded from UniProt on 08.05.2015.). The selected protease was trypsin or LysC; up to 2 missed cleavages were allowed. Cys carbamidomethylation was set as fixed modification, while Asn deamidation and Met oxidation were set as variable modifications. The precursor mass tolerance was set to 5 ppm and fragment mass tolerance was 0.5 Da.

2.5. Characterisation of the hydrolytic action of the concentrated HT-CFS of *P. fluorescens* PS19 on bovine CN fractions

The hydrolytic activity on α_s, β and κ bovine CNs was tested by incubating single protein fraction in 20 mM Tris-HCl (pH 6.7) with the 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-MS/MS analysis for peptide identification. Samples, consisting of single CNs or ultrafiltered HT-CFS were used as controls.

2.6. Separation and identification of peptides from hydrolysis of CN fractions

Separation of peptides in CN hydrolysates was performed on an Alliance 2695 HPLC system (Waters, Milford, MA, USA) equipped with a Waters 2996 diode array detector (DAD). Sampleswere 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% (v/v) trifluoroacetic acid (TFA) in MilliQ-treated water, and solvent B, 0.1% (v/v) TFA in acetonitrile. The elution gradient, expressed as the solvent B proportion, was as follows: 0–5 min, 5%; 5–65 min, 55%; 65–70 min, 95%; 70–72 min, 95%, 72–76 min, 5%. The flow rate was 0.2 mL min^{-1}, and run-to-run time, 90 min. Absorbance was recorded at 210 nm, and data were processed using the Empower software package (Waters).

Identification of peptides in hydrolysates of CN fractions was carried out by UPLC coupled to high resolution tandem mass spectrometry (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 ionisation (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, USA) kept at 40 °C. The eluents were: 5% acetonitrile, 0.08% formic acid (FA), 0.01% TFA in MilliQ-treated water (solvent A) and 5% water, 0.08% FA, 0.01% TFA in acetonitrile (solvent B). The UPLC separation was performed by using a linear elution gradient (0% to 80% of solvent B in 32 min) at a flow rate of 0.3 mL min^{-1}. The The ion source and interface MS conditions were: spray voltage 3.5 kV, sheath gas flow rate 35, auxiliary gas flow rate 15 and temperature 300 °C, ion transfer tube temperature 350 °C. The UPLC eluate was analysed by MS using Full scan (400–2000 m/z) and data dependent MS^2 analysis of ten the most intense ions [$ddMS^2$(Top10)]. The resolution was set at 70K and 17.5K 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 ms 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).

3. Results and discussion

3.1. Evaluation of the HT-CFS caseinolytic activity from *P. fluorescens* PS19 isolated from bulk bovine raw milk

In a previous work (Decimo et al., 2014), 63 strains of psycrothrophic bacteria were isolated from bulk bovine raw milk. Among them, 19 strains of *P. fluorescens* were identified. One of them, named PS19, possessed the highest proteolytic activity *in vitro* at both 7 °C and 22 °C. In this work, its proteolytic activity on bovine CNs was further characterised. To this aim, the CFS was prepared and heated to select for thermostable proteases (Marchand, Coudijzer, Heyndrickx, Dewettinck & De Block, 2008; Marchand et al., 2009). The heat-treated CFS (HT-CFS) was used for futher investigations. The CFS was obtained after bacterial growth in a MSM supplemented with 1 mM $CaCl_2$, without milk addition. This procedure was adopted in view to characterize the proteolytic activity of the HT-CFS on single CN fractions. In this regard, the use of the MSM permitted to obtain a crude enzyme extract depleted of interferring milk CNs and derived peptides, as well as of any pre-existing endogenous or exogenous proteolytic activities potentially present in milk. Nonetheless, preliminary azocasein assay did not show any difference between the proteolytic activity of HT-CFS deriving from bacteria grown in MSM or in reconstituted skimmed milk powder (10% w/v) (data not shown).

The HT-CFS from the strain cultured in MSM was further characterised by CN zymography. The caseinolytic activity of the HT-CFS was associated 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 of 40–50 kDa molecular weight (Dufour et al., 2008; Koka & Weimer, 2000; Marchand et al., 2009; Martins et al., 2015; Mateos et al., 2015; Nicodème et al., 2005). After concentration by UF at 10 kDa, the HT-CFS of the strain PS19 was used for further analysis of the proteolytic extracellular activity. To this aim, zymography with either sodium caseinate or single CN fractions (α_s, β and κ) was carried out (Figure 1). Despite of protein substrates, two proteolytic bands with molecular masses of approximately 15 kDa and 25 kDa appeared on zymography gels togheter with the 45 kDa protease (Figure 2). The presence of some low molecular weight proteases on zymogram was also reported by Rajmohan et al. (2002) when these enzymes were concentrated by UF of supernatans of *P. fluorescens* isolated from milk. Also Marchand et al. (2009) reported some *P. fluorescens* strains to display 2 or 3 clearance zones of low (<45–50 kDa) molecular weight on casein zymography. These authors assumed the appearance of low molecular weight extracellular proteases to be likely attributable to substrate depletion, by which the protease of 45–50 kDa was degraded into smaller active

fragments being the only protein source left. Similarly, Nicodème et al. (2005) described the presence of different protease bands for some strains of *Pseudomonas* spp., as revealed by zymography. More recently, together with the major 50 kDa AprX protease, Martins et al. (2015) identified several other lower molecular weight extracellular proteases from supernatants of *P. fluorescens* 041 strain isolated from raw milk.

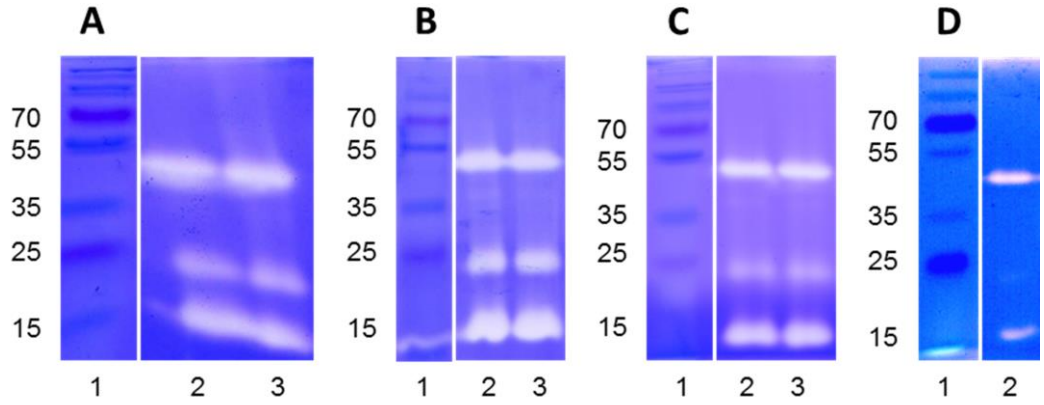

Fig. 1. Caseinolytic activity of the concentrated HT-CFS of *P. fluorescens* PS19 on sodium caseinate (A), α_s-CN (B), β-CN (C) and κ-CN (D). Lanes: 1, protein molecular weight ladder, 10–250 kDa; 2 and 3, concentrated HT-CFS.

3.2. Proteomic characterization of the extracellular proteases present in the concentrated HT-CFS of *P. fluorescens* PS19

Although the production of both 45–50 kDa and lower molecular mass proteases by single strain of *P. fluorescens* has been already reported, the molecular identification of these proteases has been studied to a lesser extent. Based on this, the 15 kDa, 25 kDa, and 45 kDa proteases present in the HT-CFS of *P. fluorescens* PS19 were further subjected to proteomic analysis by nLC-MS/MS after both *in gel* and *in solution* digestions to obtain higher identification coverage. According to, the band of 45 kDa on CN zymogram was attributed to the AprX metalloprotease of *P. fluorescens* (UniProtKB Acc. No. C9WKP6) with sequence coverage of 55.14% (Figure 2). 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 & Deeth, 2003; Dufour et al., 2008). These proteases are encoded by a gene *aprX*, which has been

characterised in several strains of *Pseudomonas* spp. isolated from raw milk, in particular in *P. fluorescens* (Marchand et al., 2009; Martins et al., 2005). The PS19 strain also possessed the *aprX* gene as resulted from 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 amino acid subtitutions differentiated the protease of this study, UniProtKB Acc. No. C9WKP6, and the AprX, UniProtKB Acc. No. Q7X4S5 (Figure 2). According Mateos et al. (2015), amino acid sequences of serralysin proteases are highly conserved within *Pseudomonas* spp.: a 76–99% similarity for AprX proteases was observed for *P. fluorescens* group. Our findings confirm this high similarity for AprX proteases from *P. fluorescens* PS19 and *P. fluorescens* F (Figure 2).

The results obtained from the digestions of the band at 15 kDa matched different fragments present in the amino acid 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 amino acid long protease with a molecular mass of 29.6 kDa. In our study, the 15 kDa band can be likely assumed as a fragment of the 45 kDa AprX metalloprotease, UniProtKB Acc. No. C9WKP6. This hypothesis is supported by findings of Kumura, Murata, Hoshino, Mikawa & Shimazaki (1999), who 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.

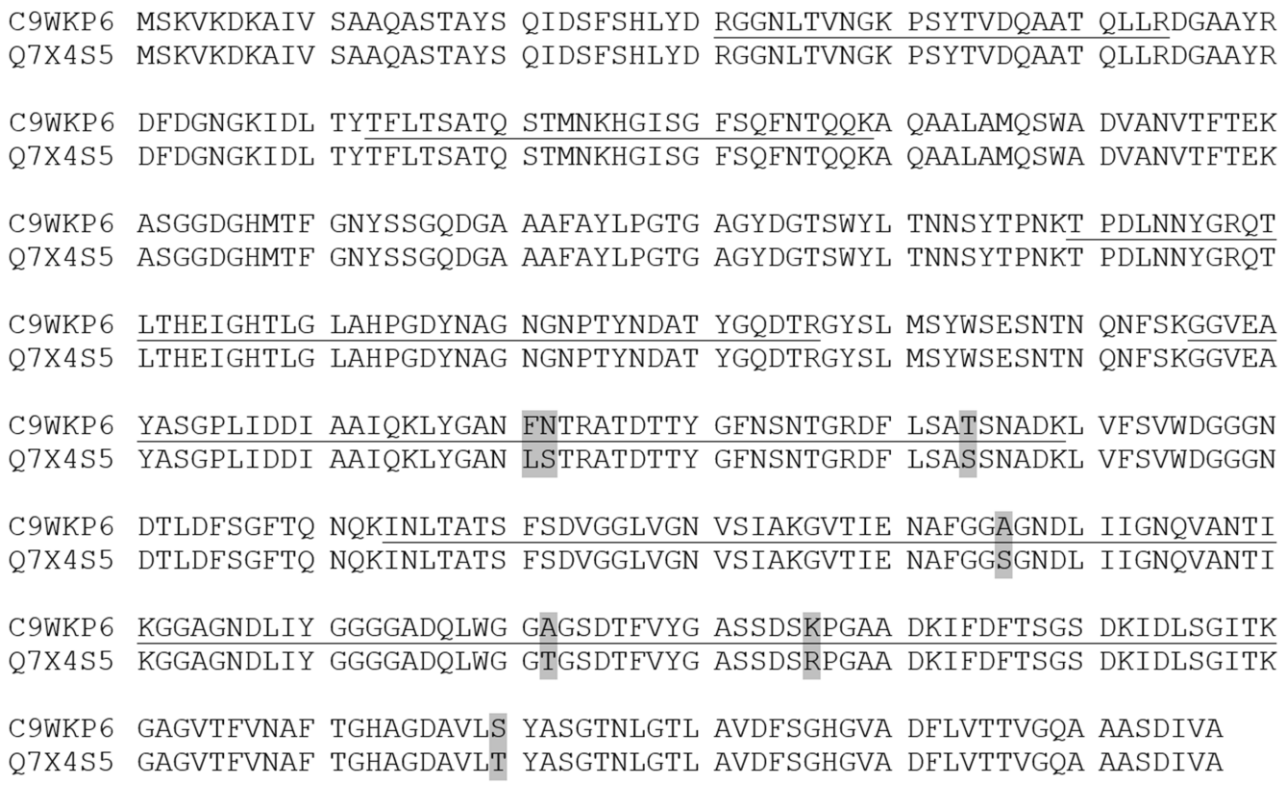

Fig. 2. Alignment of primary sequences of the protease AprX from *P. fluorescens* PS19 (UniProtKB Acc. No. C9WKP6) (this study) and protease AprX form *P. fluorescens* F (UniProtKB Acc. No. Q7X4S5) (Baglinière et al., 2013). The differences in amino acid residues are indicated by gray shading. The amino acid sequences identified by nLC-MS/MS after *in gel* digestion of protease AprX from *P. fluorescens* PS19 are underlined.

3.3. Identification of the peptides released from bovine CN fractions proteolysed with the concentrated HT-CFS of *P. fluorescens* PS19

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 were studied after HPLC separation and UV detection of CN hydrolysates. The single CN fractions were incubated 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 CN fractions to occur when incubation was carried out at 22 °C (Figure 3), 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 & Ewings, 1985). Indeed, intact β- and κ-CNs were not revealed 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 3). At 22 °C, the strongest proteolysis was again observed for κ- and β-CNs, which were no longer present in the HPLC patterns at 24 h sampling (Figure 3). Almost complete degradation of α_s-CN was observed after 96 h incubation at the same temperature. Overall, extracellular proteases present in the HT-CFS of *P. fluorescens* PS19 cleaved β-=κ->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 & Weimer (2000) found the protease isolated from *P. fluorescens* RO98 to preferentially hydrolyse κ-CN. Similarly, Costa, Gomez, Molina, Simpson & Romero (2002) demonstrated that the extract of *P. fluorescens* RV10 culture mainly proteolysed κ- and β-CN. Also Nicodème (2006) showed *Pseudomonas* sp. to hydrolyse CNs in the following order κ-> β-> α_s-CN. Finally, studying the destabilisation of UHT milk caused by the AprX protease from *P. fluorescens* F, Baglinière et al. (2013) found the enzyme to hydrolyse all CNs with a preference for β-CN.

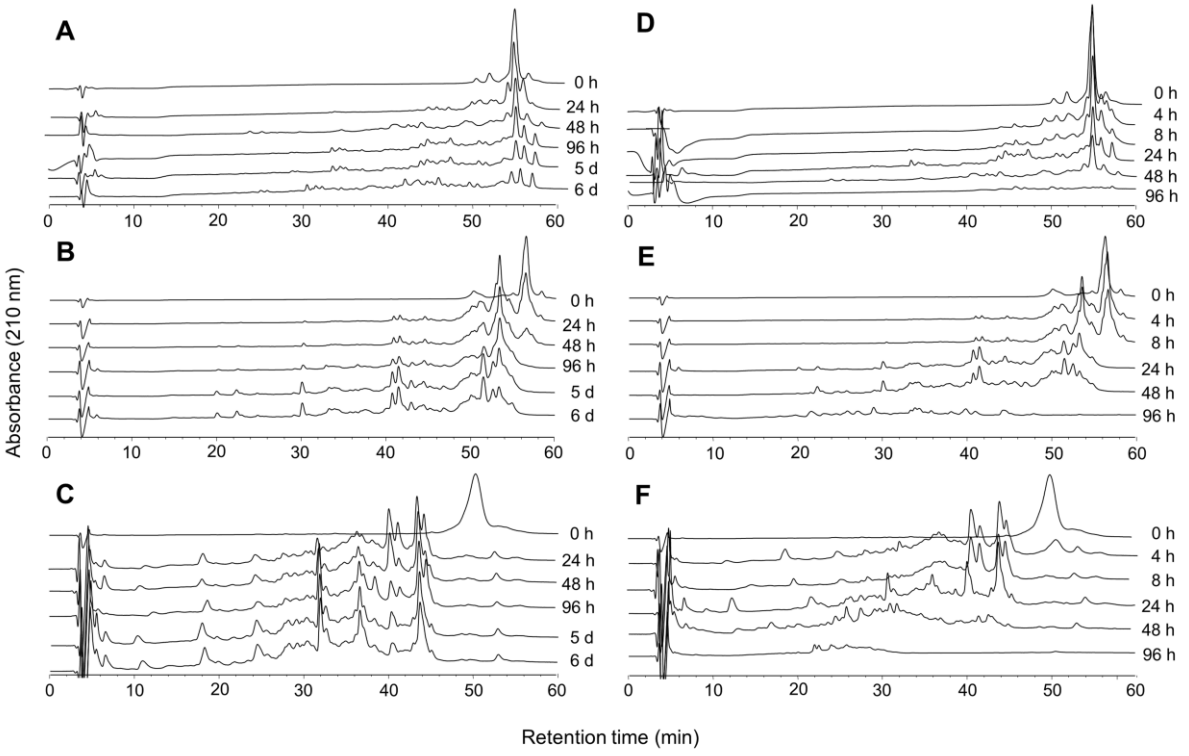

Fig. 3. HPLC patterns of peptides generated from hydrolysis of single CN fractions by the concentrated HT-CFS of *P. fluorescens* PS19 after different hydrolysis times at 7 °C (A, α_s-CN; B, β-CN; C, κ-CN) and 22 °C (D, α_s-CN; E, β-CN; F, κ-CN).

Identification of peptides released *in vitro* from CN fractions was performed by UPLC-MS/MS. After different times of incubation, the identified peptides varied in quantity according to temperature and digested CN fraction. In total, about 200 peptides were released from each CN fraction during incubation with the concentrated HT-CFS of *P. fluorescens* PS19 at 7 °C and 22 °C (Table S1 in the supplemental material). None of these peptides were revealed in the control samples constituted by either the HT-CFS or the single CNs alone.

Among the identified peptides, particular attention was paid to those that promptly formed and resisted further proteolysis till the end of the incubation. For instance, 14 peptides were identified 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 to be 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 incubation at 7 °C, only four 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 above-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 till 6-d refrigerated incubation. In contrary, none of the peptides, promptly formed after 2-h incubation at 22 °C, were still recovered at the end of the experiment at the same temperature.

Forty-six peptides originated from κ-CN after 24-h incubation at 7 °C. Most of the peptides which survived till the end of incubation (6 d) arose from the sequences 62–69 (n=4) and 105–114 (n=7) of this CN fraction (see Table S1 in the supplemental material). According to Gaucher et al. (2011), the κ-CN sequence

105–123 was mainly hydrolysed in UHT milk prepared from raw milk inoculated with *P. fluorescens* CNRZ 798. Some peptides released from this last sequence of κ-CN were also revealed by Baglinière et al. (2013) 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 incubation at 22 °C and two of them, the fragments 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, López-Fandiño, Olano, Olieman & Ramos, 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, Garcia Risco, Ramos & Lopez Fandino, 2000).

Strong proteolysis characterised the β-CN at 7 °C and 22 °C: several peptides arose from the C-terminal sequence 190–209 (Table S1 in the supplemental material), which has been known to contain peptide sequences conferring bitterness to dairy products (Visser et al., 1983). Only six 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).

Summarizing, 26 peptides were promptly released from CNs and they were detected till 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 derived from κ-, β-, and α_s-CN, respectively. Despite about 200 peptides were released from each CN fraction, most of those, resisted further proteolysis, 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* form single CN fractions by the AprX protease of *Pseudomonas* sp. LBSA1, a strain isolated from bulk bovine raw milk. These authors found several peptides coming 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 proteolitically-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), also some of the peptides generated from α_{s1}-CN overlapped the proteolitically-resistant sequences [f(4–7) and f(1–7)] of α_s-CN found in the present work (Table 1). The same authors found 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 (Mateos et al., 2015). In our work, these sites were cleaved upon the formation of the peptides f(190–202) and f(192–202) (Table 1). Overall, specific cleavage sites upon the action of the proteases contained in the HT-CFS of *P. fluorescens* PS19 were not identified. Nonetheless, in the case of κ- and α_s-CN, the presence of aliphatic (I, P) and basic (K, H) amino acids in the sequence of peptides likely hindered their further hydrolysis during incubation at refrigerated conditons with the HT-CFS of *P. fluorescens* PS19.

Table 1

Peptides released from α_s-, β- and κ-CNs upon the action of proteases present in the HT-CFS of *P. fluorescens* PS19 and revealed at every sampling time of incubation at 7 °C or 22 °C.

	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 (31–37) VLSRYPS	
	f (31–41) VLSRYPSYGLN	
	f (40–43) LNYY	
	f (62–66) AKPAA	
	f (65–68) AAVR	
	f (69–72) SPAQ	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 (108–111) 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 CNs 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 CN breakdown at refrigerated conditons. For this reason, these peptides could be regarded as indicators of the the proteolytic activity of *P. fluorescens* PS19 on bovine CNs and, potentially, as early markers of spoilage of refrigerated milk. Overall, this study provides a better understanding of the enzymatic activities of *P. fluorescens* in milk.

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