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Ser2 from *Serratia liquefaciens* L53: A new heat stable protease able to destabilize UHT milk during its storage

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ABSTRACT

The heat-stable protease Ser2 is secreted by the species *Serratia liquefaciens*, a psychrotrophic bacteria frequently found in raw milk. To understand the physicochemical modifications of casein micelles induced by Ser2 and to confirm its implication in UHT milk destabilization, the enzyme was purified and added to microfiltered raw milk before UHT treatment. UHT milk destabilization was investigated during 90 days of storage. A visual destabilization appeared after 8 days of storage with the presence of sediment. Zeta potential increase and formation of aggregates were observed during the storage. Using tandem mass spectrometry, numerous released peptides from the four caseins were identified at the end of storage. Caseins were hydrolyzed in the preferential order β- > α_{s1}- > κ- > α_{s2}. No specific peptidic hydrolysed bond was detected. The present study confirmed that the presence of the protease Ser2 in raw milk can be one of the main causes of UHT milk destabilization.

1. Introduction

Highly nutritious and with a neutral pH, raw milk presents ideal conditions for microbial growth. Generally, raw milk is supposed to be sterile in healthy udder cells. However, bacterial contamination takes place during milking, storage and transportation (Coorevits et al., 2008; Eneroth, Ahrné, & Molin, 2000). To limit the growth of most bacteria in raw milk, a cold storage is applied. During this storage, changes in the bacterial communities frequently occur (Raats, Offek, Minz, & Halpern, 2011; Rasolofo, St-Gelais, LaPointe, & Roy, 2010; von Neubeck et al., 2015). Indeed, representing only 10% of bacterial contamination before cold storage, psychrotrophic bacteria are predominant at the arrival in dairy industries (Sørhaug & Stepaniak, 1997). This microbiota is able to grow at low temperatures and can produce extracellular proteases. Most of psychrotrophic bacteria found in raw milk are inactivated by pasteurization and ultra-high temperature (UHT). However, these microorganisms are able to secret heat-stable proteases that can resist to these heat-treatments used in dairy industries (Cousin, 1982; Datta & Deeth, 2003; Dogan & Boor, 2003; Sørhaug & Stepaniak, 1997). *Pseudomonas fluorescens* is one of the most commonly present psychrotrophic species found in raw milk as a heat-stable enzyme producer (Dogan & Boor, 2003; Marchand et al., 2009). This species secrets the heat-stable protease AprX, one of the main cause of UHT milk destabilization during the storage (Baglinière et al., 2012, 2013; Matéos et al., 2015). This metalloprotease belongs to the serralysin family and hydrolyses the four caseins after UHT treatment (Baglinière et al., 2013).

However, some studies showed that *P. fluorescens* is not the only psychrotrophic species frequently found in raw milk as heat-stable proteases producer (Machado et al., 2015, 2016). The genus *Serratia*, especially the species *S. liquefaciens*, is also frequently encountered as psychrotrophic contaminant in raw milk.

This specie is commonly encountered on many plant species, insect, soil and water (Grimont & Grimont, 1978). However, in the last decade, recent studies showed that the genus *Serratia* was also detected in dairy processing factories (Cleto, Matos, Kluskens, & Vieira, 2012), in raw milk samples stored at 4 °C (Lafarge et al., 2004; Machado et al., 2015; Teh et al., 2011) and in bulk milk storage tank (Decimo, Morandi, Silvetti, & Brasca, 2014). Two studies carried out in Brazil showed that *S. liquefaciens* was the most found psychrotrophic species with the capacity to secret heat-stable proteases in raw milk (Machado et al., 2015,

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2016). Indeed, this species produces two extracellular metalloproteases, Ser1 and Ser2, belonging to the serralysin family (Kaibara et al., 2012). There is no information in the literature about Ser1 in milk.

Concerning Ser2, this protease hydrolyses sodium-caseinate after a heat-treatment of 95 °C during 8 min (Machado et al., 2016). This enzyme was highly heat-stable in UHT semi-skimmed milk at 75, 85 and 95 °C (Machado et al., 2016).

As AprX, this protease could be one of the main causes of UHT milk destabilization during the storage. Up to now, the proteolytic activity of the protease Ser2 in relation to UHT milk destabilization has never been specifically studied.

The objective of the present study was to investigate the impact of the heat-stable protease Ser2 on the stability of casein micelles of UHT milk at macroscopic, colloidal and molecular scales. For that, this protease secreted by the psychrotrophic strain *S. liquefaciens* L53, a highly proteolytic strain isolated from Brazilian raw milk (Machado et al., 2016), has been cloned, heterologous expressed in *Escherichia coli* strain BL21, purified to homogeneity and added in raw milk before UHT treatment.

2. Materials and methods

2.1. Purification of the protease Ser2

The coding sequence of *ser2* gene of the strain *S. liquefaciens* L53 was amplified by PCR and inserted downstream of the T7 promoter of the expression plasmid pET28 carrying an *N*-terminal 6x Histidine tag (Novagen, Cambridge, MA, USA). Plasmid harboring the coding sequence of *ser2* gene (pET28a-ser2) was transformed into the strain *E. coli* BL21 by heat-shock at 42 °C. To overexpress the protease Ser2, transformants of *E. coli* BL21 harbouring the plasmid pET28a-ser2 were grown at 37 °C with vigorous shaking (250 rpm) in LB medium containing kanamycin 30 μg/mL. d-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM when an optical density of 0.6 was reached. Afterwards the culture was incubated 6 h at 37 °C with stirring at 250 rpm. A centrifugation at 10,000*g* was performed for 20 min in order to collect the cells. Then, collected cells were re-suspended in lysis buffer (8 M urea, 300 mM NaCl, 50 mM NaH_2PO4, 10 mM Imidazole, pH 7.5) and sonicated on ice twelve times of 20 s burst at 100 W followed by 20 s cooling period between each burst with a sonicator (Vibra Cell VCX130, Newton, USA). To pellet the cellular debris, the cell solution was centrifuged for 30 min at 10,000*g* and the supernatant was collected and purified under denaturing conditions. One hundred mL of this sample was applied to an affinity chromatographic column HisTrap™ FF 20 mL (GE Healthcare, Little Chalfont, United Kingdom) previously equilibrated with the lysis buffer. Elution was performed at a linear flow rate of 3 mL/min with elution buffer (8 M urea, 300 mM NaCl, 50 mM NaH_2PO_4, 100 mM Imidazole, pH 8.0). The collected fraction was dialyzed overnight against ultra-pure water containing 0.02% (w/v) NaN_3 at 7 °C and lyophilized. In order to evaluate purity, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed. As described by Dherbecourt et al. (2010), tandem mass spectrometry was performed to identify the purified protease. In this study, the term Ser2 will be used to refer to the recombinant protein produced from the expression of pET28a-ser2 by the strain *E. coli* BL21.

2.2. Milk treatments

The experimental protocol for milk treatments was described in detail by Gaucher et al. (2011). Briefly, raw milk was skimmed with a cream separator (Elecrem, Châtillon, France) at 50 °C. In order to remove bacteria and somatic cells, skimmed milk was microfiltered using a TIA microfiltration unit (TIA, Bollène, France) with a ceramic membrane Sterilox (reference 1P1940, membrane area = 0.24 m^2, 1.02 m long, pore size = 0.8 μm, temperature of microfiltration = 50 °C, Pall-Exekia, Bazet, France). One part of the microfiltered milk was mixed with the purified protease Ser2 at a final concentration of about 200 μg/L and the second one was used as a control milk. Then, UHT treatments were performed for both milk samples (with and without Ser2) using an indirect UHT processing system (Microthermics UHT/HTST Lab 25 EDH, Microthermics, Inc., Raleigh, NC 27615, USA). Firstly, milk samples were preheated to 90 °C in a tubular heat exchanger. Secondly, these milk samples were heated to 140 °C in a second heat exchanger and maintained at 140 °C for 4 s. All glass bottles were aseptically filled after quick cooling to 17 °C. This experiment was performed in duplicate with two different batches of raw milk.

2.3. Physico-chemical characterization of UHT milks during storage

The different UHT milk samples were stored at about 17 °C and analyzed after 1, 8, 30, 60 and 90 days of storage. Before analysis, enumeration on a plate count agar medium (3 days of incubation at 30 °C) was performed for each milk samples to verify the absence of bacterial contamination. Each analysis, described below, was performed in triplicate, expect the mass spectrometry analysis. This analysis was performed once.

2.3.1. Non protein (NPN) and non casein (NCN) nitrogen contents

The NCN and NPN contents were determined, using the Kjeldhal method according to IDF standard 20–4 (IDF, 2001), after precipitation and filtration at pH 4.6 for NCN fraction and after precipitation at 12% (v/v) trichloroacetic and filtration for NPN fraction. The experimental error was ±0.1 g/kg of protein.

2.3.2. Analysis of NCN fraction by RP-HPLC (Reversed phase High-Performance liquid Chromatography) coupled to ESI-MS/MS (ElectroSpray Ionisation-Mass Spectrometry/Mass Spectrometry)

Mass spectrometry (MS) analysis was adapted from Nyemb et al. (2016). Briefly, a nano-RSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a nanoelectrospray ion source was used. Peptide separation was performed at a flow rate of 0.3 μL/min using solvents A [2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA (Trifluoroacetic acid) in deionized water] and B [95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water]. The elution gradient first rose from 5 to 35% solvent B over 87 min, then up to 85% solvent B over 3 min before column reequilibration. The mass spectra were recorded in positive mode using the *m*/*z* range 250–2000. The resolution of the mass analyzer for *m*/*z* of 200 a.m.u (atomic mass unit) was set in the acquisition method to 70,000 for MS and 17,500 for MS/MS. For each MS scan, the ten most intense ions were selected for MS/MS fragmentation and excluded from fragmentation for 15 s.

Peptides were identified from the MS/MS spectra using the X! TandemPipeline software (http://pappso.inra.fr) against an in house database dealing with major milk proteins which represents a portion of the Swissprot database (http://www.expasy.org). Database search parameters were specified as previously (Nyemb et al., 2016). The possible modifications were serine or threonine phosphorylation and methionine oxidation. Peptides identified with an e-value <0.01 were automatically validated. With these parameters a peptide false discovery rate of 0.2% was achieved.

2.3.3. Zeta potentials of milk particles

A Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom) was used to measure zeta potentials of particles. Firstly, milk samples were diluted 1/100 in their corresponding ultrafiltrate obtained by using Vivaspin filter (cut off of 10 kDa, Vivascience, palaiseau, France). Secondly, a voltage of 50 V with a dielectric constant of 80 was applied to the samples and then measurements were performed at a wavelength of 633 nm and a scattering angle of 173°. Based on electrophoretic mobility, the Smoluchowski (1917) was used to estimate the negative charge of casein micelles. The experimental error was ±1 mV.

2.3.4. Size distribution of milk particles

A Mastersizer 2000 granulometer (Malvern Instruments Ltd., Worcestershire, United Kingdom) was used to determine the size distribution of milk particles by laser light scattering. Milk samples were directly introduced into a dispersion cell containing deionized water under stirring at 1500 rpm. The refractive indexes used were 1.333 for water and 1.570 for casein micelles (Griffin & Griffin, 1985). The experimental error was ±5 nm.

3. Results

3.1. Expression and purification of Ser2

In order to check the purity of the protease, a SDS-PAGE was performed and showed, after Coomassie blue staining, only one homogeneous band with an apparent molecular mass of about 52 kDa (Fig. 1 – Lane 2). Zymography was carried out and showed an active protease (Fig. 1 – Lane 1). By tandem mass spectrometry using UniprotKB, the electrophoresis band was confirmed to be the protein F7J699 corresponding to the Serralysin-like metalloprotease Ser2 of *S. liquefaciens* (data not shown).

3.2. Visual observation of UHT milk samples after 3 months of storage

After 90 days of storage, the control UHT milk remained liquid and homogeneous. No sign of destabilization was observed (data not shown). For UHT milk containing 200 μg/mL of Ser2, a destabilization in two phases with the presence of white sediment was observed after 8 days of storage (data not shown).

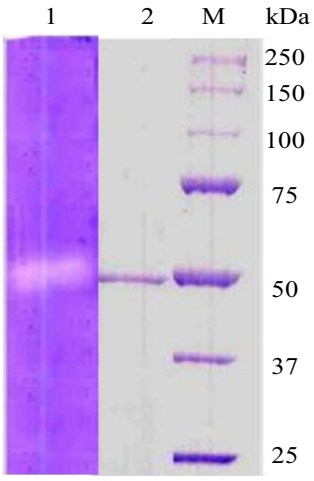

Fig. 1. Zymogram and coomassie-stained SDS-PAGE on 12% Polyacrylamide gels visualizing recombinant Ser2. Lane 1: sodium caseinate zymogram of purified Ser2; lane 2: SDS-PAGE of purified Ser2; Lane M: molecular mass standard (Biorad, Hercules, CA, USA)

3.3. Changes in NCN and NPN contents during storage of UHT milk samples

Immediately after UHT treatment, the NCN content of control UHT milk and UHT milk samples containing 200 μg/L of purified Ser2, were about 3.3 and 3.4 g/kg respectively (Fig. 2A). As described by Gaucher et al. (2011), these values were low and normal compared to raw milk (7–8 g/kg) because of the association of denatured whey proteins (α-lactalbumin, β-lactoglobulin and immunoglobulins) with casein molecules during UHT treatment. During the 90 days of storage, NCN content of control UHT milk remained stable and low. Oppositely, an increase of the NCN content was observed all along the storage for UHT milk samples containing 200 μg/L of purified Ser2. NCN content was about 9.1 g/kg after the first week of storage and increased up to 18.3 g/kg after 90 days of storage (Fig. 2A).

Related to the NPN content, the values obtained immediately after heat-treatment were 1.3 and 1.4 g/kg for control UHT milk and UHT milk samples containing 200 μg/L of purified Ser2, respectively (Fig. 2B). These contents are due to the presence of small molecules containing nitrogen as urea, creatine, small peptides and free amino acids.

The NPN content of control UHT milk samples remained stable during the 90 days of storage. For UHT milk samples containing the protease Ser2, the NPN was 6.3 g/kg after the first week of storage and it slightly increased to reach a value of 14.2 g/kg after 90 days of storage (Fig. 2B).

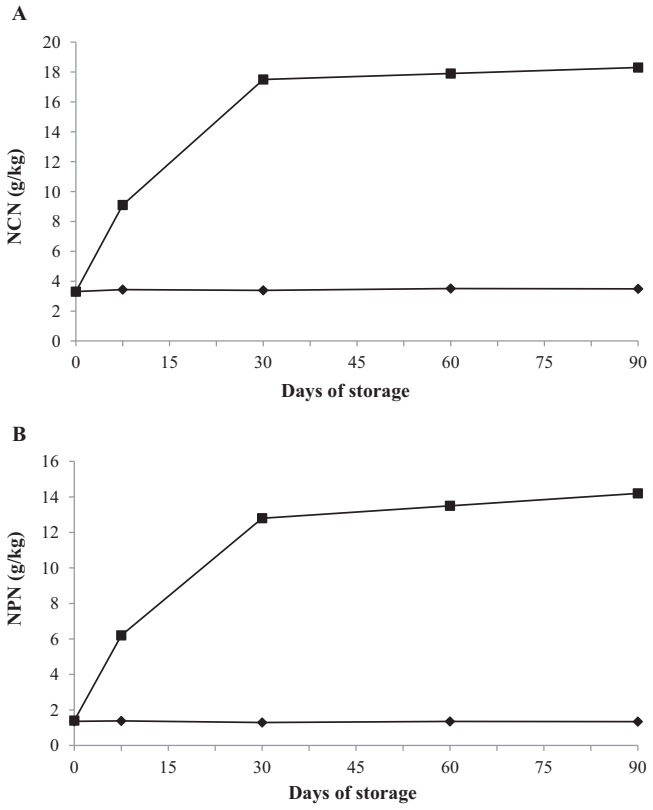

Fig. 2. Non-casein (A) and non-protein nitrogen (B) contents of control UHT milk (◆) and UHT milk manufactured from raw milk containing 200 μg/L of the purified Ser2(■) during 90 days of storage.

3.4. Separation and identification of peptides present in the NCN fractions by RP-HPLC coupled to ESI-MS/MS

The identification of the different peptides present in the NCN fractions was performed by ESI-MS/MS after their separation by RP-HPLC. After 90 days of storage, the quantity of different peptides identified in the NCN fraction of the control UHT milk was relatively high despite the absence of proteolysis. Most peptides detected come from β- and α_{s1}-caseins (Fig. 3A and Table 1). Only a few peptides coming from the α_{s2}- and κ-caseins were detected.

For the NCN fraction of UHT milk containing the protease Ser2, the number of different identified peptides was higher compared to the NCN fraction of the control UHT milk. The number of different peptides detected mainly originated from the β-casein followed by α_{s1}-, κ-, and, α_{s2}-caseins (Table 1). As for control UHT milk the different peptides detected were mainly originated from β and α_{s1}-caseins (Table 1). The amounts of different peptides detected for these two caseins were twice higher than in the non-destabilized milk. Qualitatively, the proteolysis was higher for β-casein than α_{s1}-casein. Nevertheless, the proteolysis of the α_{s1}-casein remained high (Table 1). Fully hydrolyzed, no specific cleavage bond was observed for these two caseins (Fig. 3A and B). For α_{s2}-casein, 58 different peptides were detected in the NCN fraction of destabilized UHT milk with the protease Ser2. It is three times higher than the non-destabilized milk (Table 1). However, this casein was less affected than the others (Table 1). Regarding to the number of different detected peptides, the main difference between the control UHT milk and UHT milk samples containing Ser2 was the κ-casein. Seventy-two peptides from this casein were detected for the destabilized milk whereas only 14 peptides were detected for the non-destabilized milk (Table 1). Peptides coming from the sequence 95–105 and 110–125 of the κ-casein were only detected for the NCN fraction of UHT milk containing Ser2. It is noteworthy that the sequence 125–169 of these casein was preferentially hydrolyzed (Fig. 4B).

Table 1
Number of different peptides identified from each casein molecules in the NCN fraction of control UHT milk and from NCN fraction of UHT milk manufactured from raw milk containing 200 μg/L of Ser2 at 90 days of storage. Peptides were identified by RP-HPLC coupled to ESI-MS/MS.

	Control	Ser2
β-casein	109	234
α_{s1}-casein	70	172
α_{s2}-casein	23	58
κ -casein	14	72

3.5. Zeta potential of particles during storage of UHT milk samples

For the control UHT milk, the zeta potential of particles was -17.1 ± 1 mV after heat-treatment and remained constant during the 90 days of storage (Fig. 5A). For the UHT milk samples containing the protease Ser2, the zeta potential of particles was

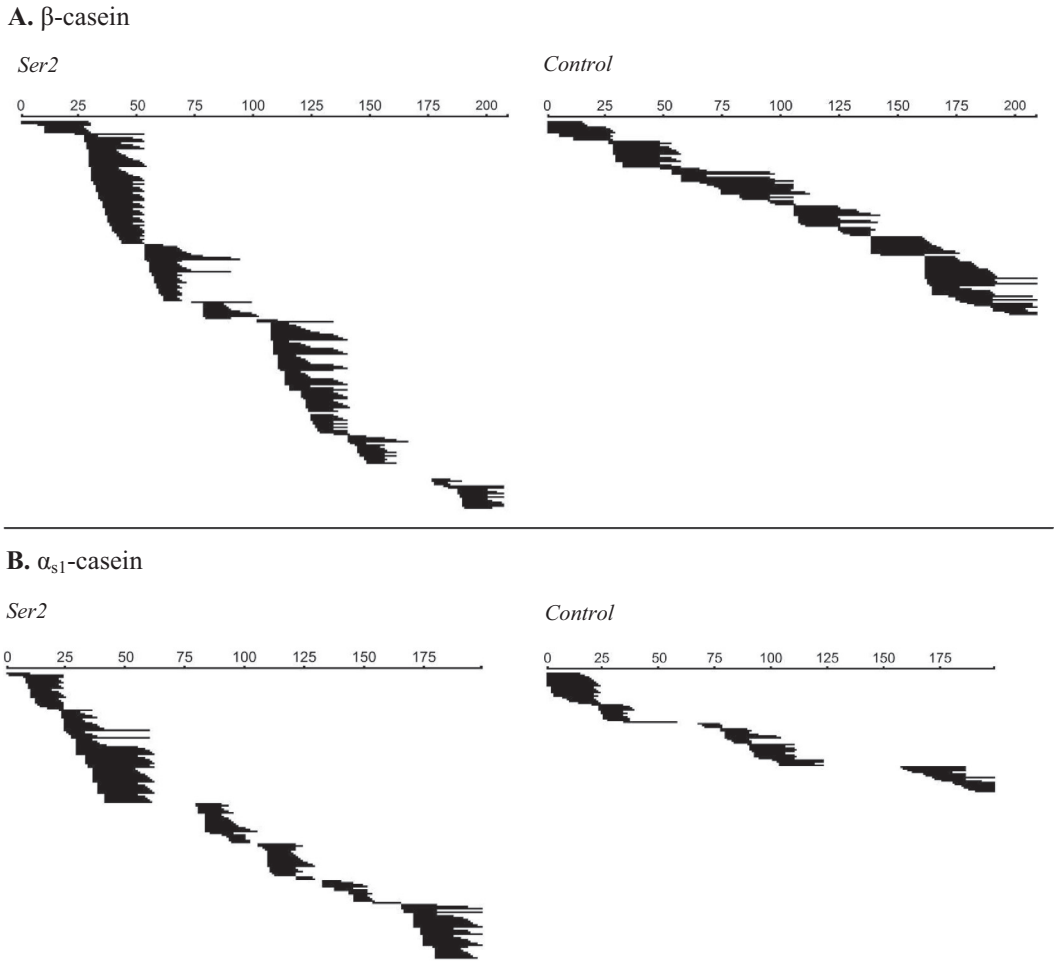

Fig. 3. Localization of the peptides identified by ESI-MS/MS in the sequence of (A) β-casein, and (B) α_{S1}-casein. These peptides were detected in the NCN fraction of UHT milk containing Ser2 and of control UHT milk at 90 days of storage

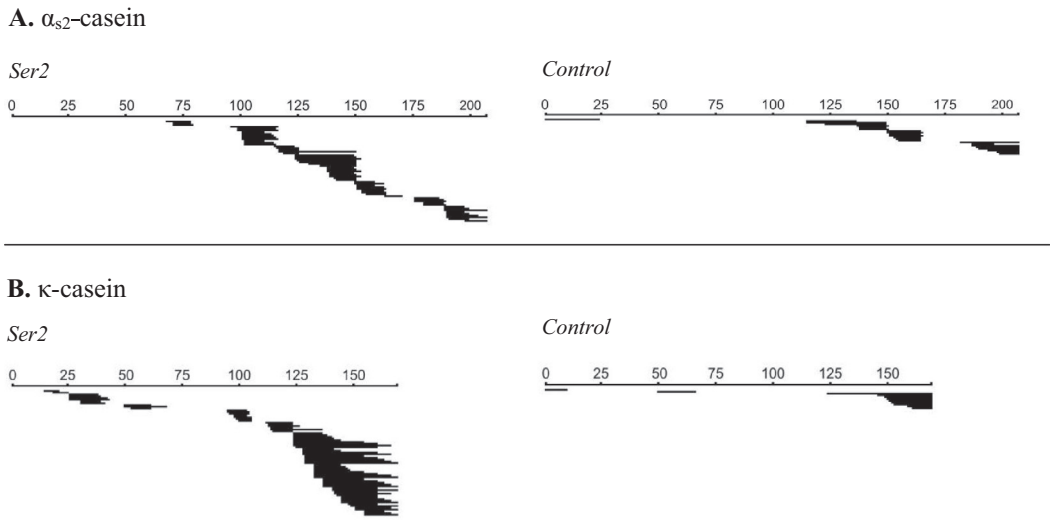

Fig. 4. Localization of the peptides identified by ESI-MS/MS in the sequence of (A) α_{s2}-casein, and (B) κ-casein. These peptides were detected in the NCN fraction of UHT milk containing Ser2 and of control UHT milk at 90 days of storage

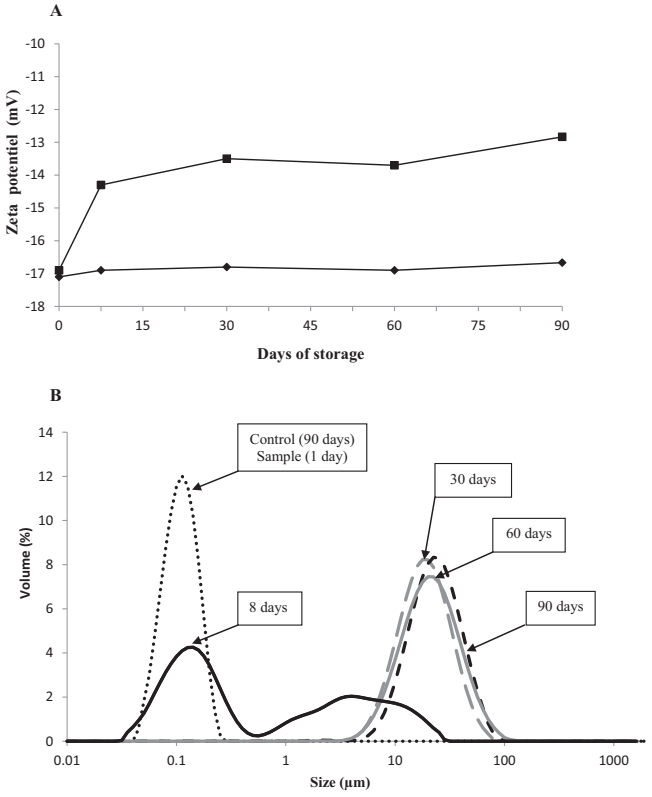

Fig. 5. Zeta potential (A) of particles from control UHT milk (◆) and UHT milks containing Ser2 (■) during 90 days of storage and particle size distribution (B) of control UHT milk (after 90 days of storage) and UHT milks containing 200 μg of Ser2 during 90 days of storage.

−16.9 ± 1 mV after heat-treatment, and decreased progressively as a function of time to reach a zeta potential of −12.8 mV at the end of storage (Fig. 5A).

3.6. Size distribution of particles during storage of UHT milk samples

A monomodal particles size distribution ranging from 0.05 to 0.32 μm was observed for the control UHT milk along the 90 days of storage (Fig. 5B). The average particle size of this population was 0.2 μm and corresponded to casein micelles.

For the UHT milk containing 200 μg/L of the protease Ser2, a new population with a particle size between 0.7 and 15 μm appeared on the first week of storage. The average particle size of 0.2 μm corresponding to stable casein micelles was still present, but at a lower level. After 30 days of storage, a new population with a diameter between 2 and 100 μm was observed. The average particle size of 0.2 μm corresponding to casein micelles was absent. These particles corresponded to aggregates. However, the nature of this new population was not determined in the current study. Similar profiles of particle size distribution were observed for the UHT milk containing Ser2 after 30, 60 and 90 days of storage.

4. Discussion

Identifying the cause of UHT milk destabilization is a scientific and technological challenge. The causes of this phenomenon are multiple. One factor of this phenomenon is the presence of psychrotrophic bacteria in raw milk secreting heat-stable protease, particularly *P. fluorescens* and the protease AprX. However, another species, *S. liquefaciens*, frequently found in raw milk, can also secrete a heat-stable protease Ser2. As for the AprX, the presence of this protease in raw milk could be one of the main causes of UHT milk destabilization. This protease could modify the physicochemical properties of casein micelles leading to the destabilization of UHT milk during its storage. To confirm this hypothesis, Ser2 enzyme was purified, identified and added to microfiltered skimmed milk at a final concentration of 200 μg/L and then an UHT treatment was performed. The stability and physicochemical modifications of casein micelles of UHT milk containing the protease Ser2 were investigated during 90 days of storage.

The addition of Ser2 at a final concentration of 200 μg/L led to the UHT milk destabilization. This destabilization, resulting in the formation of sediment (data not shown), appeared after 8 days of storage and it was due to the proteolysis of casein micelles by the heat-stable protease Ser2. The proteolysis of casein micelles was confirmed by the increase of NCN (Fig. 2A) and NPN (Fig. 2B) contents during the storage. It is noteworthy that a lower increase in NPN was observed during the storage compared with the increase in NCN content (Fig. 2A and B). This result can be

explained by the fact that large casein fragments were generated by the proteolysis and retained in the NCN fraction but not in the NPN fraction. The physicochemical modifications of casein micelle of the destabilized milk, due to the proteolysis by Ser2, were confirmed by the determination of a decrease of zeta potential (Fig. 5A). This result can explain the presence of aggregates (Fig. 5B) and the visual destabilization of UHT milk. Indeed, when a decrease of casein micelle charge occurs, the repulsions between micelles are reduced, resulting in the formation of aggregates (Fig. 5B). In a previous study (Baglinière et al., 2013), the same experiments were conducted with the protease AprX and showed similar results with kinetics of destabilization equivalent. After 90 days of storage, the UHT milk containing AprX was destabilized, with the presence of aggregates. Increases of NCN, NPN contents (Fig. 2A and B) and decreases in the casein micelles zeta potential (Fig. 5A) observed during the storage were really close for the destabilized UHT milk containing Ser2 and the destabilized UHT milk containing AprX.

Relating to the mass spectrometry analysis, the number of different peptides detected was in the following order β- > α_{s1}- > κ- > α_{s2} (Table 1). Compared to the other casein molecules, the particular free position and the higher concentration in the aqueous phase of β-casein in casein micelles could explain the preferential proteolysis of β-casein (Baglinière et al., 2012). Similar results were described by Baglinière et al. (2013) with the protease AprX. However, in the present study, the quantity of different peptides detected was significantly higher than in the study carried out by Baglinière et al. (2013) with the enzyme AprX. These differences in terms of number of detected peptides could be explained by the fact that the spectrometer used in the present study was more sensitive and able to detect peptides at a lower concentration levels.

It is important to discuss the number of different identified peptides coming from the κ-casein (Fig. 4B). This number was three times lower than in the β-casein. The low concentration of this casein in milk (about 3.5 g/L against about 10 g/L for β-casein) and the covalent interaction induced by the UHT treatment between β-lactoglobulin and κ- casein in the external part of the micelle could explain this difference. The access of the protease to κ-casein can be limited by this interaction. Nevertheless, qualitatively, κ-casein was highly hydrolysed in this study. The detection of various different peptides from the sequence 95–104 and 95–105 showed that the peptide bonds 104–105 and 105–106 were hydrolysed, generating the release of negatively charged Casein Macro Peptide (CMP). Casein micelles stability was negatively affected by this hydrolysis, leading to their destabilization and thus UHT milk destabilization. Indeed, it is well known that in cheese making, the hydrolysis of the peptide bond 105–106 of the κ-casein by rennet releases the CMP in the aqueous phase. This phenomenon is responsible for casein micelle destabilization and its coagulation (Recio, López-Fandiño, Olano, Olieman, & Ramos, 1996). In the study with the protease AprX (Baglinière et al., 2013), the number of different peptides coming from the κ-casein was lower. However, the main peptides detected came from the sequence 105–125, showing that peptide bond 104–105 was also hydrolysed, generating the release of CMP.

The qualitative differences of proteolysis observed between AprX and Ser 2 could be related to the difference in the structure and consequently in the respective activities of both enzymes.

UHT milk without addition of Ser2 (control) was not destabilized after 90 days of storage. UHT milk remained liquid and homogenous (data not shown). The absence of important proteolysis was confirmed by a constant and low NCN and NPN contents during the storage (Fig. 2A and B). A relatively high number of different peptides was detected by RP-HPLC coupled with ESI/MS-MS (Table 1) despite the absence of proteolysis during the storage. These peptides could come from a proteolysis before the UHT treatment. In our experimental design, the use of microfiltration did not remove the possible proteases present in raw milk. Thus, exogenous proteases could hydrolyse casein micelles during 24 h of cold storage before their inactivation by UHT treatment. These results are different than those described by Baglinière et al. (2013). In their study, only a few peptides coming from the four caseins were detected for control UHT milk after 90 days of storage.

5. Conclusion

The present study confirms that the protease Ser2 secreted by the specie *S. liquefaciens* can be one of the main causes of UHT milk destabilization. This protease is able to resist to UHT treatment and then to hydrolyze the four casein molecules, leading to the UHT milk destabilization. Similar patterns of released peptides identified by RP-HPLC coupled to mass spectrometry were observed for this protease and the protease AprX secreted by *P. fluorescens* and confirmed that Ser2 has also a large activity spectrum. From these results, it is interesting to detect and quantify their activities in raw milk to avoid a possible destabilization of UHT milk during its storage.

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