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Proteolysis in Irish farmhouse Camembert cheese during ripening

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Abstract

Proteolysis in an Irish farmhouse Camembert cheese was studied during 10 weeks of ripening. Urea-polyacrylamide gel electrophoresis of pH 4.6-insoluble fractions of cheese showed the degradation of caseins, initially due to the action of chymosin and plasmin and later due to *Penicillium camemberti* proteinases. Proteolytic specificities of *Penicillium camemberti* proteinases on the caseins in milk hydrolysates were determined and 64, 6, 28, and 2 cleavage sites were identified in α_{s1}-, α_{s2}-, β-, and κ-casein, respectively. Proteolysis in cheese was studied and peptides produced were determined and compared to the cleavage specificities of *Penicillium camemberti* proteinases. Regions most susceptible to proteolysis were 1–40, 79–114, and 168–199 in α_{s1}-casein; 42–79 and 97–116 in α_{s2}-casein; 40–57, 101–125, 143–189, and 165–209 in β-casein; and 31–81 and 124–137 in κ-casein. The present study describes in detail the proteolytic action of proteinases from *Penicillium camemberti* in Camembert cheese during ripening.

Practical applications Camembert cheese is a major international cheese variety, made in many countries around the world. The ripening of the cheese involves many biochemical changes and this study provides new information on peptides produced during ripening. *Penicillium camemberti* is an important mold used in the production of this type of cheese and detailed information is provided on the action of its

enzymes on the caseins. Data reported in this study furthers the understanding of the ripening of Camembert cheese.

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Keywords

Camembert cheese; cheese ripening; peptides; proteolysis

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1. INTRODUCTION

Camembert cheese is a widely known soft, mold-ripened cheese, originating from France (Scott, 1986; Sousa & McSweeney, 2001). The distinct appearance and taste of Camembert is due to the action of mold *Penicillium camemberti*, added to the milk or cheese curds during cheese manufacture. Wolfe, Button, Santarelli, and Dutton (2014) studied the complex microbiota of Camembert; microorganisms, other than *P. camemberti*, responsible for changes in texture, taste, flavor, color, and sensory characteristics were mainly lactic acid bacteria, yeasts (*Kluyveromyces lactis, Debaryomyces hansenii*), *Geotrichum candidum*, and coryneform bacteria. High moisture content in the cheese causes rapid growth of the mold and results in fast ripening (Schlesser, Schmidt, & Speckman, 1992). Yeasts commonly grow on the cheese surface, favored by a low-pH environment created by lactic acid bacteria (Baroiller & Schmidt, 1990; Leclercq-Perlat, Oumer, Bergere, Spinnler, & Corrieu, 1999). The lactic acid produced is consequently consumed by the yeasts and molds resulting in an increased pH (Wolfe et al., 2014). A pH gradient between the surface of the cheese and center is caused by consumption of lactic acid and production of ammonia (Vassal et al., 1986). *G. candidum* also prepares the curd for the growth of *P. camemberti* by hydrolyzing the proteins and fat (Leclercq-Perlat et al., 1999; Morel et al., 2015).

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Ripening of Camembert cheese involves similar basic biochemical processes as in hard cheeses. Various proteinases act on the cheese during ripening, from the coagulant, milk, starter, and the mold *P. camemberti* (Spinnler, 2017). The increase in pH during ripening caused by lactate metabolism decreases the solubility of calcium phosphate which precipitates at the surface. This creates a gradient of calcium ions and lactate and they migrate from the center toward the surface causing softening of the cheese at the center (Spinnler, 2017).

A high level of proteolysis is observed in Camembert cheese during ripening (Sousa & McSweeney, 2001). Formation of peptides and free amino acids contributes to the unique flavor of Camembert cheese (Fox & McSweeney, 1996). Proteolysis is due to the presence of three agents: rennet, plasmin, and *P. camemberti* proteinases which are dominant later in the ripening (Guizani, Kasapis, Al-Attabi, & Al-Ruzeiki, 2002). High levels of chymosin are retained in the curd as syneresis is driven by acidification; thus, curds and whey are separated at a relatively low pH favoring chymosin retention (Bansal, Fox, & McSweeney, 2009). The proteinase activity at the center is initially very low; however, in the outer region, it increases rapidly after 6–7 days of ripening, when the mold begins to grow. Concentrations of both aspartyl- and metalloproteinases are maximal after about 15–21 days, then slowly decrease as ripening proceeds (Lenoir, 1984; Spinnler, 2017). The proteolytic potential of the proteinases is mainly due to the production of an appreciable quantity of a metallo- and aspartylproteinases (Lenoir, 1984; Spinnler, 2017); the pH optima

of these proteinases is between pH 3.5 and 5.5. The enzymes hydrolyse α_{s1}-casein better than β- and κ-caseins with a relative activity ratio of 1:0.7:0.6 (Gripon, 1993). Both aspartyl proteinase and metalloproteinase are synthesized and their levels in cheese are maximal after about 15 days and then decrease slowly (Lenoir, 1984; Spinnler, 2017). Other enzymes that have been characterized from *P. camemberti* include an acid carboxypeptidase, which is a serine enzyme with an optimum pH of 3.5, able to reduce the bitterness of a casein hydrolyzate by releasing hydrophobic amino acids (Ahiko, Iwasawa, Ulda, & Nigata, 1981) and an alkaline aminopeptidase, with a pH optimum of 8.0–8.5 (Matsuoka, Fuka, Kaminogawa, & Yamauchi, 1991).

The objective of the current study was to determine the action of *P. camemberti* proteinases on the caseins and to evaluate the extent of proteolysis occurring in Camembert cheese during ripening of 10 weeks. Peptides produced by action of *P. camemberti* proteinases and other enzymes during ripening were also identified.

2. MATERIAL AND METHODS

2.1. Sample

Whole Irish farmhouse Camembert cheeses, manufactured according to the traditional cheese-making procedure of the manufacturer were obtained frozen from the manufacturer (300 g) from three separate batches were at 2 days, molding (MLD) at 4 days, wrapping (WRP) at ~2, 4, 6, 8, and 10 weeks of ripening. These were thawed, crumbled and were mixed thoroughly, together with the rind.

2.2. Determination of the proteolytic specificity of mold proteinases on caseins

P. camemberti culture SWING® PC, (Chr. Hansen, Hørsholm, Denmark) used to manufacture the Camembert cheese were obtained in lyophilized condition from and stored at 4°C. The lyophilized mold spores (1 g) were hydrated in 10-ml sterile distilled water containing 0.01% Tween 80 (Sigma Aldrich Co, St. Louis, MO, USA) prior to inoculation. Inoculation was carried out aseptically in 1:1 suspension of 100-ml potato dextrose broth (Sigma Aldrich Co., St. Louis, MO, USA) (Le Dréan et al., 2010) and-100 ml (10%) reconstituted milk from low-heat skimmed milk (LHSM) powder (Kerry, Listowel, Ireland). The suspension was incubated in an orbital (shaking) incubator (Stuart Scientific, Stone, Staffordshire, United Kingdom) for 7 days at 25°C (Le Dréan et al., 2010) at speed of 100 rpm. On the seventh day of incubation, the entire mold biomass was centrifuged at 9,000 g for 45 min at 4ºC in a Sorvall RC5C plus centrifuge (Kendro Laboratory Products, Newtown, CT, USA). The filtrate obtained was filtered through Whatman paper number 113 and stored frozen in aliquots at −20°C. To determine the proteolytic specificity of *P. camemberti* aliquots were thawed and filtered for peptide analysis by UPLC which was a Waters Acquity UPLC H-Class Core System with a Waters Acquity UPLC TUV Detector (dual wavelength) and Acquity Column Heater 30-A, the system was interfaced with Empower 3 software (Waters Corp., Milford, MA, USA). The column used was an Acquity UPLC BEH C-18 column (Waters Corp., Milford, MA, USA) and Q-TOF LCMS analyzed on a quadrupole Orbitrap mass spectrometer (Q-Exactive, Thermo Scientific, Waltham, MA, USA) equipped with a reversed-phase NanoLC UltiMate 3000 HPLC system (Dionex LC Packings, now Thermo Scientific) as described below. Only peptides derived from the caseins were considered.

2.3. Compositional analysis and proteolysis

The pH of a cheese slurry (made from 25-g cheese and 50 g of deionized water) was determined using a calibrated pH meter. The same slurry was used to prepare pH 4.6-soluble and -insoluble fractions of cheeses at all time points as described by Kuchroo and Fox (1982). Compositional analysis of the cheeses comprised of determination of moisture content using an oven-drying method (International Dairy Federation [IDF], 1982). Protein

(N × 6.38) and nitrogen contents of the cheeses and of the pH 4.6-soluble extracts were determined by the macro-Kjeldahl method (IDF, 1986). Percentage fat was determined by the Gerber method (Institute for Industrial Research & Standards, 1955) and percentage of NaCl was measured by a titrimetric method using potentiometric end-point determination as described by Fox (1963). Experimental analysis were repeated in triplicate for each sample and data were analyzed using R© 16 (R version 3.4.0; the R foundation for Statistical Computing, University of Auckland, New Zealand). Differences in means between the samples were tested by (one-way ANOVA) analysis of variance at significance level, a, of .05 (*P* value ≤.05), throughout the study.

Proteolysis during ripening was studied by urea-polyacrylamide gel electrophoresis (urea-PAGE) of freeze-dried pH 4.6-insoluble fractions (Andrews, 1983; O'Mahony, Lucey, & McSweeney, 2005; Shalabi & Fox, 1987). The gels were stained with Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977) and destained by several distilled water washes.

Individual free amino acids (FAAs) were determined according to Fenelon and Guinee (2000). Frozen pH 4.6-soluble extracts of triplicate cheeses at 6 and 10 weeks of ripening were used for analysis. Samples were deproteinized prior to analysis by mixing equal volumes of 24% (w/v) trichloroacetic acid (TCA) to the soluble extracts.
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Peptides were resolved using ultra-performance liquid chromatography (UPLC) and mass spectroscopy (MS). Samples for UPLC were prepared from pH 4.6-soluble extracts by filtering through 0.22-μm cellulose acetate filters (Sartorious GmbH, Gottingen, Germany) and maintained at 4°C during analysis. Analysis was carried out as described by Mane, Ciocia, Beck, Lillenvang, and McSweeney (2019).

2.4. Peptide identification

For mass spectrometry (MS), casein hydrolysates and pH 4.6-soluble extracts from cheese were acidified by trifluoroacetic acid (TFA), desalted with C18 STAGE tips (Rappsilber, Mann, & Ishihama, 2007), and resuspended in 0.5% acetic acid (AA) in 2.5% acetonitrile (ACN). Peptide fractions were analyzed on a quadrupole Orbitrap mass spectrometer (Q-Exactive, Thermo Scientific, Waltham, MA, USA) equipped with a reversed-phase NanoLC UltiMate 3,000 HPLC system (Dionex LC Packings, now Thermo Scientific). Peptide samples were loaded onto a C18 reversed-phase column (10 cm length, 75 μm inner diameter) and eluted with a linear gradient from 2% to 97% ACN containing 0.5% AA in 60 min at a flow rate of 250 nl/min. The injection volume was 5 μL. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS2 acquisition. Survey full scan MS spectra (m/z 300–2,000) were acquired in the Orbitrap. The 12 most intense ions were sequentially isolated and fragmented by higher energy C-trap dissociation.

Raw data from MS was processed using MaxQuant version 1.5.5.1 (Cox & Mann, 2008; Tyanova, Temu, & Cox, 2016), incorporating the Andromeda search engine (Cox et al., 2011). To identify peptides and proteins, MS/MS spectra were matched to the custom database of bovine proteins previously identified in the milk proteome containing 1,059 entries. All searches were performed with unspecific digest. The database searches were performed with no fixed modification but with acetylation (protein N terminus) and oxidation (M) as variable modifications. Mass spectra were searched using the default setting of MaxQuant namely a false discovery rate of 1% on the peptide level. For the generation of ion intensities for peptide profiles, signals of corresponding peptides in different nano-HPLC MS/MS runs were matched by MaxQuant (Cox & Mann, 2008; Cox et al., 2011) applying a mass accuracy of at least 20 ppm and a maximum time window of 1 min. Perseus statistical software (version 1.5.5.3) was used to analyze the peptide ion current intensities (Tyanova, Temu, Sinitcyn, et al., 2016).

3. RESULTS AND DISCUSSION

3.1. Compositional analysis

The physicochemical composition of an Irish farmhouse Camembert cheese was determined during 10 weeks of ripening and pH, moisture, fat, salt (NaCl), protein and nitrogen contents in cheese and pH 4.6-soluble nitrogen (SN) as a percentage of total nitrogen (TN) (% pH 4.6-SN/TN) are reported in Table 1. Moisture levels (~55%–59%) showed no significant increase as ripening proceeded and values found are typical for this variety of cheese (Guizani et al., 2002; Khidr, 1995; Sousa & McSweeney, 2001). The % NaCl was almost same throughout ripening (1.66%–1.77%), with no significant increase; likewise there was no significant difference in % fat. pH values showed a significant increase over the 10-week ripening (4.9–7.3). The increase in pH during ripening results from metabolism of lactic acid, release of NH_3 due to deamination of free amino acids, both caused by action of the mold (Grippon, 1993). The increase was slow initially but pH of the interior rapidly equilibrated with the outer part after 30 days (Sousa & McSweeney, 2001). Crude protein and %N values of cheese showed a significant increase (12.66%–18.15% and 1.99%–2.84%, respectively). These values were similar to those reported earlier (Sousa & McSweeney, 2001) although the reason for the increase during ripening is unclear. Overall, the gross composition was observed to be typical of Camembert cheese (Guizani et al., 2002; Sousa & McSweeney, 2001).

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Table 1 Compositional analysis of Irish farmhouse Camembert cheese during ripening of 10 weeks

	pH	% moisture	% fat	% salt	% N (Cheese)
2 d	4.99 ± 0.09^{a}	55.44 ± 0.70^{a}	28.00 ± 0.50^{a}	1.73 ± 0.03^{ab}	1.98 ± 0.05^{a}
MLD	4.98 ± 0.07^{a}	58.05 ± 1.41^{a}	28.67 ± 1.04^{ab}	1.75 ± 0.01^{b}	2.14 ± 0.02^{ab}
WRP	5.06 ± 0.01^{a}	58.01 ± 0.59^{a}	28.17 ± 0.58^{a}	1.66 ± 0.07^{a}	2.09 ± 0.06^{a}
4 w	6.00 ± 0.14^{b}	55.95 ± 1.38^{a}	31.83 ± 0.29^{c}	1.77 ± 0.02^{b}	2.30 ± 0.03^{b}
6 w	6.33 ± 0.56^{b}	55.51 ± 1.27^{a}	29.50 ± 1.00^{ab}	1.74 ± 0.01^{ab}	2.59 ± 0.04^{c}
8 w	7.05 ± 0.11^{c}	56.08 ± 1.12^{a}	30.17 ± 0.58^{bc}	1.75 ± 0.01^{ab}	2.71 ± 0.09^{cd}
10 w	7.33 ± 0.04^{c}	59.45 ± 0.43^{b}	31.83 ± 0.29^{c}	1.75 ± 0.01^{ab}	2.84 ± 0.09^{d}

Note

Samples 2 days: (2d); Molding: (MLD); Wrapping: (WRP); 4 weeks: (4 w); 6 weeks: (6w); 8 weeks: (8w) and 10 weeks: (10w) of ripening, where %*N* = Nitrogen%, %P = protein% in Irish farmhouse Camembert cheese, total SN = pH 4.6-soluble nitrogen. (SN) as a percentage of total nitrogen, %FDM = fat in dry matter and % MNFS = moisture in nonfat solids, represented by mean ± standard deviation and different superscript letters represent a significant difference. ($p < .05$). Superscript letters in the same column show no significant difference at $p < .05$, where $n = 3$ for all the averages and *SD*.

3.2. Proteolysis

pH 4.6-SN/TN (%) is commonly used as an index of proteolysis and this fraction is mainly produced in Camembert by the action of chymosin, plasmin, lactocepins (LacCeps) and proteinases from the mold on the caseins (Gripon, Desmazeaud, Lebars, & Bergere, 1975; Guizani et al., 2002). Levels of pH 4.6-SN/TN increased from 12.90% to 25.64% (Table 1), indicating extensive proteolysis during ripening. Reported values from the current study were slightly higher than the values (~18%) of the outer part of the same Irish Camembert cheese studied at 4 weeks by Sousa and McSweeney (2001).

Proteolysis in Camembert cheese was also evaluated by urea-PAGE of freeze-dried pH 4.6-insoluble fractions (Figure 1). Extensive breakdown of α_{s1}- and β-casein was observed in electrophoretograms from 6 weeks onward, in agreement with earlier studies (Sousa & McSweeney, 2001). Breakdown of β-caseins into γ-caseins by the action of plasmin was observed on electrophoretograms of Camembert; plasmin action was favored by the high pH of the cheese (Figure 1; Gripon, 1993; Trieu-Cuot & Gripon, 1982). The significant increase in proteolysis begins at the surface of the cheese at MLD (day 6 of ripening) (Boutrou et al., 1999; Boutrou & Guéguen, 2005; Boutrou, Kerriou, & Gassi, 2006; Guizani et al., 2002), and is mainly associated with action of chymosin and plasmin up to 4 weeks and later also due to *P. camemberti* proteinases from 6 weeks onward.

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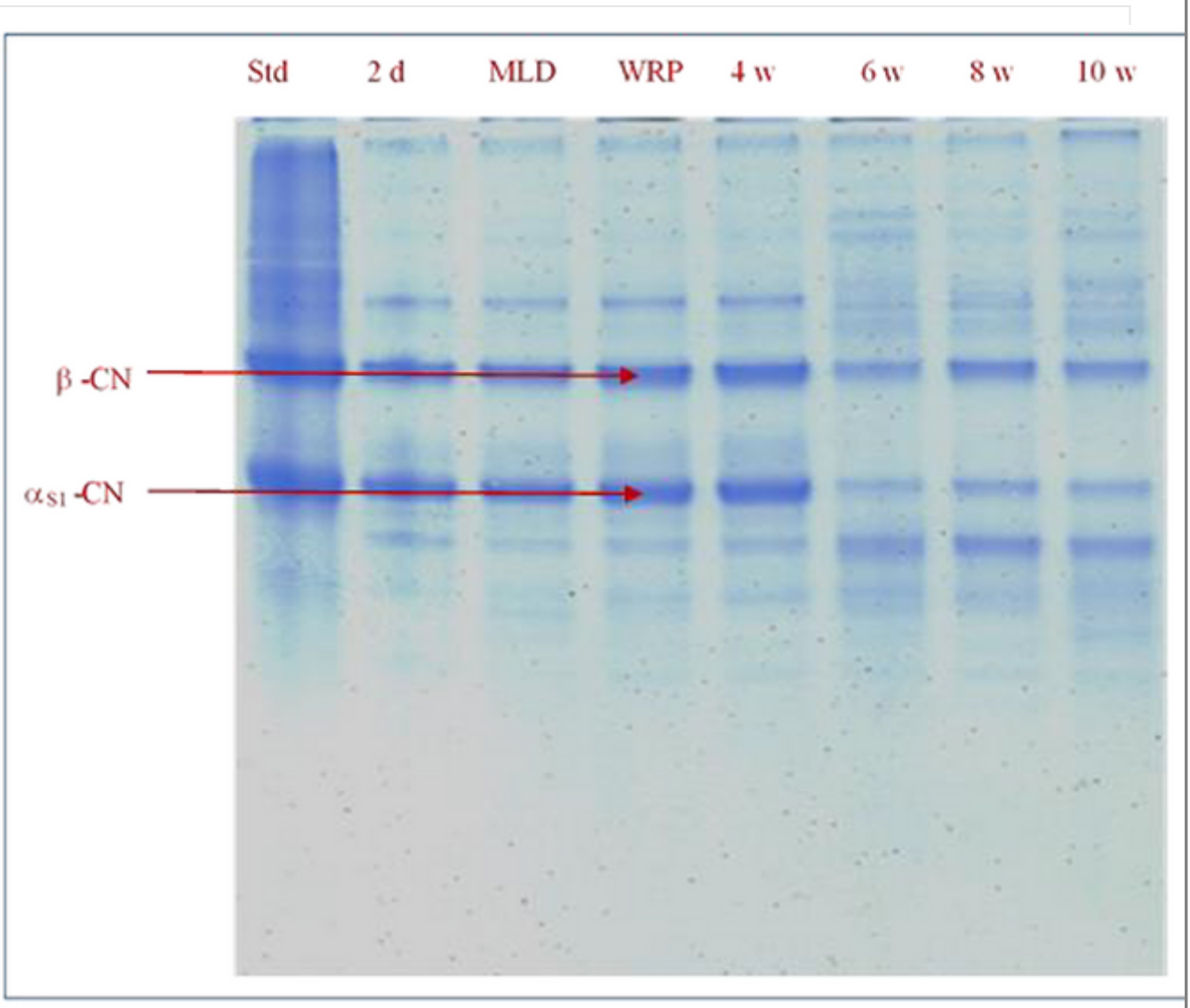

Fig. 1 Urea-polyacrylamide gel electrophoretograms of sodium caseinate: (Std) and pH 4.6-insoluble extracts of Irish farmhouse Camembert at 2 days: (2d); Molding

(MLD); Wrapping (WRP); 4 weeks: (4 w); 6 weeks: (6 w); 8 weeks: (8 w); and 10 weeks (10 w) of ripening

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Production of a large number peptides as a result of proteolysis was observed from the UPLC chromatograms of the pH 4.6-soluble extracts (Figure 2). The complexity of the chromatograms indicated a high number of peptides. Less complexity at later time points suggested degradation of peptides into very short peptides or free amino acids which have early elution times. Early eluting peptides (hydrophilic) at retention times from 2 to 30 min and 2 to 24 min for cheeses at 2 days, MLD and WRP, had the highest peak intensities compared to those 4 weeks onward. Peptides eluting later (hydrophobic) from 27 to 45 min were at higher concentration early in ripening (2 days, MLD and WRP) as compared to late ripening. The peptides were progressively hydrolyzed from 4 weeks and later time points of ripening, probably due to the combined effect of aspartyl and metalloproteinases. These observations were similar to those of an earlier study on the same variety of cheese (Sousa & McSweeney, 2001).

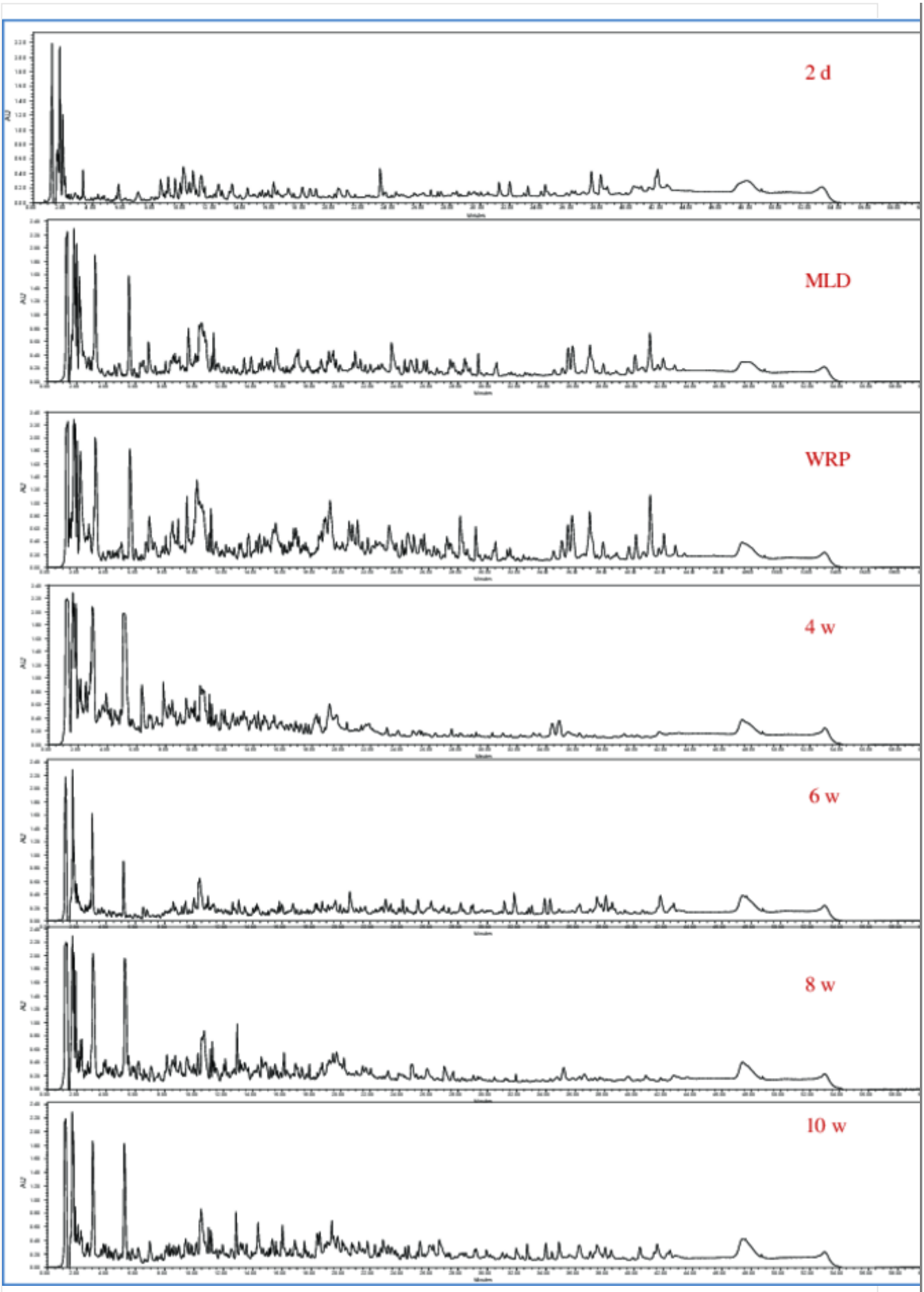

Fig. 2 UPLC (C_8 column) chromatograms of pH 4.6-soluble extracts of Irish farmhouse Camembert; 2 days: (2d); Molding (MLD); Wrapping (WRP); 4 weeks: (4 w); 6 weeks: (6w); 8 weeks: (8w); and 10 weeks (10w) of ripening, at wavelength of 214 nm

Total amino acid contents of 1997 and 3,048 μg/g of cheese were found at 6 and 10 weeks, respectively, indicating extensive proteolysis. Results of previous studies on Camembert (Boutrou et al., 1999, 2006; Boutrou & Guéguen, 2005; Guizani et al., 2002; Lee & Bae, 2018; Mei, Guo, Wu, Li, & Yu, 2015; Sousa & McSweeney, 2001) are in agreement with the concentrations of free amino acids found in the present work. Free amino acid profiles of pH 4.6-soluble extracts from cheeses are shown in Figure 3. Concentration of all individual amino acids was observed to be higher at 10 weeks than at 6 weeks. Amino acids with the highest concentration at 10 weeks were glutamic acid (406.16 μg/g) of cheese, lysine (355.72 μg/g), proline (273.29 μg/g), leucine (272.03 μg/g), valine (223.51 μg/g), phenylalanine (200.67 μg/g), and isoleucine (203.275 μg/g) of cheese. High concentrations of lysine, leucine, valine, phenylalanine, and proline have previously been found in Camembert (Kubiková & Grouch, 1998). High concentration of glutamic acid in Camembert and other mold ripened cheeses can be explained by its presence in high quantities in caseins or by production from α-ketoglutarate by action of aminotranferases (Vicente, Ibàñez, Barcina, & Barron, 2001). Results for Camembert were similar to the amino acid profiles of blue cheeses (Mane et al., 2019; Zarmpoutis, McSweeney, & Fox, 1997). The difference in the concentrations of amino acids between 6- and 10-week cheese samples indicated extensive proteolysis. Levels of free amino acids also tend to increase with ripening of Camembert cheese (Bergamini, Hynes, & Zalazar, 2006; Tejada, Abellan, Cayuela, Martinez-Cacha, & Fernandez-Salguero, 2008), which is in agreement with the current results.

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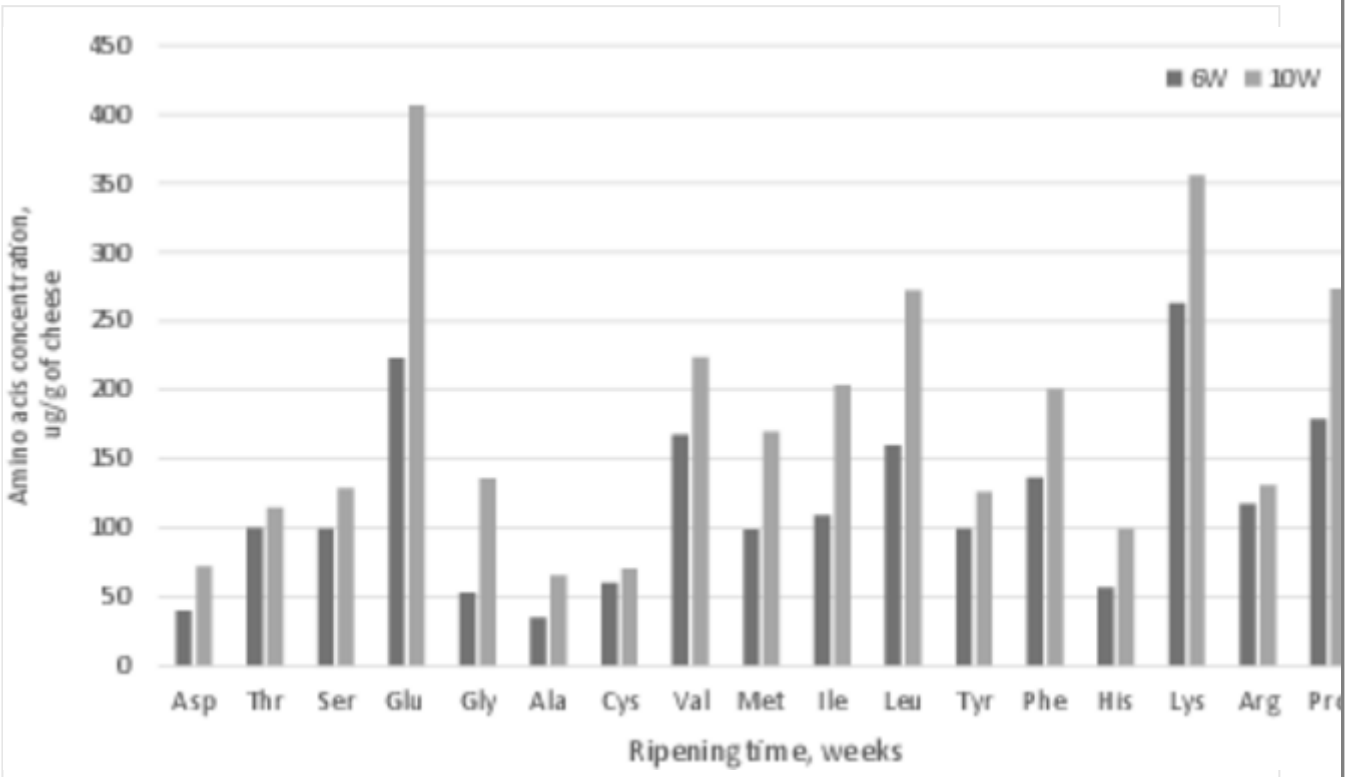

Fig. 3 Individual free amino acids contents of Irish farmhouse Camembert 6 weeks: (6w) and 10 weeks (10w) of ripening

3.3. Action of *Penicillium camemberti* enzymes on caseins

Proteolysis during ripening of Camembert cheese is catalyzed by chymosin, plasmin, starter proteinases, peptidases, and mold (aspartyl, metallo-) proteinases. Specificities of these enzymes, apart from mold proteinases, have been reviewed by Upadhyay, McSweeney,

Magboul, and Fox (2004) and Ardo, McSweeney, Magboul, Upadhyay, and Fox (2017). However, detailed specificities of proteinases from *Penicilium camemberti* (PC proteinases) on the caseins are unknown. Thus, an experiment was designed to determine where the mold enzymes cleave the caseins. Hydrolyzates produced from centrifuged culture filtrates (potato dextrose broth + 10% LHSMP) at the end of the growth, contained a large number of peptides produced by the proteolytic action of mold proteinases on the caseins (Figure 4); first 100 casein-derived peptides with the highest values of relative intensity from LCMS data were sorted into those from α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein and cleavage sites were plotted (Supplementary data Table S1). Peptides in the hydrolyzate not derived from the caseins were ignored.

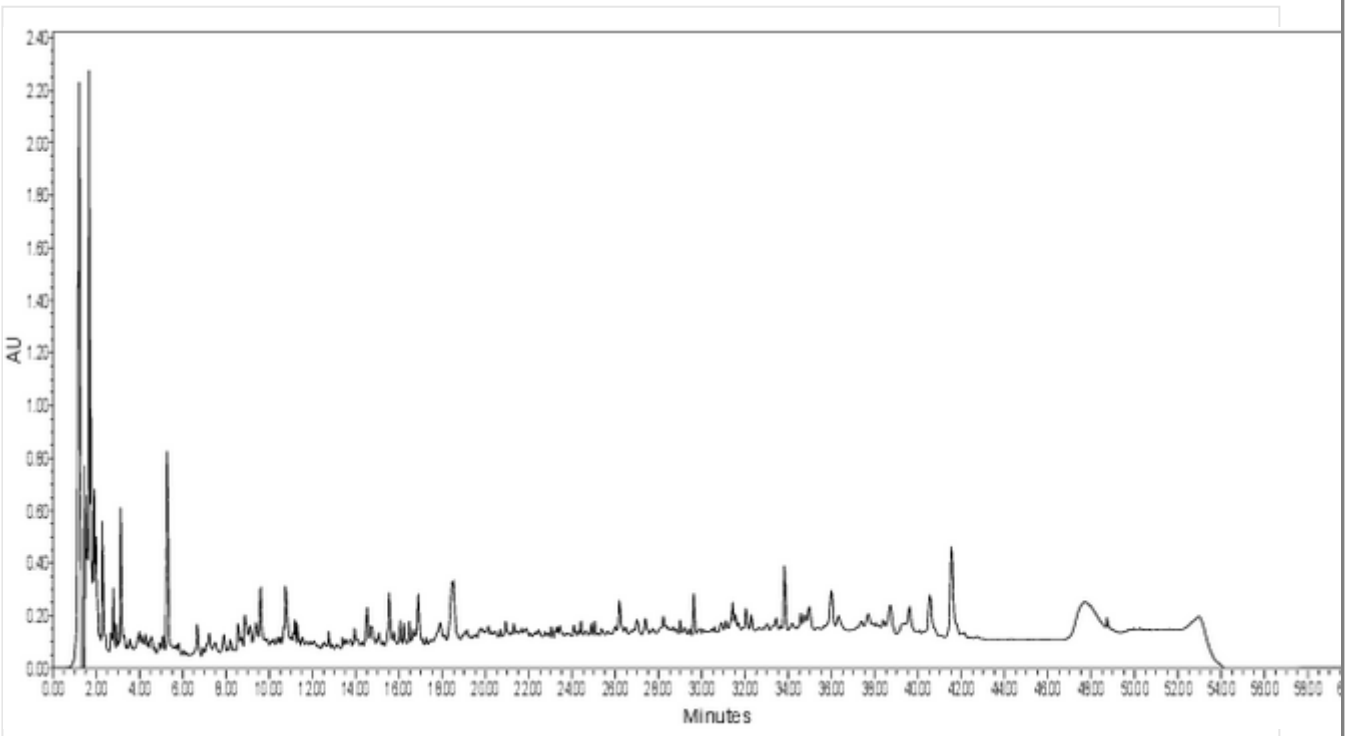

Fig. 4 Ultra-performance liquid chromatograms (C_8 column) peptide profiles of cell free supernatants of *Penicillium camemberti* strain PC in 1:1 ratio of 10% (low-heat skimmed milk powder) LHSMP and potato dextrose broth for 7 days

Certain regions of the caseins were found to be more susceptible to proteolysis. Regions that were most susceptible to the action of mold proteinases from the culture hydrolyzates in α_{s1}-casein were from residues 1–42, 79–96, and 101–145; in α_{s2}-casein were from residues 100–125; in β-casein were from residues 34–59, 110–130, and 145–204. Major cleavage sites in α_{s1}-casein produced by the action of PC proteinases were Glu_{30}–Val_{31}, Arg_{119}–Leu_{120}, Leu_{120}–His_{121}, His_{121}–Ser_{122}, and Phe_{179}–Ser_{180}. Cleavage sites common to action of both PC proteinases and chymosin (Ardo et al., 2017) in α_{s1}-casein (Figure 5a) were Phe_{23}–Phe_{24}, Phe_{24}–Val_{25}, Met_{54}–Glu_{55}, Lys_{79}–His_{80}, His_{80}–Ile_{81}, Lys_{103}–Tyr_{104}, Lys_{105}–Val_{106}, and Asp_{181}–Ile_{182}. Sites cleaved by the action lactocepins (Ardo et al., 2017) and PC proteinases were Gln_{13}–Glu_{14}, Leu_{16}–Asn_{17}, and Phe_{32}–Gly_{33}. Cleavage sites in common to action of PC proteinases, chymosin, and lactocepins (Fox & McSweeney, 1996) in α_{s1}-casein were Gln_{13}–Glu_{14}, Phe_{23}–Phe_{24}, and Phe_{32}–Gly_{33}.

Fig. 5 Click here to Correct

(a-d) The primary structure of bovine α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein showing the peptides from cell free supernatants of *Penicilium camemberti PC* incubated in 1:1 milk (10% low-heat skimmed milk powder) and potato dextrose broth suspension on shaking incubation for 7 days

In case of α_{s2}-casein (Figure 5b), PC proteinases cleaved at sites Leu_{99}–Tyr_{100}, Tyr_{100}–Gln_{101}, Gln_{101}–Gly_{102}, Arg_{114}–Asn_{115}, Pro_{118}–Ile_{119}, and Lys_{152}–Leu_{153}. These sites are not known to be cleaved by plasmin or lactocepin.

In β-casein (Figure 5c), PC proteinases cleaved at sites Gln_{34}–Ser_{35}, Gln_{46}–Asp_{47}, Lys_{107}–Glu_{108}, Pro_{110}–Phe_{111}, Trp_{143}–Met_{144}, Met_{144}–His_{145}, Lys_{169}–Val_{170}, Phe_{190}–Leu_{191}, Tyr_{193}–Gln_{194}, Gln_{194}–Glu_{195}, and Pro_{196}–Val_{197}. Plasmin (Ardo et al., 2017) and PC proteinases both cleaved at sites Lys_{107}–Glu_{108} and Arg_{183}–Asp_{184}. Trieu-Cuot, Archieri-Haze, and Gripon (1982) showed that PC proteinases hydrolyzed Lys_{97}–Val_{98}, Lys_{99}–Glu_{100}, and Lys_{29}–Ile_{30} faster than other bonds in β-casein; these cleavage sites were also identified in this study.

The only cleavage sites for PC proteinases in κ-casein (Figure 5d) were at Pro_{156}–Pro_{157} and Pro_{157}–Glu_{158}.

Action of PC proteinases produced peptide fragment α_{s1}-CN (f80-89) produced by cleavage at Lys_{79}–His_{80}; this peptide is known to have antioxidant activity (Table 2) (Baum, Fedorova, Ebner, Hoffmann, & Pischetsrieder, 2013). Likewise, peptide fragment α_{s2}-CN (f148–161) produced by cleavage at Phe_{147}–Thr_{148} is a bioactive peptide (Table 2) with angiotensin-converting enzyme (ACE) inhibitory activity, (Sistla, 2013).

Table 2 List of bioactive peptides in casein from pH 4.6-soluble extracts Irish farm-house Camembert cheese during 10 weeks of ripening compared to those reported in literature

Protein	Peptide sequence	Name	Source	Fragment	Reference
α_{s1}-CN	FPEVFGK	Casokinin-7. (ACE)	Milk	28–34	Sun and Jenssen (2012)
α_{s1}-CN	HIQKEDVPSER	Antioxidant	Milk	80–90	Baum et al. (2013)
α_{s2}-CN	TKKTKLTEEEKNRL	ACE	Milk	148–161	Sistla (2013)
β-CN	DKIHPF	ACE	Cheddar, Manchego	47–52	Gómez-Ruiz, Taborda, Amigo, Recio and Ramos (2006)
β-CN	KVLPVPQ	Antihypersensitive	Milk	169–175	Park (2009)

Protein	Peptide sequence	Name	Source	Fragment	Reference
β-CN	YQEPVLGPVRGPFPI	Casecidin-15. (antimicrobial)	Milk, Roquefort	192–206	Rizzello et al. (2005)

The action of proteinases from *P. roqueforti* PR-R (Mane et al., 2019) and *P. camemberti* (Supplementary Data Table S1) on the caseins was compared. Cleavages sites in common between enzymes from the mold species in α_{s1}-casein were Leu_{16}–Asn_{17}, Lys_{36}–Val_{37}, Glu_{63}–Pro_{64}, Ser_{88}–Glu_{89}, Tyr_{91}–Leu_{92}, Arg_{119}–Leu_{120}, His_{121}–Ser_{122}, Leu_{156}–Asp_{157}, and Lys_{193}–Thr_{194}. In case of β-casein, PR-R and PC proteinases both cleaved at sites Asn_{48}–Glu_{49}, Leu_{176}–Pro_{177}, Val_{183}–Tyr_{184}, and Tyr_{203}–Val_{204}.

3.4. Peptide identification in Irish farmhouse Camembert cheese

Identification of peptides produced during the 10-week ripening of Irish farmhouse Camembert in pH 4.6-soluble extracts was a major objective of the current study. Very little has been so far been reported in the literature about generation of peptides during the ripening of this particular variety of cheese, which is commercially very important.

A large number of peptides was produced during ripening (Supplementary Data Table S2). First 100 peptides with highest values of relative intensity, obtained from LC-MS data were considered at each time point and were then sorted into fragments from α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein (Supplementary Data Table S2). Peptides identified were compared with cleavage specificities of PC proteinases determined earlier and with known specificities of chymosin, plasmin, and lactocepins (Ardo et al., 2017; Breen, Fox, & McSweeney, 1995; Fernandez et al., 2005; Singh, Fox, & Healy, 1995; Singh, Fox, & Healy, 1997; Singh, Fox, Højrup, & Healy, 1994; Upadhyay et al., 2006). The peptides were plotted and cleavage sites noted (Figure 6a-d). A large number of peptides derived from α_{s1}-casein were observed as this protein was strongly degraded throughout cheese ripening.

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Fig. 6 Click here to Correct

(a-d) The primary structure of bovine α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein showing the peptides, showing the peptides derived from pH 4.6-soluble extracts, by action of PC proteinases, chymosin, plasmin, and cell envelope proteinases/lactocepins (LacCeps) from Irish farmhouse Camembert cheese, during 10 weeks of ripening

Major α_{s1}-casein (Figure 6a) fragments produced during ripening were derived from residues 1–40, 79–114, and 168–199 (Figure 6a). Certain regions in a casein were highly susceptible to enzymatic hydrolysis and contained common cleavage sites for PC–proteinases, chymosin, and lactocepins (Ardo et al., 2017). Major peptide fragments identified were α_{s1}-CN (f14–23), α_{s1}-CN (f17–23/24), α_{s1}-CN (f 24–34), and α_{s1}-CN (f 25–34). The common fragment, α_{s1}-CN (f1–23) produced by the action of chymosin (Fox & McSweeney, 1996), was absent at all stages of ripening. The reason for this is likely the action of cell envelope-associated proteinases or lactocepins and endopeptidases of starter and nonstarter bacteria, which can hydrolyze α_{s1}-CN(f1–23) rapidly (Figure 1) at the N-terminal bonds Gln_{13}–Glu_{14}, Glu_{14}–Val_{15}, Leu_{16}–Asn_{17},

Asn17–Glu18, Glu18–Asn19, and Arg22–Phe23 (Ardo et al., 2017). Peptides produced by action of lactocepins and/or chymosin at their N- or C-termini (Ardo et al., 2017) included αs1-CN (f30–38), αs1-CN (f31–37/38), αs1-CN (f33–40), αs1-CN (f154–164), αs1-CN (f165–193), αs1-CN (f169–189/190/197/198), αs1-CN (f170–189/190/192/198), and αs1-CN (f180–198/199). Lactocepin-derived peptide fragment αs1-CN (28–34), produced by cleavage at Pro27–Phe38, is a reported bioactive peptide, with casokinin 7-ACE inhibitory activity (Sun & Jenssen, 2012), and was found at all the time points of ripening.

The regions from which most peptides were derived in αs2-casein (Figure 6b), were from residues 42–79 and 97–116 (Figure 6b). Peptides produced during ripening were mainly due to the action of plasmin and lactocepins (Ardo et al., 2017). Fragments produced by the action of PC–proteinases, plasmin, and lactocepins at their N-termini were αs1-CN (f115–125) and αs2-CN (f115–126). Action of plasmin and lactocepins (Ardo et al., 2017) on αs2-casein helped to produce fragments αs2-CN (f80–88), αs2-CN (f150–161/162), αs2-CN (f151–156/158/161/162/163), and αs2-CN (f196–208), which were also found in Camembert at all stages of ripening (Table 2).

In case of β-casein, regions that were most susceptible to proteolysis were from residues 40–57, 101–125, 143–189, and 165–209 (Figure 6c). These susceptible regions of the casein resulted in one fragment, β-CN (f108–124) produced by cleavage at sites common to action of PC proteinases and plasmin (Ardo et al., 2017; Fox et al., 1994). Peptide fragments derived from action of PC proteinases and lactocepins at their N- or C-termini (Ardo et al., 2017) were β-CN (f47–52/55/56), β-CN (f191–204), and β-CN (f194–206). These peptide fragments were found from wrapping stage to end of the ripening.
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A bioactive peptide fragment derived from the action lactocepins at its N-terminus, β-CN (f192–206) (Table 2), produced by cleavage at Leu191–Leu192; this peptide possesses casecidin-15 antimicrobial activity (Rizzello et al., 2005). The peptide fragment was found at all the time points of ripening (Supplementary Data Table S2).

A small number of peptides from κ-caseins (Figure 6d), was found in low concentrations during ripening. Regions most susceptible to proteolysis were from residues 31–81 and 124–137 (Figure 6d). Fragments produced from pH 4.6-soluble extract, by action of lactocepins at their N- or C-terminus (Ardo et al., 2017) were κ-CN (f31–41), κ-CN (f60–80), κ-CN (f71–82), κ-CN (f72–84), and κ-CN (f102–111), produced by cleavage at Tyr30–Val31, Pro59–Tyr60, Pro70–Ala71, Ala71–Gln72, and Pro101–His102. κ-CN (f96–105) was produced by chymosin cleaving at its C-terminus (Phe105-Met106); (Ardo et al., 2017).

4. CONCLUSION

The current study investigated proteolysis with in Camembert cheese respect levels of pH 4.6-soluble nitrogen that increased during 10-week ripening. Composition and pH4.6-SN/TN showed a significant difference after 4 weeks of ripening indicating action of mold enzymes and extensive proteolysis. Urea-PAGE, UPLC, and LC-MS analyses provide qualitative and quantitative differences in cheese during 10-week ripening. Major cleavage sites of *P. camemberti* proteinases identified in αs1-casein were Glu30–Val31, Arg119–Leu120, Leu120–His121, His121–Ser122, Phe179–Ser180; in αs2-casein were Leu99–Tyr100, Tyr100–Gln101, Gln101–Gly102, Arg114–Asn115, Pro118–Ile119, and Lys152–Leu153, in β-casein were Gln34–Ser35, Gln46–Asp47, Lys107–Glu108, Pro110–Phe111, Trp143–Met144, Met144–His145, Lys169–Val170, Phe190–Leu191, Tyr193–Gln194, Gln194–Glu195, and Pro196–Val197; and in κ-caseins were Pro156–Pro157 and Pro157–Glu158. Peptides identified in pH 4.6-soluble extracts from Irish farmhouse Camembert cheese with the highest values of relative intensity were plotted and regions

most susceptible to action of chymosin, plasmin, lacocepins, and/or PC proteinases identified in α_{s1}-casein were 1–40, 79–114, and 168–199; in α_{s2}-casein were 42–79 and 97–116; in β-casein were 40–57, 101–125, 143–189, and 165–209; and in κ-casein were 31–81 and 124–137 were reported. The peptides were compared for matches with bioactive peptides. The overall study helps to understand and elucidate the process of ripening and action of enzymes in Camembert cheese.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Material

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