International Dairy Journal 97 (2019) 191–200

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Proteolysis in Danish blue cheese during ripening

A. Mane [a], F. Ciocia [a], T.K. Beck [b], S.K. Lillevang [b], P.L.H. McSweeney [a, *]

[a] Food for Health Ireland, School of Food and Nutritional Sciences, University College Cork, T12 Y337, Cork, Ireland
[b] Arla Foods, Amba, Bregnehøjvej 16, 6500, Vojens, Denmark

ARTICLE INFO

Article history:
Received 21 September 2018
Received in revised form
28 May 2019
Accepted 28 May 2019
Available online 12 June 2019

ABSTRACT

Proteolysis in Danish blue cheese was studied during 9 weeks' ripening. Levels of pH 4.6-soluble N as a percentage of total N increased from 7.2% to 25%, indicating extensive proteolysis. Urea-polyacrylamide gel electrophoretograms confirmed the extent of proteolysis through chymosin and plasmin action early in ripening, but later the action of *Penicillium roqueforti* proteinases became apparent. The proteolytic specificity of *Penicillium roqueforti* PR-R proteinases on α_{S1}- and β-casein was determined in a model system. Regions most susceptible to proteinase action in α_{S1}-casein were 6–40, 69–99, 124–147 and 155–199, with a total of 91 cleavage sites identified; regions in β-casein susceptible to proteolysis were 43–87, 101–119, 161–185 and 192–209 with a total of 118 cleavage sites identified. A large number of peptides was identified cheese extracts during 9 weeks ripening, principally from α_{S1}-casein regions 1–40, 105–136 and 150–176 and β-casein regions 6–14, 46–68, 101–140 and 193–209.

1. Introduction

Danish blue is a semi-soft cheese variety with a white-to-yellowish curd and a thin edible rind. The cheese is ripened at ~10 °C and a relative humidity 85–90% (Kinsella & Hwang, 1976b). It has a fat content of 25–30% (~50–60% in dry matter) and is aged for eight to twelve weeks (EU, 2017). The principal biochemical changes during the ripening of Danish Blue and related varieties involve proteolysis, lipolysis and metabolism of residual lactose and of lactate and citrate. The aroma of blue cheese is dominated by *n*-methyl ketones, produced from fatty acids via the first four steps of β-oxidation brought about by *Penicillium roqueforti* (Kinsella & Hwang, 1976b). Reduced firmness/hardness of cheese texture (softening) generally occurs as ripening progresses due to proteolysis and de-acidification.

Proteolysis during cheese ripening has been an area of great interest and thus has been extensively reviewed (Ardo, McSweeney, Maghboul, Upadhayay, & Fox, 2017; Fox & McSweeney, 1996; Fox, Singh, & McSweeney, 1995; Sousa, Ardo, & McSweeney, 2001; Upadhyay, McSweeney, Maghboul, & Fox, 2004). Blue cheese undergoes extensive proteolysis during ripening (Gripon, Desmazeaud, Le Bars, & Bergère, 1977; Hewedi & Fox, 1984; Kinsella & Hwang, 1976a). An inter-varietal comparison of blue cheese was conducted by Zarmpoutis, McSweeney, and Fox (1997) where these cheeses exhibited much more extensive degradation of the caseins than in Cheddar.

Proteolytic enzymes (proteinases and peptidases) from starter cultures and the mould degrade the caseins extensively, thereby causing textural changes and aroma development by producing precursor compounds (peptides and amino acids) for further metabolism (Ardo, McSweeney, Magboul, Upadhyay, & Fox, 2017; Sousa, Ardö, & McSweeney, 2001). The extensive proteolysis in Danish blue cheese is largely associated with the principal secondary microorganism, *Penicilium roqueforti*, through the action of extra- and intracellular enzymes, including metalloproteinases, aspartic-proteinases, aminopeptidases, and acid carboxypeptidases (Madkor, Fox, Shalabi, & Metwalli, 1987; Zarmpoutis, McSweeney, Beechinor, & Fox, 1996; Zarmpoutis et al., 1997). Aspartyl and metalloproteinases act specifically on α_{S1}- and β-caseins and have been characterised (Gripon, 1993). Maximum protease activity in the mycelium and in the extracellular medium occurs when the mycelium has attained full growth (Cantor, van den Tempel, Hansen, & Ardo, 2004). The activity of these enzymes (mould aspartyl- and metalloproteases) is maximum in blue cheese at the time of sporulation of the mould (Cantor, van den Tempel, Hansen, & Ardö, 2004), which is around ~4 w in case of Danish blue cheese. The metalloprotease is active from pH 4.5 to 8.5, and aspartyl proteases ranges from 3.5 to 6.5 (Trieu-Cuot, Archieri-Haze, & Gripon, 1982b) which corresponds to the pH of Danish blue cheese (pH 4.8–5.8) during ripening (Zarmpoutis et al., 1997). The

* Corresponding author. Tel.: +353 21 490 2011.
E-mail address: p.mcsweeney@ucc.ie (P.L.H. McSweeney).

https://doi.org/10.1016/j.idairyj.2019.05.017
0958-6946/

metalloprotease and aspartyl proteases have broad specificity and hydrolyse both α_{S1}- and β-caseins. The metalloprotease cleaves β-casein at Pro_{90}-Glu_{91}, which is not often hydrolysed by proteases because of its proline residue, and also at Lys_{28}-Lys_{29}, which is also cleaved by plasmin (Le Bars & Gripon, 1981; Trieu-Cuot et al., 1982b; Trieu-Cuot & Gripon, 1983). Aspartyl proteases hydrolyse the caseins only into high molecular weight peptides including α_{S1}-CN (f24–199) produced by cleavage at Phe_{23}–Phe_{24} in α_{S1}-casein which is also the major chymosin cleavage site in this protein (Ardo et al., 2017; Trieu-Cuot, Archieri-Haze, & Gripon, 1982a). Aspartyl proteases produce high molecular weight peptides by cleaving β-caseins at Arg_1-Lys_{29}, Lys_{29}-Val_{209}, Lys_{97}-Val_{209} (Le Bars & Gripon, 1981; Trieu-Cuot et al., 1982b; Trieu-Cuot & Gripon, 1983). However, the full and precise action of mould enzymes on the caseins remain to elucidated.

The objective of this study was to track the extensive proteolysis occurring during the ripening of Danish Blue cheese for 9 weeks. The cleavage sites of the proteinases of *Penicillium roqueforti* on the major caseins were also determined.

2. Materials and methods

2.1. Cheese manufacture

Samples of commercial Danish Blue cheese were obtained up to 9 weeks of ripening from Arla Foods, Vojens, Denmark. The cheeses were manufactured in three independent batches ~2–3 days apart. Milk was pasteurised at 74 °C for 15 s, $CaCl_2$ and mould culture (*Penicillium roqueforti* strain PR-R) were added post pasteurisation followed by addition of mesophilic starter culture to initiate acidification. Rennet was added and the coagulated curd was cut and stirred to release whey. The pH was monitored for 24 h after the addition of starter culture. Cheese curd was then filled into moulds with a diameter of 20 cm. The curds were turned 4 times in 24 h. The cheeses were ripened at standard ripening conditions for blue cheese and were sampled at day 0 [out of salt (OOS)], 2 weeks, 4 weeks, 7 weeks and 9 weeks. Samples were kept frozen at −20 °C until analysis.

2.2. Sample preparation and compositional analysis

The frozen wedges (~1.5–2 kg) with a very thin rind were thawed, crumbled and mixed thoroughly. The moisture content of the cheeses was determined using an oven-drying method (IDF, 1982). A calibrated pH meter was used to measure the pH of cheese slurries made from 30 g cheese and 60 g of deionised water. pH 4.6-soluble and -insoluble fractions of cheeses at all time points were prepared as described by Kuchroo and Fox (1982). Crude protein and nitrogen contents of the cheeses and of pH 4.6-soluble extracts were determined by the macro-Kjeldahl method (crude protein is reported as N × 6.38; IDF, 1986), percentage fat was determined by the Gerber method (IIRS, 1955).

2.3. Analysis of proteolysis

Proteolytic breakdown was studied by urea-polyacrylamide electrophoresis (urea-PAGE) of freeze-dried pH 4.6-insoluble fractions (Andrews, 1983; O'Mahony, Lucey, & McSweeney, 2005; Shalabi & Fox, 1987). Gels were stained using Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977) and de-stained by several distilled water washes.

Individual free amino acid (FAA) contents were determined according to Fenelon and Guinee (2000) from frozen pH 4.6-soluble extracts from cheeses at 7 w and 9 w of ripening. Samples were first de-proteinised by mixing equal volumes of 24% (v/v) trichloroacetic acid (TCA).

Peptide profiling was done using ultra-performance liquid chromatography (UPLC) and mass spectroscopy (MS). For UPLC samples were prepared by filtering pH 4.6-soluble extracts through 0.22 μm cellulose acetate filters (Sartorious GmbH, Gottingen, Germany) and maintained at 4 °C during analysis. Peptide profiles were determined using 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.). Elution was monitored at 214 nm with a mobile phase of two solvents A: 0.1% (v v^{-1}) formic acid (Sigma–Aldrich, Darmstadt, Germany) in Milli-Q water and B: 0.1% (v v^{-1}) formic acid in acetonitrile (Sigma–Aldrich) with a flow rate of 0.2 mL min^{-1} over run time of 60 min.

Peptides in pH4.6-soluble extracts were determined by LC-MS using a Waters Acquity G2 Q-TOF LC-MS, model XEVO-G2QTOF#YBA051, coupled to a Waters Acquity UPLC. Samples were filtered through 0.22 μm cellulose acetate filters and a volume of 10 μL was injected into a Waters Acquity UPLC H-Class Core System. Samples were eluted at 214 nm using a mobile phase composed of two solvents. Solvent A was 0.1% (v/v) formic acid in Milli-Q water and Solvent B was 0.1% (v/v) formic acid in acetonitrile. Analysis was performed at a capillary source temperature of 120 °C, de-solvation temperature at 450 °C and spray voltage of 3.0 kV. Samples were analysed in the resolution mode with a mass range from 50 to 2000 Da. Samples and solvents were pumped through a Waters Acquity SDS pump with high pressure limit of 1031.24 bar at a flow rate of 0.2 mL min^{-1}, passing through Acquity UPLC BEH C-18 column. Detection of peptides was carried out by Waters Acquity UPLC LG, using a photodiode array detector (PDA). Elution was using the solvents described above but with a gradient of 97% solvent A, 3% solvent B at 0.0 min; 60% A, 40% B at 47 min; 15% A, 85% B up to 51 min and 97% A, 3% B up to end of the run, with a flow rate of 0.20 mL min^{-1}.

Raw data acquired were processed by Mass Lynx v4.1 software also used to control the instrument during the sample runs and compared through Protein Lynx Global Server (PLGS) software v2.4 (Waters Corp., Milford, MA, USA) for running comparative sequence database searches for bovine casein α_{S1}-casein and β-casein. Data obtained from LC-MS was compared with the peak area values from UPLC analysis by setting the PDA detector (LCMS) at a bandwidth of 1.2 nm resolution and a 3D channel, to provide a high quality spectra and best mimic the dual tuneable UV/Vis detector (TUV) detector on the basis of retention time values as both the techniques were run with similar instrumental settings.

2.4. Determination of the specificity of *Penicillium roqueforti* PR-R on the caseins

Penicillium roqueforti PR-R, the strain used to make cheese, was obtained as an inoculum concentrate and was stored at 4 °C. The inoculum (1 g) was hydrated in 10 mL distilled water containing 0.01% Tween 80 (Sigma, France) prior to inoculation 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 milk from 10% low heat skimmed milk (LHSM) powder (Kerry, Listowel, Ireland). This suspension was incubated in an orbital incubator (Stuart Scientific, Staffordshire, UK) for 7 days at 25 °C (Le Dréan et al., 2010) at speed of 100 rev min^{-1}. When grown, the entire mycelial biomass was centrifuged at 9000× *g* for 45 min at 4 × C in Sorvall centrifuge. The filtrate was filtered through Whatman paper number 113. The filtrate was stored frozen in aliquots at −20 °C.

Peptides derived from the major caseins present in this hydrolysate were identified by Q-ToF LCMS as described above.

2.5. Statistical analysis

Each experimental analysis was repeated in triplicate for each cheese sample. All statistical analyses were performed using R® 16 (R version 3.4.0; the R Foundation for Statistical Computing, University of Auckland, Auckland, New Zealand). Differences in means between batches and ripening time points were tested by analysis of variance (one way-ANOVA) at significance level, α, of 0.05 ($P \leq 0.05$), throughout the study.

3. Results and discussion

3.1. Compositional analysis of Danish blue cheese

The physico-chemical composition of Danish blue cheese is reported and values for pH, moisture, fat, salt, protein and nitrogen in cheese, moisture in non-fat solids, fat in dry matter and pH 4.6-soluble nitrogen (SN) as a percentage of total nitrogen (total SN), are shown in Table 1. Moisture levels ranged between 43.2 and 47.6%. Moisture in non-fat solids (MNFS) increased from 61.8% to 69% during ripening. Fat content was from 29.0% to 32.6%, with no significant change except at the last time point of ripening (9 w). Fat-in-dry matter (FDM) ranged from 51.7% to 59.7%, with no significant difference. Values of moisture, fat, MNFS and FDM were similar to the blue cheeses previously reported in the literature (Diezhandino, Fernández, González, McSweeney, & Fresno, 2015; Wolf, Perotti, & Zalazar, 2010; Zarmpoutis et al., 1997). Crude protein content (% N × 6.38) remained around 18.0% up to 7 weeks and significantly increased to 24% by 9 weeks of ripening. Total nitrogen in cheese ranged between 2.83% and 3.78% during the 9 week ripening. The pH increased significantly from 4.87 to ~5.7–5.8 during ripening. Increase in pH results from lactate metabolism resulting in production of CO_2 and H_2O (Fox, Lucey, & Cogan, 1990; Gori, Ryssel, Arneborg, & Jespersen, 2012).

3.2. Proteolysis

Levels of pH 4.6-soluble nitrogen as a percentage of total nitrogen (pH4.6-SN/TN) in Danish blue cheeses during ripening increased significantly from 7.6% to 29.9% (Table 1), indicating an increased extent of proteolysis up to 9 w of ripening. Values were low for the OOS and 2 w cheeses where the mould growth was low to moderate, highest values were found at the fourth week and later, reflecting the growth of moulds from 2 to 5 weeks. The marked increase in proteolysis in Danish blue cheese after 4 w reflects the extensive proteolytic action of *Penicillium roqueforti*, which appeared around that stage of ripening (Trieu-Cuot & Gripon, 1983; Zarmpoutis et al., 1996). The value (29.9%) found at 9 w, (age at which Danish blue cheese is commercially packed) in this study was comparable with other samples of commercial ready blue cheeses including, the Irish blue cheese, Chetwynd (~32%–36%; Zarmpoutis, McSweeney, Beechinor, & Fox, 1996), but were lower than the values reported for Gorgonzola and Stilton cheeses (45%–55%; Zarmpoutis et al., 1996).

Proteolysis in Danish blue cheese was also evaluated by urea-PAGE of freeze-dried pH 4.6-insoluble fractions as shown in Fig. 1. Electrophoretograms showed extensive degradation of α_{S1}- and β-casein from 4 w and onwards, with initial breakdown of β-casein was by the action of plasmin forming the γ-caseins (Zarmpoutis et al., 1997) followed by high rate of proteolysis by the action of mould enzymes. A significant increase in proteolysis was observed at 4 w and later in the present study, when the mould had become visible (Gripon et al., 1977; Le Bars & Gripon, 1981). Greater numbers of peptides were generated at 4 w and later stages of ripening and reflected the development of *Penicillium roqueforti* and its proteinases in the cheeses.

A high number of peptide fragments were generated during ripening as can be observed in the UPLC chromatograms (Fig. 2) of pH 4.6-soluble extracts of OOS, 4 w and 9 w-old cheeses. A clear increase in complexity of the peptide profiles was seen at 4 w and 9 w, as compared with OOS. Peaks with highest intensity were observed from retention times 2–10 min in all chromatograms. During ripening, the number (both hydrophobic and hydrophilic) and size of peaks increased from the early (OOS) to intermediate (4 w) to end of ripening (9 w). Many of the later-eluting peptides appeared (36–43 min retention time) to be hydrolysed progressively after 4 w of ripening. A large number of peptides (Fig. 2) was produced during ripening. The highest number of peptide peaks were observed at 4 w of ripening and coincided with the growth of *Penicillium roqueforti*; its enzymes are very active at the pH of this cheese (~4.8–5.8; Cantor et al., 2004). As observed in other blue-veined cheese varieties like Gorgonzola and Silton (Zarmpoutis et al., 1997), chromatograms point towards extensive proteolysis during of ripening.

pH 4.6-soluble extracts from cheeses at 7 w and 9 w (Fig. 3) contained 20.57 and 35.59 μmol g^{-1} cheese of total FAAs, respectively. Concentration of individual amino acids at 9 w, were (μmol g^{-1} cheese): glutamic acid, 5.41; leucine, 4.84; lysine 4.12. Histidine and valine also had comparatively higher values at 9 w (2.67 μmol g^{-1} cheese) than those at 7 w (2.41 μmol g^{-1} cheese), as shown in Fig. 3. A high content of glutamic acid, could be explained by its presence in high amounts in the caseins or perhaps formation from α-ketoglutarate that acts as a co-substrate for aminotransferases (Vincente, Ibàñez, Barcina & Barron, 2001). High concentrations of leucine (4.84 μmol g^{-1} cheese), phenylalanine (2.3 μmol g^{-1} cheese) and valine (4.12 μmol g^{-1} cheese) at 9 w suggested preferential cleavage of peptide bonds involving hydrophobic residues also observed in studies of Zarmpoutis et al. (1997) and Diana, Rafecas, Arco, and Quilez (2014) on blue cheese. The lower concentration of certain FAAs, e.g., cysteine and tryptophan,

Table 1
Compositional analysis of Danish blue cheese during ripening time of 9 weeks.[a]

Ripening time	pH	Moisture (%)	N (%)	N × 6.38 (%)	pH4.6-SN(%)	MNFS(%)	FDM(%)
OOS	4.87 ± 0.23^{a}	43.91 ± 0.89^{a}	3.01 ± 0.06^{a}	18.77 ± 0.38 ^{ab}	9.67 ± 0.14^{a}	61.84 ± 1.16^{a}	51.71 ± 0.45^{a}
2 w	5.11 ± 0.34^{b}	45.24 ± 0.86^{ab}	2.82 ± 0.08^{a}	18.00 ± 0.50^{a}	7.57 ± 0.12^{a}	63.72 ± 1.17^{a}	52.96 ± 1.06^{a}
4 w	5.82 ± 0.41^{e}	47.62 ± 0.45^{b}	2.89 ± 0.15^{a}	18.45 ± 0.97^{ab}	28.83 ± 1.33^{c}	69.03 ± 0.24^{a}	59.19 ± 1.23^{a}
7w	5.36 ± 0.27^{c}	43.2 ± 2.65^{a}	3.33a±0.10^{b}	21.25 ± 0.66^{bd}	21.79 ± 0.46^{b}	62.30 ± 2.50^{a}	54.03 ± 1.46^{ab}
9 w	5.69 ± 0.25^{d}	45.13 ± 1.39^{ab}	3.78 ± 0.37^{b}	24.14 ± 2.38 ^{de}	29.94 ± 2.82^{c}	67.12 ± 1.55^{a}	59.65 ± 1.25^{b}

[a] Abbreviations are: OOS, samples out of salt (time zero); 2 w, 2 weeks; 4 w, 4 weeks; 7 w, 7 weeks; 9 w, 9 weeks; N, nitrogen; N × 6.38, crude protein in blue cheese; pH4.6-SN, pH 4.6-soluble nitrogen as a percentage of total nitrogen; FDM, fat in dry matter; MNFS, moisture in non-fat solids. Values are means ± standard deviation of three cheese-making batches of Danish blue cheese; different superscript letters indicate significant difference ($P < 0.05$).

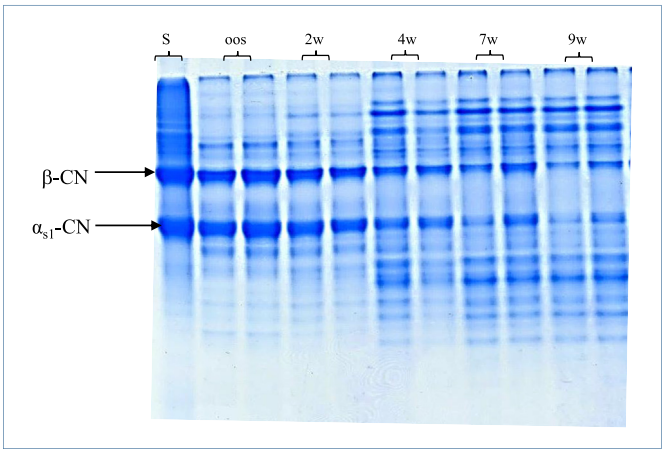

Fig. 1. Urea polyacrylamide gel electrophoretograms of pH 4.6-insoluble nitrogen fraction of Danish blue cheese at different ripening periods: S, sodium caseinate; other lanes show pH 4.6-insoluble nitrogen fraction separated from cheese ripened for 0 weeks (OOS, out of salt), 2 weeks (2 w), 4 weeks (4 w), 7 weeks (7 w), 9 weeks (9 w) showing the breakdown of the caseins indicating extensive proteolysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

is likely due to their presence in low quantities in regions of caseins extensively degraded (Eren-Vapur & Ozcan, 2012; Ganesan & Weimer, 2007; Lauer & Baker, 1977). Levels of FFAs were comparable with those found in studies on other blue cheese varieties like Gorgonzola and Stilton (Zarmpoutis et al., 1997).

3.3. Action of enzymes from *Penicillium roqueforti* PR-R on the caseins

The enzymes that catalyse proteolysis during the ripening of Danish blue cheeses are the coagulant, plasmin, mould proteinases and various starter proteinases and peptidases. The specificity of chymosin, plasmin and lactococcal proteinases (lactocepins) on the caseins is known (Ardo et al., 2017; Upadhyay, McSweeney, Magboul, & Fox, 2004), but the specificity of aspartyl and metallo-proteinases from *Penicillium roqueforti* on the caseins remains to be determined in detail. Thus, an experiment was designed to determine the exact specificities of mould proteinases on the caseins. Complexity of the hydrolysate was apparent from UPLC chromatograms (Fig. 4). Casein-derived peptides identified in the PR-R hydrolysate were used to map the cleavage sites (Fig. 5A, B). Only peptides with highest LCMS relative intensity values for α_{S1}-casein and β-casein (Supplementary material, Table S1) were used to determine the cleavage sites. N- and C- termini of the peptide fragments along with their sequence are listed in Supplementary material, Table S1. The peptides produced in the PR-R hydrolysates were compared with peptides known to be produced in hydrolysates by action of chymosin, plasmin and lactocepins.

Certain regions of α_{S1}-casein (residues 5–40, 69–99, 124–147 and 155–199) contained multiple cleavage sites and were cleaved extensively by PR-R proteinases. The major cleavage sites of PR-R on α_{S1}-casein were Pro_5–Ile_6, Lys_7–His_8, Leu_{11}–Pro_{12}, Glu_{14}–Val_{15}, Phe_{23}–Phe_{24}, Phe_{24}–Val_{25}, Glu_{30}–Val_{31}, His_{79}–Ile_{80}, Gly_{126}–Ile_{127}, Met_{135}–Ile_{136}, Gln_{155}–Leu_{156}, Leu_{157}–Asp_{158}, Trp_{164}–Tyr_{165}, Asn_{184}–Pro_{185}, Asn_{190}–Ser_{191} and Met_{196}–Pro_{197}. A major chymosin-derived peptide α_{S1}-CN(f1–23) (Fox & McSweeney, 1996) produced by cleavage of Phe_{23}–Phe_{24}, was absent from the hydrolysate in this study, as it could have been hydrolysed quickly into smaller fragments. Also mould aspartyl proteases (Claverie-Martin & Vega-Hernandez, 2007) showed similar specificity to that of chymosin (Ardo et al., 2017; Breen, Fox, & McSweeney, 1995; Fernandez, Singh, & Fox, 1998; Singh, Fox, Højrup, & Healy, 1994; Singh, Fox, & Healy, 1995, 1997; Upadhyay et al., 2004) and peptides α_{S1}-CN (f6–13/14/15) and α_{s1}-CN (f8–14) were produced in the PR-R hydrolysates by cleavage at the bonds Gln_{13}–Glu_{14}, Glu_{14}–Val_{15} and Leu_{16}–Asn_{17}; these sites are also cleaved by chymosin activity (McSweeney, Pochet, Fox, & Healy, 1994). Similarly, PR-R proteinases cleaved bonds at Pro_5–Ile_6, Lys_7–His_8, Phe_{23}–Phe_{24}, Ser_{66}–Ser_{67} and Ala_{175}–Pro_{176}, which were also cleaved by cell-envelope associated proteinases or lactocepins (Breen et al., 1995; Fernandez et al., 1998; Singh et al., 1994, 1995, 1997). Sites Glu_{84}–Asp_{85} and Ile_{81}–Glu_{82}, are also susceptible to the action of both chymosin and lactocepins and PR-R proteinases (Supplementary material Table S1). The N terminal fragment α_{S1}-CN(f165–189), produced by cleavage at Ala_{164}–Trp_{165} which is a major cleavage site of PR-R proteinases on α_{S1}-casein; this site is also cleaved by the action of chymosin and lactocepins (Fox & McSweeney, 1996). The peptide α_{S1}-CN(f157–164), which was identified in the PR-R hydrolysate, has ACE inhibitory action and is a notable bioactive peptide (Sanchez-Rivera et al., 2014) and was found in the PR-R hydrolysate.

In case of β-casein, regions 47–67, 101–119, 161–185 and 192–209 were cleaved extensively by PR-R proteinases. The cleavage sites of PR-R identified in β-casein were Glu_5–Leu_6, Glu_{14}–Ser_{15}, Ile_{66}–His_{67}, Glu_{100}–Ala_{101}, Phe_{119}–Thr_{120}, Asn_{132}–Leu_{133}, Thr_{154}–Val_{155}, Ser_{168}–Lys_{169}, Lys_{176}–Ala_{177}, Pro_{186}–Ile_{187}, Tyr_{193}–Ile_{194} and Ile_{207}–Ile_{208}. Certain cleavage sites identified in β-casein in the PR-R hydrolysate were the same as those of plasmin (Ardö, Lilbæk, Kristiansen, Zakora, & Otte, 2007; Ardö, Pripp, & Lillevang, 2009; Rehn, Petersen, Hallin Saedén, & Ardö, 2010) as follows: Lys_{97}–Val_{98}, Lys_{105}–His_{106}, Lys_{113}–Tyr_{114}, Lys_{169}–Val_{170}, Lys_{176}–Ala_{177} and Arg_{202}–Gly_{203}. The bonds Thr_{78}–Gln_{79}, Thr_{154}–Val_{155}, Ser_{164}–Leu_{165}, Leu_{191}–Leu_{192} and Pro_{200}–Val_{201} cleaved by PR-R proteinases were also cleaved by action of lactocepins (Ardo et al., 2017), producing peptide fragments β-CN(f79–85), β-CN(f155–161), β-CN(f165–168/169/170), β-CN(f165–176) and β-CN(f192–197), β-CN(f193–197), β-CN(f201–207). It was observed that PR-R proteinases produced a number of fragments by cleavage at sites containing proline (Gly_{203}–Pro_{204}, Tyr_{180}–Pro_{181}, Ala_{103}–Pro_{104}, Leu_{137}–Pro_{138}) or isoleucine (Ile_{49}–His_{50}, Ile_{66}–His_{67}, Ile_{207}–Ile_{208}) as a part of scissile bond, which was also noted earlier (Cunningham & O'Connor, 1997; Fox & McSweeney, 1996). β-Casein peptide fragments in PR-R hydrolysates with reported bioactivity included opioid peptide fragment β-CN(f60–66), produced by cleavage at Val_{59}–Tyr_{60}, β-casomorphin-7 peptide fragment β-CN(f114–119) produced by cleavage at Lys_{113}–Tyr_{114} and angiotensin I-converting enzyme (ACE) inhibitor peptide fragment β-CN(f47–52) produced by cleavage at Gln_{46}–Asp_{47} (Sanchez-Rivera et al., 2014).

3.4. Peptide identification in Danish blue cheese

The major objective of the current study was identification of peptides in the pH 4.6-soluble extracts of Danish blue cheeses produced during 9 w of ripening. A large amount of peptide data was obtained from LCMS and UPLC analyses of the pH 4.6-soluble extracts. LCMS analyses found 922 (4 w), 664 (7 w) and 749 (9 w) α_{S1}-casein fragments and 1049 (4 w), 714 (7 w) and 804 (9 w) β-casein fragments. A total of 3270 α_{S1}-casein fragments and 3668 β-casein fragments were produced at the end of 9 w ripening. However, only the 50 peptide fragments with the highest value of relative intensity from α_{S1}-casein and β-casein (Supplementary material Table S2) were considered (Fig. 6A, B). Relative intensity values quantify the amount of an ion produced in relation with the

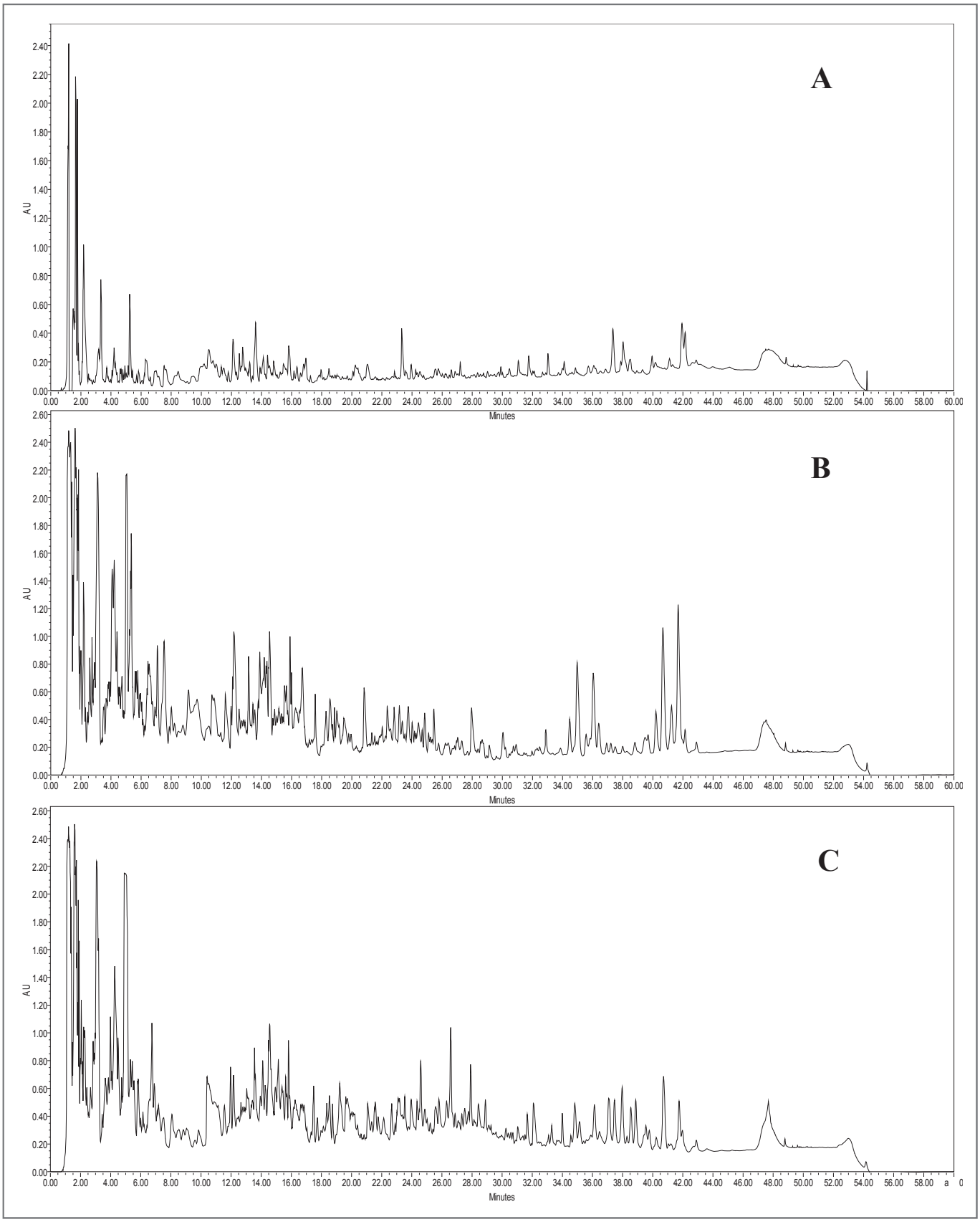

Fig. 2. Ultra performance liquid chromatograms (C_8 column) of pH 4.6-soluble extracts from (A) zero weeks (out of salt), (B) 4 weeks and (C) 9 weeks of ripening in Danish blue cheese, at wavelength of 214 nm.

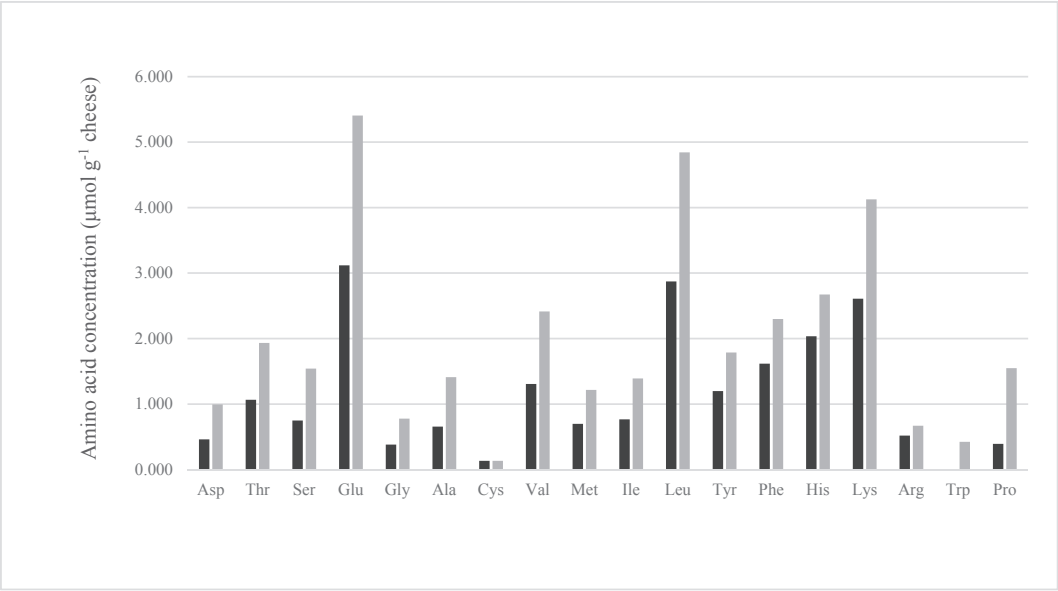

Fig. 3. Concentration (µ mol g^{-1} of cheese) of free amino acids in pH 4.6-soluble extracts from Danish blue cheese at 7 weeks (■) and 9 weeks (■) of ripening.

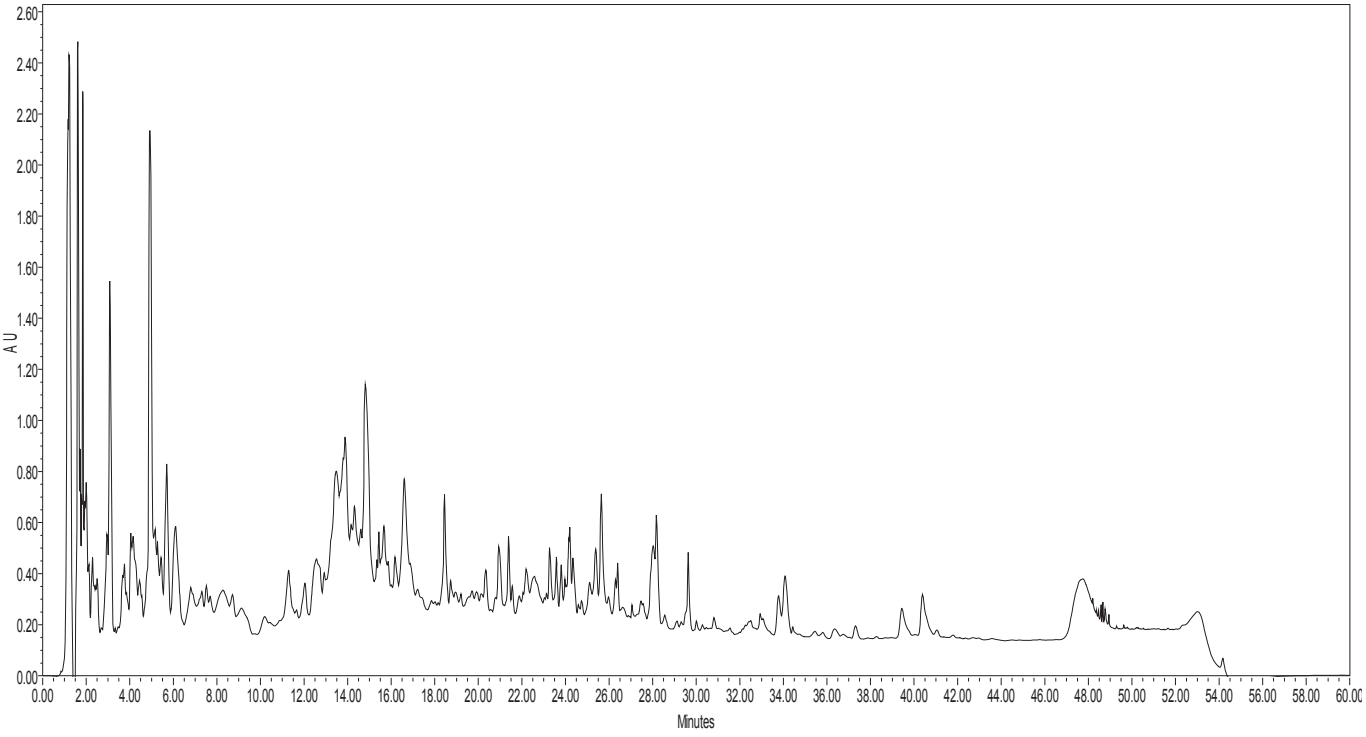

Fig. 4. Ultra performance liquid chromatograms (C_8 column) peptide profiles of cell-free supernatants of *Penicillium roqueforti* strain PR-R grown in 1:1 ratio of 10% (low heat skimmed milk powder) LHSMP and potato dextrose broth for 7 d at 25 °C, at wavelength of 214 nm.

most abundant ion (Zhang et al., 2010) and are thus related to concentration. Highest number of peptide fragments were produced at 4 w and 9 w. Peptides identified in current study were compared with the cleavage specificities of PR-R proteinases (Section 3.3) together with known specificities of chymosin, plasmin, and lactocepins (Ardo et al., 2017; Fernandez et al., 1998; Upadhyay et al., 2004).

The major α_{S1}-casein fragments produced during ripening were α_{S1}-CN(f1–16), (f8–14), (f10–16), (f10–18), (f14–20), (f31–40), (f37–63), (f73–85), (f109–128), (f112–135), (f115–136), (128–158), (f157–164), (f166–172) and (f166–176) and the major β-casein fragments were β-CN(f23/25–34), (f61–75), (f102/113/130–136), (f164–175). Most peptides from α_{S1}-casein (Fig. 6A) were derived from residues 1–40, 105–136 or 150–176. In case of β-casein, regions most susceptible to proteolysis were between residues 6–14, 46–68, 101–140 and 193–209 (Fig. 6B).

Peptide fragments produced from α_{S1}-casein by action of PR-R proteinases included α_{S1}-CN(f14–20), (f31–40), (f37–63),

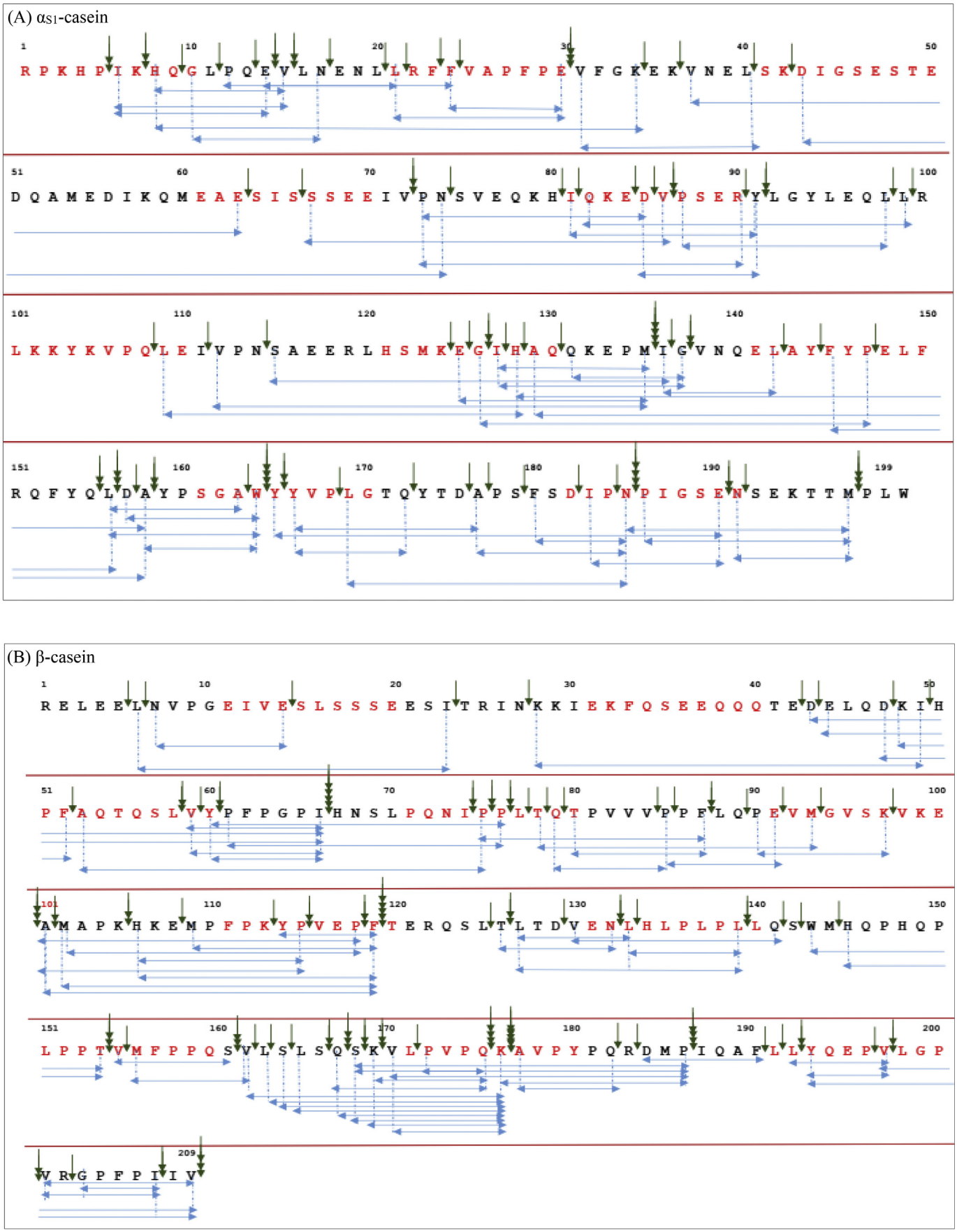

Fig. 5. The primary structure of (A) bovine α_{S1}-casein and (B) bovine β-casein showing the peptides produced by proteinases from cell free supernatants of *Penicilium roqueforti*, PR-R incubated in 1:1 milk (10% low heat skimmed milk powder) and potato dextrose broth suspension on shaking incubation for 7 days at 25 °C.

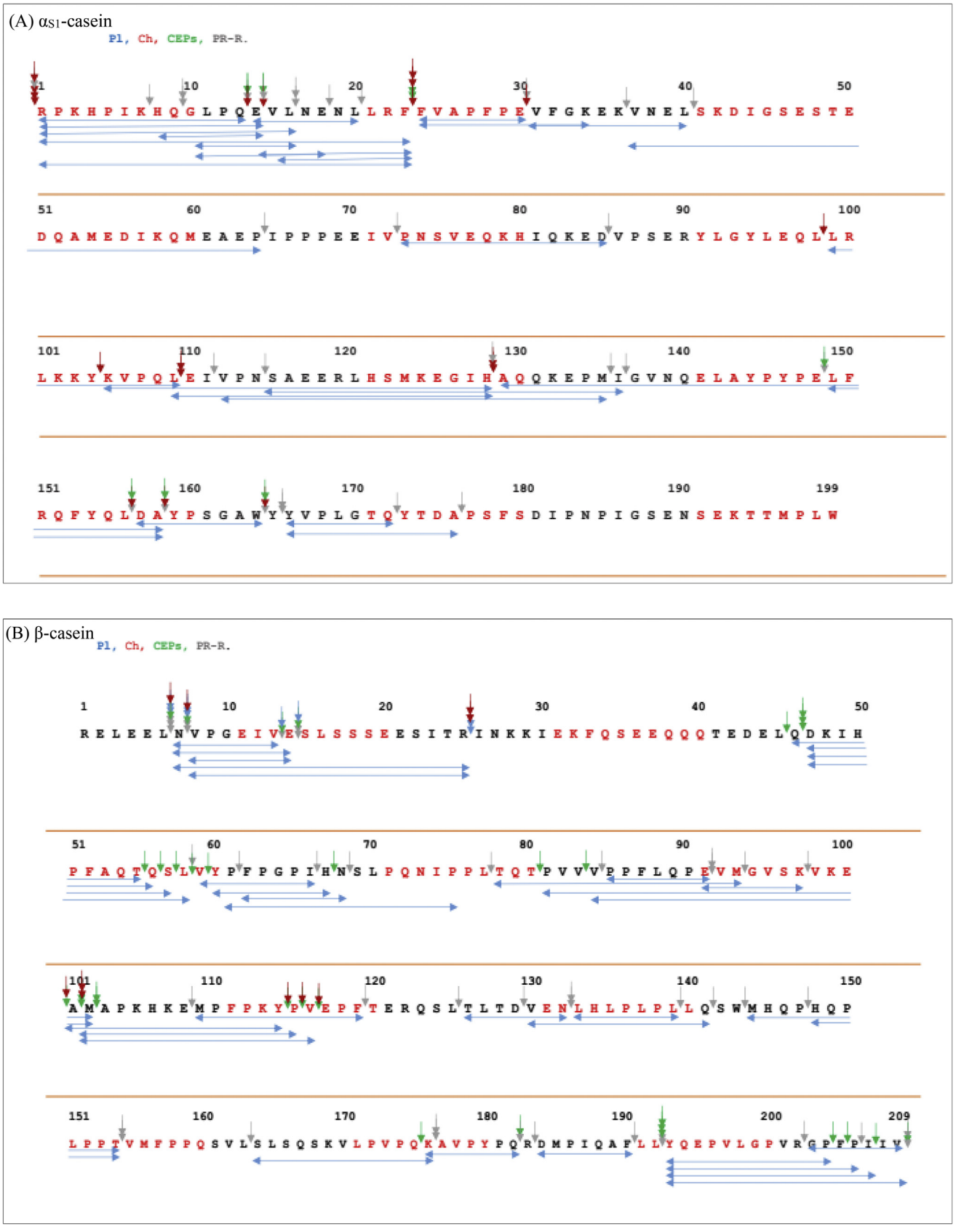

Fig. 6. The primary structure of (A) bovine α_{S1}-casein and (B) bovine β-casein, showing cleavage sites (vertical arrows: plasmin, blue; chymosin, red; lactocepins, green; PR-R proteinases, grey) and peptides in pH 4.6 soluble extracts of Danish blue cheeses, during 9 weeks of ripening. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(f73–85), (f109–128), (f112–135), (f115–136), (f129–158), (f166–172) and (f166–176). It is unclear why the major chymosin-derived fragment, α_{s1}-CN(f1–23) (Fox & McSweeney, 1996), was absent in early stages (OOS and 2 w) of ripening but was found at 7 w and 9 w. Peptides produced by the action of chymosin and/or lactocepins included α_{S1}-CN(f8–14), (f24–30), (f10–16/18), (f149–158) and (f157–164). In the earlier stages of ripening, fragment α_{S1}-CN(f12–25) was observed at OOS and 2 w. Plasmin hydrolysed caseins producing peptide fragments including α_{S1}-CN(f33–47) and α_{S1}-CN(f105–128) (Breen et al., 1995; Fernandez et al., 1998; Singh et al., 1994; 1995; 1997; Upadhyay et al., 2004). Action of several peptidases along with action of PR-R proteinases and other proteolytic enzymes was also observed.

Fragments produced by the action of PR-R proteinases included β-CN(f59–66), (f85–91), (f78–93), (f109–119), (f126–132), (f130–141), (f177–182), (f184–190) and (f193–207/209). β-Casein was hydrolysed at Lys_{97}–Val_{98} which is a major cleavage site of PR-R proteinases (Section 3.3) and other mould proteinases (Le Bars & Gripon, 1981; Trieu-Cuot et al., 1982a; b). More susceptible regions of the casein resulted into fragments produced by the action of PR-R proteases and plasmin (Fox & McSweeney, 1996); peptides produced were β-CN(f28–40/45/55), (f100–116/119/131), (f184–190). Peptides β-CN(f46–55), (f47–56/57/58), (f81/84–102) and (f102–115/116) were produced by the action of lactocepins. The pH conditions (5–5.8; Møller, Rattray, & Ardo, 2012; Mulvihill & Fox, 1978; Pelissier, Mercier, & Ribadeau-Dumas, 1974; Visser & Slangen, 1977) in Danish blue cheeses during later ripening stages allow action of chymosin on β-casein which produces peptide fragments including β-CN(f7/8–25) and (f101/102–114/115/116).

Among the peptides identified, ACE-inhibitory activity was reported by Sanchez-Rivera et al. (2014) in fragments α_{S1}-CN(f157–164) and β-CN(f133–139). These peptides were found throughout the 9 w ripening of Danish blue cheese. Many peptides from both α_{S1}- and β-casein could not be ascribed to the action of PR-R proteinases, chymosin, plasmin and lactocepins; these peptides may have been processed further by the action of peptidases.

4. Conclusions

The current study investigated in detail proteolysis in Danish blue cheese, including determination of the sites of action of *P. roqueforti* proteinases on α_{S1}- and β-casein and identification of numerous peptides produced during the 9 w of ripening. Levels of pH 4.6-soluble nitrogen increased significantly after 4 weeks of ripening and was indicative of extensive primary proteolysis. Urea-PAGE, FAAs analysis, LCMS and UPLC peptide profiles of the pH 4.6-soluble extracts showed considerable qualitative and quantitative differences during ripening of the cheese. Cleavage sites specific to action of PR-R proteinases on α_{S1}-casein and β-casein in hydrolysates were determined and used to help identify which peptides were produced in the cheese during ripening by the action of these enzymes. This study could be useful for understanding the complex mechanisms of proteolysis during ripening of blue cheese and clarifying the relationship of proteolytic activity in the cheese to enzymes from *Penicillium roqueforti* specifically in Danish blue cheese.

Acknowledgements

This project was funded by Food for Health, Ireland (FHI). The authors would also like to thank Arla Foods Denmark for all their help and support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.05.017.

References

Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on caseins. *Journal of Dairy Research, 50*, 45–55.
Ardö, Y., Lilbæk, H., Kristiansen, K. R., Zakora, M., & Otte, J. (2007). Identification of large phosphopeptides from β-casein that characteristically accumulate during ripening of the semi-hard cheese Herrgård. *International Dairy Journal, 17*, 513–524.
Ardo, Y., McSweeney, P. L. H., Magboul, A. A. A., Upadhyay, V. K., & Fox, P. F. (2017). Biochemistry of cheese ripening: Proteolysis. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.), *Cheese: Chemistry, physics & microbiology* (4th ed., Vol. 1, pp. 445–482). London, UK: Academic Press.
Ardö, Y., Pripp, A. H., & Lillevang, S. K. (2009). Impact of heat-treated *Lactobacillus helveticus* on bioactive peptides in low-fat, semi-hard cheese. *Australian Journal of Dairy Technology, 64*, 58–62.
Blakesley, R. W., & Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry, 82*, 580–582.
Breen, E. D., Fox, P. F., & McSweeney, P. L. H. (1995). Fractionation of peptides in a 10 kDa ultrafiltration retentate of a water-soluble extract of Cheddar cheese. *International Journal of Food Science, 7*, 211–220.
Cantor, M. D., van den Tempel, T., Hansen, T. K., & Ardö, Y. (2004). Blue cheese. In P. F. Fox, P. L. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), *Cheese: Chemistry, physics and microbiology. Major cheese groups* (3rd ed., Vol. 2, pp. 175–198). London, UK: Academic Press.
Claverie-Martin, F., & Vega-Hernandez, M. C. (2007). Aspartic proteases in cheese making. In J. Polaina, & A. P. Maccabe (Eds.), *Industrial enzymes* (pp. 207–219). Dordrecht, The Netherlands: Springer.
Cunningham, D. F., & O'Connor, B. (1997). Proline specific peptidases. *Biochimica and Biophysica Acta, 1343*, 160–186.
Diana, M., Rafecas, M., Arco, C., & Quílez, J. (2014). Free amino acid profile of Spanish artisanal cheeses: Importance of gamma-aminobutyric acid (GABA) and ornithine content. *Journal of Food Composition and Analysis, 35*, 94–100.
Diezhandino, I., Fernández, D., González, L., McSweeney, P. L. H., & Fresno, J. M. (2015). Microbiological, physico-chemical and proteolytic changes in a Spanish blue cheese during ripening (Valdeón cheese). *Food Chemistry, 168*, 134–141.
Eren-Vapur, U., & Ozcan, T. (2012). Determination of free amino acids in whole-fat Turkish White Brined cheese produced by animal and microbial milk-clotting enzymes with and without the addition of starter culture. *Mljekarstvo, 62*, 241–250.
EU. (2017). Council regulation (EC) No 510/2006 amendment application according to article 9 'Danablu' EC No: DK-PGI-0217-0328-21.02.2011. *Official Journal of the European Union*, 12–15.
Fenelon, M. A., & Guinee, T. P. (2000). Primary proteolysis and textural changes during ripening in Cheddar cheeses manufactured to different fat contents. *International Dairy Journal, 10*, 151–158.
Fernandez, M., Singh, T. K., & Fox, P. F. (1998). Isolation and identification of peptides from the diafiltration permeate of the water-soluble fraction of Cheddar cheese. *Journal of Agricultural and Food Chemistry, 46*, 4512–4517.
Fox, P. F., Lucey, J. A., & Cogan, T. M. (1990). Glycolysis and related reactions during cheese manufacture and ripening. *Critical Reviews in Food Science and Nutrition, 29*, 237–253.
Fox, P. F., & McSweeney, P. L. H. (1996). Proteolysis in cheese during ripening. *Food Reviews International, 12*, 457–509.
Fox, P. F., Singh, T. K., & McSweeney, P. L. H. (1995). Biogenesis of flavour compounds in cheese. In E. L. Malin, & M. H. Tunick (Eds.), *Chemistry of structure/function relationships in cheese* (pp. 59–98). New York, NY, USA: Plenum Press.
Ganesan, B., & Weimer, B. C. (2007). Amino acid metabolism in relationship to cheese flavour development. In B. C. Weimer (Ed.), *Improving the flavour of cheese* (pp. 70–101). Cambridge, UK: Woodhead/CRC Press.
Gori, K., Ryssel, M., Arneborg, N., & Jespersen, L. (2012). Isolation and identification of the microbiota of Danish farmhouse and industrially produced surface-ripened cheeses. *Microbial Ecology, 65*, 602–615.
Gripon, J. C. (1993). Mould-ripened cheeses. In P. F. Fox (Ed.), *Cheese: Chemistry, physics and microbiology* (2nd ed., pp. 111–136). London, UK: Chapman and Hall.
Gripon, J. C., Desmazeaud, M. J., Le Bars, D., & Bergère, J. L. (1977). Role of proteolytic enzymes of *Streptococcus lactis*, *Penicillium roqueforti* and *Penicillium caseicolum* during cheese ripening. *Journal of Dairy Science, 60*, 1532–1538.
Hewedi, M. M., & Fox, P. F. (1984). Ripening of blue cheese: Characterization of proteolysis. *Milchwissenschaft, 39*, 198–201.
IDF. (1982). *Cheese and processed cheese; determination of the total solids content. Standard 4A:1982*. Brussels, Belgium: International Dairy Federation.
IDF. (1986). *Determination of the nitrogen content (Kjeldahl method) and calculation of crude protein content. Standard 20a:1986*. Brussels, Belgium: International Dairy Federation.
IIRS. (1955). *Determination of the percentage of fat in cheese. Irish Standard 69:1955*. Dublin: Institute for Industrial Research and Standards.

Kinsella, J. E., & Hwang, D. H. (1976a). Biosynthesis of f1avours by *Penicillium roqueforti*. *Biotechnology and Bioengineering, 58*, 927–938.
Kinsella, J. E., & Hwang, D. H. (1976b). Enzymes of *Penicillium roqueforti* involved in the biosynthesis of cheese flavour. *CRC Critical Reviews in Food Science & Nutrition, 8*, 191–228.
Kuchroo, C. N., & Fox, P. F. (1982). Soluble nitrogen in cheddar cheese: Comparison of extraction procedures. *Milchwissenschaft, 37*, 331–335.
Lauer, B. H., & Baker, B. E. (1977). Amino acid composition of casein isolated from the milks of different species. *Canadian Journal of Zoology, 55*, 231–236.
Le Bars, D., & Gripon, J. C. (1981). Role *of Penicillim roqueforti* proteinases during Blue cheese ripening. *Journal of Dairy Research, 48*, 479–487.
Le Dréan, G., Mounier, J., Vasseur, V., Arzur, D., Habrylo, O., & Barbier, G. (2010). Quantification of *Penicillium camemberti* and *Penicillium roqueforti* mycelium by real-time PCR to assess their growth dynamics during ripening cheese. *International Journal of Food Microbiology, 138*, 100–107.
Madkor, S., Fox, P. F., Shalabi, S. I., & Metwalli, N. H. (1987). Studies on the ripening of Stilton cheese: Proteolysis. *Food Chemistry, 25*, 13–29.
McSweeney, P. L. H., Pochet, S., Fox, P. F., & Healy, A. (1994). Partial identification of peptides from the water-insoluble fraction of Cheddar cheese. *Journal of Dairy Research, 61*, 587–590.
Møller, K. K., Rattray, F. P., & Ardö, Y. (2012). Camel and bovine chymosin hydrolysis of bovine α_{S1}- and β-caseins studied by comparative peptide mapping. *Journal of Agriculture and Food Chemistry, 60*, 11421–11432.
Mulvihill, D. M., & Fox, P. F. (1978). Proteolysis of bovine β-casein by chymosin: Influence of pH, urea and sodium chloride. *Irish Journal of Food Science & Technology, 2*, 135–139.
O'Mahony, J. A., Lucey, J. A., & McSweeney, P. L. H. (2005). Chymosin-mediated proteolysis, calcium solubilisation, and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science, 88*, 3101–3114.
Pelissier, J.-P., Mercier, J.-C., & Ribadeau-Dumas, B. (1974). Etude de la proteolyse des caseines α_{S1}- et β-bovines par la presure. *Annales de Biologie Animale, Biochimie, Biophysique, 14*, 343–362.
Rehn, U., Petersen, M. A., Hallin Saedén, K., & Ardö, Y. (2010). Ripening of extra hard cheese made with mesophilic dl-starter. *International Dairy Journal, 20*, 844–851.
Sanchez-Rivera, L., Diezhandino, I., Gomez-Ruiz, J. A., Fresno, J. M., Miralles, B., & Recio, I. (2014). Peptidomic study of Spanish blue cheese (Valdeon) and changes after simulated gastrointestinal digestion. *Electrophoresis, 35*, 1627–1636.
Shalabi, S. I., & Fox, P. F. (1987). Electrophoretic analysis of cheese: Comparison of methods. *Irish Journal of Food Science & Technology, 11*, 135–151.
Singh, T. K., Fox, P. F., & Healy, A. (1995). Water-soluble peptides in cheddar cheese: Isolation and identification of peptides in the UF retentate of water-soluble fractions. *Journal of Dairy Research, 62*, 629–640.
Singh, T. K., Fox, P. F., & Healy, A. (1997). Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. *Journal of Dairy Research, 64*, 433–443.
Singh, T. K., Fox, P. F., Højrup, P., & Healy, A. (1994). A scheme for the fractionation of cheese nitrogen and identification of principal peptides. *International Dairy Journal, 4*, 111–122.
Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal, 11*, 327–345.
Trieu-Cuot, P., Archieri-Haze, M. J., & Gripon, J. C. (1982a). Effect of aspartyl proteinases of *Penicillium caseicolum* and *Penicillium roqueforti* on caseins. *Journal of Dairy Research, 49*, 487–500.
Trieu-Cuot, P., Archieri-Haze, M. J., & Gripon, J. C. (1982b). Etude comparative de l'action des métalloprotéases de *Penicillium caseicolum* et *Penicillium roqueforti* sur les caséines alpha sl et bèta. *Lait, 62*, 234–249.
Trieu-Cuot, P., & Gripon, J. C. (1983). Etude électrophorétique de la proteolyse au cours del'affinage des fromages à pâte persillee du type Bleu d'Auvergne. *Lait, 63*, 116–128.
Upadhyay, V. K., McSweeney, P. L. H., Magboul, A. A. A., & Fox, P. F. (2004). Proteolysis in cheese during ripening. In P. F. Fox, P. L. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), *Cheese: Chemistry, physics & microbiology* (3rd ed., pp. 391–433). London, UK: Elsevier.
Vicente, M. S., Ibàñez, F. C., Barcina, Y., & Barron, L. J. R. (2001). Changes in the free amino acid content during ripening of Idiazabal cheese: Influence of starter and rennettype. *Food Chemistry, 72*, 309–317.
Visser, S., & Slangen, K. J. (1977). On the specificity of chymosin (rennin) inits action on bovine β-casein. *Netherland Milk Dairy Journal, 31*, 16–30.
Wolf, I. V., Perotti, M. C., & Zalazar, C. A. (2010). Composition and volatile profiles of commercial Argentinean blue type cheeses. *Journal of the Science of Food and Agriculture, 91*, 385–393.
Zarmpoutis, I. V., McSweeney, P. L. H., Beechinor, J., & Fox, P. F. (1996). Proteolysis in the Irish farmhouse blue cheese, Chetwynd. *Irish Journal Agriculture Food Research, 35*, 25–36.
Zarmpoutis, I. V., McSweeney, P. L. H., & Fox, P. F. (1997). Proteolysis in blue-veined cheeses: An intervarietal study. *Irish Journal Agriculture Food Research, 36*, 219–229.
Zhang, G., Ueberheide, B. M., Waldemarson, S., Myung, S., Molloy, K., Eriksson, J., et al. (2010). Protein quantitation using mass spectrometry. *Methods in Molecular Biology, 673*, 211–222.