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Proteolysis in Danish Blue cheese during ripening

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Proteolysis in Danish blue cheese was studied during 9 weeks of 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 the action of chymosin and plasmin early in ripening, but later the action of *Penicillium roqueforti* proteinases became apparent. The proteolytic specificity of *Penicillium roqueforti* PR-R proteinases on αs1- and β-casein was determined in a model system. Regions most susceptible to action of fungal proteinases in αs1-casein were 6-40, 69-99, 124-147 and 155-199, with a total of 91 cleavage sites identified in this protein and 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 in cheese extracts during 9 weeks ripening, principally from αs1-casein regions 1-40, 105-136 and 150-176 and β-casein regions 6-14, 46-68, 101-140 and 193-209. This study determined the proteolytic specificity of *Penicillium roqueforti* proteinases on the major caseins and identified numerous peptides produced in Danish blue cheese during ripening.
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 (Official Journal of the European Union, 2014). 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 heavily reviewed (Fox, Singh & McSweeney., 1995; Fox & McSweeney, 1996; Sousa, Ardo & McSweeney, 2001; McSweeney, 2004; Upadhyay, McSweeney, Maghboul & Fox, 2004; Ardo, McSweeney, Maghboul, Upadhayay & Fox, 2017). Blue cheese undergoes extensive proteolysis during ripening (Kinsella & Hwang, 1976a; Gripon, Desmazeaud, Le Bars & Bergère, 1977; Hewedi & Fox, 1984). An inter-varietal comparison of blue cheese was conducted by Zarmpoutis, McSweeney & 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 (Sousa, Ardo & Mcsweeney, 2001; Ardo, McSweeney, Maghboul, Upadhay & Fox, 2017). The extensive proteolysis in Danish blue cheese is largely associated with the principal secondary micro-
organisms, *Penicilium roqueforti* through the action of their exo and intracellular enzymes, including metalloproteinases, aspartic-proteinases, aminopeptidases, and acid carboxypeptidases (Madkor, Shalabi & Metwalli, 1987a; Zarmpoutis, McSweeney & Fox, 1996, 1997). Aspartyl and metalloproteinases act specifically on αs1- 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 & Ardo, 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- 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, McSweeney & Fox, 1997). The metalloprotease and aspartyl proteases have broad specificity and hydrolyse both αs1- and β-caseins. The metalloprotease cleaves β-casein at Pro90-Glu91, which is not often hydrolysed by proteases because of its proline residue, and also at Lys28-Lys29, which is also cleaved by plasmin (Le Bars & Gripon, 1981; Trieu-Cuot, Archieri-Haze & Gripon, 1982b; Trieu-Cuot & Gripon, 1983). Aspartyl proteases hydrolyse the caseins only into high molecular weight peptides including αs1-CN (f24-199) produced by cleavage at Phe23-Phe24 in αs1-casein which is also the major chymosin cleavage site in this protein (Trieu-Cuot, Archieri-Haze & Gripon, 1982a; Larsen et al., 1998; Ardo, McSweeney, Maghboul, Upadhayay & Fox, 2017). Aspartyl proteases produce high molecular weight peptides by cleaving β-caseins at Arg1-Lys29, Lys29-Val209, Lys97-Val209 (Le Bars & Gripon, 1981; Trieu-Cuot, Archieri-Haze & Gripon, 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, CaCl₂ 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 (out of salt (OOS or zero day), 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 to 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 & 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 x 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; Shalabi & Fox 1987; O’Mahony, Lucey & McSweeney, 2005). 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 et al. (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⁻¹) 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 (Sartorius GmbH, Gottingen, Germany) and maintained at 4ºC during analysis. Peptide profiles were determined by 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., Milford, MA, USA). 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 120ºC, de-solvation temperature at 450ºC 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 was 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 αs1-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, USA) (Le Dréan, Mounier, Vasseur,
Arzur, Habrylo & Barbier 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, OSA, UK) for 7 days at 25°C (Le Dréan, Mounier, Vasseur,
Arzur, Habrylo & Barbier, 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 analyses 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, \(\alpha\), of 0.05 (P value \(\leq 0.05\)), 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 to 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 (Zarmpoutis, McSweeney & Fox, 1997; Wolf, Perotti & Zalazar, 2010; Diezhandino, Fernández, González, McSweeney & Fresno, 2015). Crude protein content (% N x 6.38) remained around 18.0% up to 7 weeks and significantly increased to 24% by 9 weeks of ripening. % Nitrogen in cheese ranged between 2.83% to 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 CO₂ and H₂O (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, McSweeney & Fox, 1996). The value (29.9 %) found at 9 w, (age at which Danish blue cheese is commercially packed) in this study was comparable to other samples of commercial ready blue cheeses like, the Irish blue cheese, Chetwynd (~32% to 36%; Zarmpoutis, McSweeney & Fox, 1996), but were lower than the values reported for Gorgonzola and Stilton cheeses (45% to 55%; Zarmpoutis, McSweeney & Fox, 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 αs1- 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, McSweeney & Fox, 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, Desmazeaud, Le Bars & Bergère, 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 to OOS. Peaks with highest intensity were observed from retention times 2 to 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 to 43 min retention time) to be hydrolysed progressively after 4 w of ripening. A large number of peptides (Fig. 2) were 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, van den Tempel, Hansen & Ardo, 2004). As observed in other blue-veined cheese varieties like Gorgonzola and Silton (Zarpoutis, McSweeney & Fox, 1997), chromatograms point towards extensive proteolysis during ripening.

pH 4.6-soluble extracts from cheeses at 7 w and 9 w (Fig. 3) contained 20.57 μmol g⁻¹ cheese and 35.59 μmol g⁻¹ cheese of total FAAs, respectively. Concentration of individual amino acids at 9 w, were glutamic acid 5.41 μmol g⁻¹ cheese, leucine 4.84 μmol g⁻¹ cheese and lysine 4.12 μmol g⁻¹ cheese. 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 which acts as a co-substrate for aminotransferases (Vincente, Ibañez, 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 Zarpoutis McSweeney & Fox, (1997) and Diana, Rafecas, Arco & Quilez, (2014) on blue cheese. The lower concentration of certain FAAs, e.g., cysteine and tryptophan, is likely due to their presence in low quantities in regions of caseins extensively degraded (Lauer & Baker 1977; Ganesan & Weimer, 2007; Eren-Vapur & Ozcan, 2012). Levels of FFAs were comparable to those found in studies on other blue cheese varieties like Gorgonzola and Stilton (Zarpoutis, McSweeney & Fox, 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 (Upadhyay, McSweeney, Maghboul & Fox, 2004; Ardo, McSweeney, Maghboul, Upadhyay & Fox, 2017), 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. This was evident through the complexity of the UPLC chromatograms (Fig. 4). Casein-derived peptides identified in the PR-R hydrolysate were used to map the cleavage sites (Fig. 5 [A] and [B]). Only peptides with highest LCMS relative intensity values for αs1-casein and β-casein (Supplementary data Table 1) were used to determine the cleavage sites. N- and C- termini of the peptide fragments along with their sequence are listed in Supplementary data Table 1. The peptides produced in the PR-R hydrolysates were compared to peptides known to be produced in hydrolysates by action of chymosin, plasmin and lactocepins.

Certain regions of αs1-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 αs1-casein were Pro5-Ile6, Lys7-His8, Leu11-Pro12, Glu14-Val15, Phe23-Phe24, Phe24-Val25, Glu30-Val31, His79-Ile80, Gly126-Ile127, Met135-Ile136, Gln155-Leu156, Leu157-Asp158, Trp164-Tyr165, Asn184-Pro185, Asn190-Ser191 and Met196-Pro197. A major chymosin-derived peptide αs1-CN(f1-23) (Fox & McSweeney, 1996) produced by cleavage of Phe23-Phe24, 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 (Singh, Fox, Højrup & Healy; Singh, Fox & Healy, 1994, 1995, 1997; Breen, Fox & Mcsweeney, 1995; Fernandez, Singh & Fox, 1998; Upadhyay, McSweeney, Maghboul & Fox, 2004; Ardo, McSweeney, Maghboul, Upadhayay & Fox, 2017) and peptides αs1-CN (f6-13/14/15) and αs1-CN (f8-14) were produced in the PR-R hydrolysates by cleavage at the bonds Gln13-Glu14, Glu14-Val15 and Leu16-Asn17; these sites are also cleaved.
by chymosin activity (McSweeney, Pochet, Fox & Healy, 1994). Similarly, PR-R proteinases cleaved bonds at Pro5-Ile6, Lys7-His8, Phe23-Phe24, Ser66-Ser67 and Ala175-Pro176, which were also cleaved by cell-envelope associated proteinases or lactocepins (Singh, Fox, Højrup & Healy; Singh, Fox & Healy, 1994, 1995, 1997; Breen, Fox & McSweeney, 1995; Fernandez, Singh & Fox, 1998). Sites Glu84-Asp85 and Ile81-Glu82, are also susceptible to the action of both chymosin and lactocepins and PR-R proteinases (Supplementary data Table 1). The N terminal fragment αs1-CN(f165-189), produced by cleavage at Ala164-Trp165 which is a major cleavage site of PR-R proteinases on αs1-casein; this site is also cleaved by the action of chymosin and lactocepins (Fox & McSweeney, 1996). The peptide αs1-CN(f157-164), which was identified in the PR-R hydrolysate, has ACE inhibitory action and is a notable bioactive peptide (Sanchez-Rivera, Diezhandino, Gomez-Ruiz, Fresno, Miralles & Reico, 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 Glu5-Leu6, Glu14-Ser15, Ile66-His67, Glu100-Ala101, Phe119-Thr120, Asn132-Leu133, Thr154-Val155, Ser168-Lys169, Lys176-Ala177, Pro186-Ile187, Tyr193-Ile194 and Ile207-Ile208. 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: Lys97-Val98, Lys105-His106, Lys113-Tyr114, Lys169-Val170, Lys176-Ala177 and Arg202-Gly203. The bonds Thr78-Gln79, Thr154-Val155, Ser164-Leu165, Leu191-Leu192 and Pro200-Val201 cleaved by PR-R proteinases were also cleaved by action of lactocepins (Ardo, McSweeney, Maghboul, Upadhayay & Fox, 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 (Gly203-Pro204, Tyr180-Pro181, Ala103-Pro104,}


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 (Fox & McSweeney, 1996; Cunningham & O’Connor, 1997). 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, Diezhandino, Gomez-Ruiz, Fresno, Miralles & Reico, 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 data Table 2) were considered (Fig. 6 A & B). Relative intensity values quantify the amount of an ion produced in relation with the most abundant ion (Zhang, Ueberheide, Waldemarson, Myung, Molloy 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 to the cleavage specificities of PR-R proteinases (Section 3.3) together with known specificities of chymosin, plasmin, and lactocepins (Fernandez, Singh & Fox, 1998; Upadhyay, McSweeney, Maghboul & Fox, 2004; Ardo, McSweeney, Maghboul, Upadhyay & Fox, 2017).
The major αs1-casein fragments produced during ripening were αs1-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 αs1-casein (Fig. 6[A]) 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. 7[B]).

Peptide fragments produced from αs1-casein by action of PR-R proteinases included αs1-CN(f14-20), (f31-40), (f37-63), (f73-85), (f109-128), (f112-135), (f115-136), (f129-158), (f166-172) and (f166-176). It is unclear why the major chymosin-derived fragment, αs1-CN(f1-23) (Fox and 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 αs1-CN (f8-14), (f24-30), (f10-16/18), (f149-158) and (f157-164). In the earlier stages of ripening, fragment αs1-CN(f12-25) was observed at OOS and 2 w. Plasmin hydrolysed caseins producing peptide fragments including αs1-CN(f33-47) and αs1-CN(f105-128) (Singh, Fox, Højrup & Healy; Singh, Fox & Healy, 1994, 1995, 1997; Breen, Fox & McSweeney, 1995; Fernandez, Singh & Fox, 1998; Upadhyay, McSweeney, Maghboul & Fox, 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 hydrolyzed at Lys97-Val98 which is a major cleavage site of PR-R proteinases (Section 3.3) and other mould proteinases (Le Bars & Gripon, 1981; Trieu-Cuot, Archieri-Haze & Gripon, 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-
Peptides β-CN (f46-55), (f47-56/57/58), (f81/84-102) and (f102-115/116) were produced by the action of lactocepins. pH conditions (5-5.8) (Pelissier, Mercier & Ribadeau-Dumas, 1974; Visser & Slangen, 1977; Mulvihill & Fox, 1978; Møller, Rattray & Ardo, 2012) 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 αs1-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 αs1- 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 \( \alpha_{s1} \)- and \( \beta \)-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 \( \alpha_{s1} \)-casein and \( \beta \)-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

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References


Exterkate, F. A., & Alting, A. C. (1993). The conversion of the $\alpha_{s1}$-casein-(1-23)-fragment by the free and bound form of the cell-envelope proteinase of *Lactococcus lactis* subsp. cremoris under conditions prevailing in cheese. *Systematic and Applied Microbiology, 16*, 1-8.


Results

Table 1

Compositional analysis of Danish blue cheese during ripening time of 9 weeks.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>% moisture</th>
<th>% N</th>
<th>% (Nx6.38)</th>
<th>pH4.6 SN</th>
<th>% MNFS</th>
<th>% FDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>OOS</td>
<td>4.87±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.91±0.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.01±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.77±0.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.67±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.84±1.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.71±0.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2w</td>
<td>5.11±0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.24±0.86&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.82±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.00±0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.57±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.72±1.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.96±1.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4w</td>
<td>5.82±0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47.62±0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.89±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.45±0.97&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>28.83±1.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>69.03±0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.19±1.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7w</td>
<td>5.36±0.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43.2±2.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.33±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.25±0.66&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>21.79±0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.30±2.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.03±1.46&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>9w</td>
<td>5.69±0.25&lt;sup&gt;d&lt;/sup&gt;</td>
<td>45.13±1.39&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.78±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.14±2.38&lt;sup&gt;de&lt;/sup&gt;</td>
<td>29.94±2.82&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67.12±1.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.65±1.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Samples out of salt (OOS), 2 weeks: (2 w), 4 weeks: (4 w), 7 weeks: (7 w), 9 weeks: (9 w) of ripening, where %N = Nitrogen%, % (Nx6.38) = crude protein% in blue cheese, total SN = pH 4.6-soluble nitrogen (SN) as a percentage of total nitrogen, %FDM = fat in dry matter and % MNFS = moisture in non-fat solids, represented by mean ± standard deviation and different superscript letters represent significant difference (P<0.05), of three cheese-making batches of Danish blue cheese.

Fig. 1. Urea polyacrylamide gel electrophoretograms of pH 4.6-insoluble nitrogen fraction of Danish blue cheese at different ripening periods. Standard protein markers: sodium caseinate: (S), Lane 2-6 depicts pH 4.6-insoluble nitrogen fraction separated from cheese ripened Out of Salt (OOS), 2 weeks: (2 w), 4 weeks: (4 w), 7 weeks: (7 w), 9 weeks: (9 w) of ripening clearly shows the breakdown of the caseins indicating extensive proteolysis.
Fig. 2. Ultra performance liquid chromatograms (C₈ column) of pH 4.6-soluble extracts from Out of Salt (OOS), 4 weeks (4 w) 9 weeks (9 w) of ripening in Danish blue cheese, at wavelength of 214 nm.
Fig. 3. Concentration (μmol g⁻¹ of cheese) of free amino acids in pH 4.6-soluble extracts from Danish blue cheese at 7 weeks (7 w) and 9 weeks (9 w) of ripening.

Fig. 4. Ultra performance liquid chromatograms (C₈ column) peptide profiles of cell free supernatants of Penicillium roqueforti strain PR-R 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.
Fig 5. A) The primary structure of bovine $\alpha_{s1}$-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 25ºC.
Fig 5. B] The primary structure of bovine β-casein, , showing the peptides produced by proteinases from cell free supernatants of *Penicillium 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. A] The primary structure of bovine $\alpha_{\text{s1}}$-casein, showing the peptides produced by proteinases from pH 4.6 soluble extracts of Danish blue cheeses, during 9 weeks of ripening.
Fig. 6. B] The primary structure of bovine β-casein, showing peptides produced by proteinases from pH 4.6 soluble extracts of Danish blue cheeses, during 9 weeks of ripening.