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Proteolysis in Danish Blue cheese during ripening

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A B S T R A C T

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 α_{s1}- and β-casein was determined in a model system. Regions most susceptible to action of fungal proteinases in α_{s1}-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 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. 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 ~10ºC 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, Upadhayay & Fox, 2017). *T*he 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 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 & 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 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, Archieri-Haze & Gripon, 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 (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 Arg_1-Lys_{29}, Lys_{29}-Val_{209}, Lys_{97}-Val_{209} (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, $CaCl_2$ 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 (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).

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145 *2.3 Analysis of proteolysis*

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

150 Individual free amino acid (FAA) contents were determined according to Fenelon et al. (2000)
151 from frozen pH 4.6-soluble extracts from cheeses at 7 w and 9 w of ripening. Samples were
152 first de-proteinised by mixing equal volumes of 24% (v v^{-1}) trichloroacetic acid (TCA).

153 Peptide profiling was done using ultra-performance liquid chromatography (UPLC) and mass
154 spectroscopy (MS). For UPLC samples were prepared by filtering pH 4.6-soluble extracts
155 through 0.22 μm cellulose acetate filters (Sartorious GmbH, Gottingen, Germany) and
156 maintained at 4ºC during analysis. Peptide profiles were determined by using a Waters Acquity
157 UPLC H-Class Core System with a Waters Acquity UPLC TUV Detector (dual wavelength)
158 and Acquity Column Heater 30-A, the system was interfaced with Empower 3 software
159 (Waters Corp., Milford, MA, USA). The column used was an Acquity UPLC BEH C-18
160 column (Waters Corp., Milford, MA, USA). Elution was monitored at 214 nm with a mobile
161 phase of two solvents A: 0.1% (v v^{-1}) formic acid (Sigma-Aldrich, Darmstadt, Germany) in
162 Milli-Q water and B: 0.1% (v v^{-1}) formic acid in acetonitrile (Sigma-Aldrich) with a flow rate
163 of 0.2 mL min^{-1} over run time of 60 min.

164 Peptides in pH4.6-soluble extracts were determined by LC-MS using a Waters Acquity G2 Q-
165 TOF LC-MS, model XEVO-G2QTOF#YBA051, coupled to a Waters Acquity UPLC. Samples
166 were filtered through 0.22 μm cellulose acetate filters and a volume of 10 μL was injected into
167 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^{-1}) formic acid in Milli-Q water and Solvent B was 0.1% (v v^{-1}) 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 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, USA) (Le Dréan, Mounier, Vasseur, Arzur, Habrylo & Barbier 2010) and 100 mL milk from 10% low heat skimmed milk (LHSM)

193 powder (Kerry, Listowel, Ireland). This suspension was incubated in an orbital incubator
194 (Stuart Scientific, Staffordshire, OSA, UK) for 7 days at 25°C (Le Dréan, Mounier, Vasseur,
195 Arzur, Habrylo & Barbier, 2010) at speed of 100 rev min^{-1}. When grown, the entire mycelial
196 biomass was centrifuged at 9000 g for 45 min at 4ºC in Sorvall centrifuge. The filtrate was
197 filtered through Whatman paper number 113. The filtrate was stored frozen in aliquots at -
198 20°C. Peptides derived from the major caseins present in this hydrolysate were identified by
199 Q-ToF LCMS as described above.

200 *2.5 Statistical analysis*

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

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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 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

239 in proteolysis in Danish blue cheese after 4 w reflects the extensive proteolytic action of
240 *Penicillium roqueforti*, which appeared around that stage of ripening Trieu-Cuot & Gripon,
241 1983; Zarmpoutis, McSweeney & Fox, 1996). The value (29.9 %) found at 9 w, (age at which
242 Danish blue cheese is commercially packed) in this study was comparable to other samples of
243 commercial ready blue cheeses like, the Irish blue cheese, Chetwynd (~32% to 36%;
244 Zarmpoutis, McSweeney & Fox., 1996), but were lower than the values reported for
245 Gorgonzola and Stilton cheeses (45% to 55%; Zarmpoutis, McSweeney & Fox, 1996).

246 Proteolysis in Danish blue cheese was also evaluated by urea-PAGE of freeze-dried pH 4.6-
247 insoluble fractions as shown in Fig.1. Electrophoretograms showed extensive degradation of
248 α_{s1}- and β-casein from 4 w and onwards, with initial breakdown of β-casein was by the action
249 of plasmin forming the γ-caseins (Zarmpoutis, McSweeney & Fox, 1997) followed by high
250 rate of proteolysis by the action of mould enzymes. A significant increase in proteolysis was
251 observed at 4 w and later in the present study, when the mould had become visible (Gripon,
252 Desmazeaud, Le Bars & Bergère, 1977; Le Bars & Gripon 1981). Greater numbers of peptides
253 were generated at 4 w and later stages of ripening and reflected the development of *Penicillium*
254 *roqueforti* and its proteinases in the cheeses.

255 A high number of peptide fragments were generated during ripening as can be observed in the
256 UPLC chromatograms (Fig. 2) of pH 4.6-soluble extracts of OOS, 4 w and 9 w-old cheeses. A
257 clear increase in complexity of the peptide profiles was seen at 4 w and 9 w, as compared to
258 OOS. Peaks with highest intensity were observed from retention times 2 to 10 min in all
259 chromatograms. During ripening, the number (both hydrophobic and hydrophilic) and size of
260 peaks increased from the early (OOS) to intermediate (4 w) to end of ripening (9 w). Many of
261 the later-eluting peptides appeared (36 to 43 min retention time) to be hydrolysed progressively
262 after 4 w of ripening. A large number of peptides (Fig. 2) were produced during ripening. The
263 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 (Zarmpoutis, McSweeney & Fox, 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 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, Iba´n˜ez, 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 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 (Zarmpoutis, 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, Upadhayay & 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 α_{s1}-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 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 (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 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 (Singh, Fox, Højrup & Healy; Singh, Fox & Healy, 1994, 1995, 1997; Breen, Fox & Mcsweeney, 1995; Fernandez, Singh & Fox, 1998). 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 data Table 1). 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, 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 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, McSweeney, Maghboul, Upadhay&ay & 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 (Gly_{203}-Pro_{204}, Tyr_{180}-Pro_{181}, Ala_{103}-Pro_{104},

338 Leu_{137}-Pro_{138}) or isoleucine (Ile_{49}-His_{50}, Ile_{66}-His_{67}, Ile_{207}-Ile_{208}) as a part of scissile bond,
339 which was also noted earlier (Fox & McSweeney, 1996; Cunningham & O'Connor, 1997). β-
340 Casein peptide fragments in PR-R hydrolysates with reported bioactivity included opioid
341 peptide fragment β-CN(f60-66), produced by cleavage at Val_{59}-Tyr_{60}, β-casomorphin-7 peptide
342 fragment β-CN(f114-119) produced by cleavage at Lys_{113}-Tyr_{114} and angiotensin I-converting
343 enzyme (ACE) inhibitor peptide fragment β-CN(f47-52) produced by cleavage at Gln_{46}-Asp_{47}
344 (Sanchez-Rivera, Diezhandino, Gomez-Ruiz, Fresno, Miralles & Reico, 2014).

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346 *3.4 Peptide identification in Danish blue cheese*

347 The major objective of the current study was identification of peptides in the pH 4.6-soluble
348 extracts of Danish blue cheeses produced during 9 w of ripening. A large amount of peptide
349 data was obtained from LCMS and UPLC analyses of the pH 4.6-soluble extracts. LCMS
350 analyses found 922 (4 w), 664 (7 w) and 749 (9 w) α_{s1}-casein fragments and 1049 (4 w), 714
351 (7 w) and 804 (9 w) β-casein fragments. A total of 3270 α_{s1}-casein fragments and 3668 β-
352 casein fragments were produced at the end of 9 w ripening. However, only the 50 peptide
353 fragments with the highest value of relative intensity from α_{s1}-casein and β-casein
354 (Supplementary data Table 2) were considered (Fig. 6 A & B). Relative intensity values
355 quantify the amount of an ion produced in relation with the most abundant ion (Zhang,
356 Ueberheide, Waldemarson, Myung, Molloy et al., 2010) and are thus related to concentration.
357 Highest number of peptide fragments were produced at 4 w and 9 w. Peptides identified in
358 current study were compared to the cleavage specificities of PR-R proteinases (Section 3.3)
359 together with known specificities of chymosin, plasmin, and lactocepins (Fernandez, Singh &
360 Fox, 1998; Upadhyay, McSweeney, Maghboul & Fox, 2004; Ardo, McSweeney, Maghboul,
361 Upadhayay & Fox, 2017).

362 The major α_{s1}-casein fragments produced during ripening were α_{s1}-CN(f1-16), (f8-14), (f10-
363 16), (f10-18), (f14-20), (f31-40), (f37-63), (f73-85), (f109-128), (f112-135), (f115-136), (128-
364 158), (f157-164), (f166-172) and (f166-176) and the major β-casein fragments were β-
365 CN(f23/25-34), (f61-75), (f102/113/130-136), (f164-175). Most peptides from α_{s1}-casein (Fig.
366 6[A]) were derived from residues 1-40, 105-136 or 150-176. In case of β-casein, regions most
367 susceptible to proteolysis were between residues 6-14, 46-68, 101-140 and 193-209 (Fig. 7[B]).

368 Peptide fragments produced from α_{s1}-casein by action of PR-R proteinases included α_{s1}-
369 CN(f14-20), (f31-40), (f37-63), (f73-85), (f109-128), (f112-135), (f115-136), (f129-158),
370 (f166-172) and (f166-176). It is unclear why the major chymosin-derived fragment, α_{s1}-CN(f1-
371 23) (Fox and McSweeney, 1996), was absent in early stages (OOS and 2 w) of ripening but
372 was found at 7 w and 9 w. Peptides produced by the action of chymosin and/or lactocepins
373 included α_{s1}-CN (f8-14), (f24-30), (f10-16/18), (f.149-158) and (f157-164). In the earlier stages
374 of ripening, fragment α_{s1}-CN(f12-25) was observed at OOS and 2 w. Plasmin hydrolysed
375 caseins producing peptide fragments including α_{s1}-CN(f33-47) and α_{s1}-CN(f105-128) (Singh,
376 Fox, Højrup & Healy; Singh, Fox & Healy, 1994, 1995, 1997; Breen, Fox & Mcsweeney, 1995;
377 Fernandez, Singh & Fox, 1998; Upadhyay, McSweeney, Maghboul & Fox, 2004). Action of
378 several peptidases along with action of PR-R proteinases and other proteolytic enzymes was
379 also observed.

380 Fragments produced by the action of PR-R proteinases included β-CN(f59-66), (f85-91), (f78-
381 93), (f109-119), (f126-132), (f130-141), (f177-182), (f184-190) and (f193-207/209). β-Casein
382 was hydrolyzed at Lys_{97}-Val_{98} which is a major cleavage site of PR-R proteinases (Section 3.3)
383 and other mould proteinases (Le Bars & Gripon, 1981; Trieu-Cuot, Archieri-Haze & Gripon,
384 1982a, b). More susceptible regions of the casein resulted into fragments produced by the
385 action of PR-R proteases and plasmin (Fox & McSweeney, 1996); peptides produced were β-

386 CN(f28-40/45/55), (f100-116/119/131), (f184-190). Peptides β-CN (f46-55), (f47-56/57/58),
387 (f81/84-102) and (f102-115/116) were produced by the action of lactocepins. pH conditions
388 (5-5.8) (Pelissier, Mercier & Ribadeau-Dumas, 1974; Visser & Slangen, 1977; Mulvihill & Fox,
389 1978; Møller, Rattray & Ardo, 2012) in Danish blue cheeses during later ripening stages allow
390 action of chymosin on β-casein which produces peptide fragments including β-CN(f7/8-25)
391 and (f101/102-114/115/116).

392 Among the peptides identified, ACE-inhibitory activity was reported by Sanchez-Rivera et al.
393 (2014) in fragments α_{s1}-CN(f157-164) and β-CN(f133-139). These peptides were found
394 throughout the 9 w ripening of Danish blue cheese. Many peptides from both α_{s1}- and β-casein
395 could not be ascribed to the action of PR-R proteinases, chymosin, plasmin and lactocepins;
396 these peptides may have been processed further by the action of peptidases.

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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.

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Results

Table 1

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

	pH	%moisture	%N	%(Nx6.38)	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}
2w	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}

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.

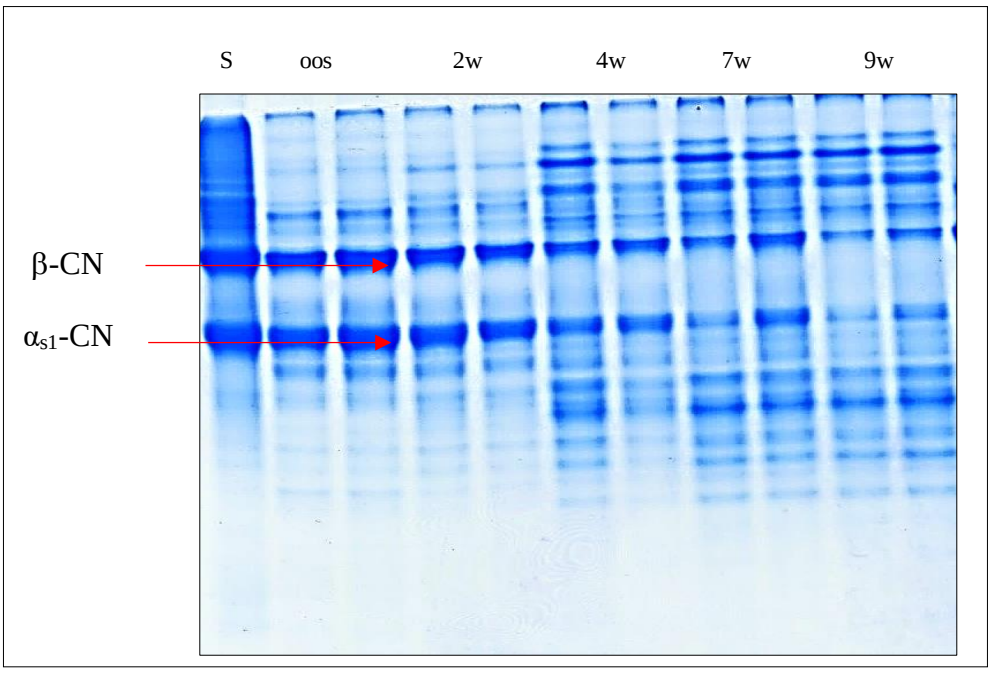

Fig. 1. Urea polyacrylamide gel electrophoretograms of pH 4.6-insoluble nitrogen fraction of Danish blue cheese at different ripening periods. Standard protein markers: sodium caseinate: (S), Lane 2-6 depicts pH 4.6-insoluble nitrogen fraction separated from cheese ripened for Out of Salt (OOS), 2 weeks: (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.

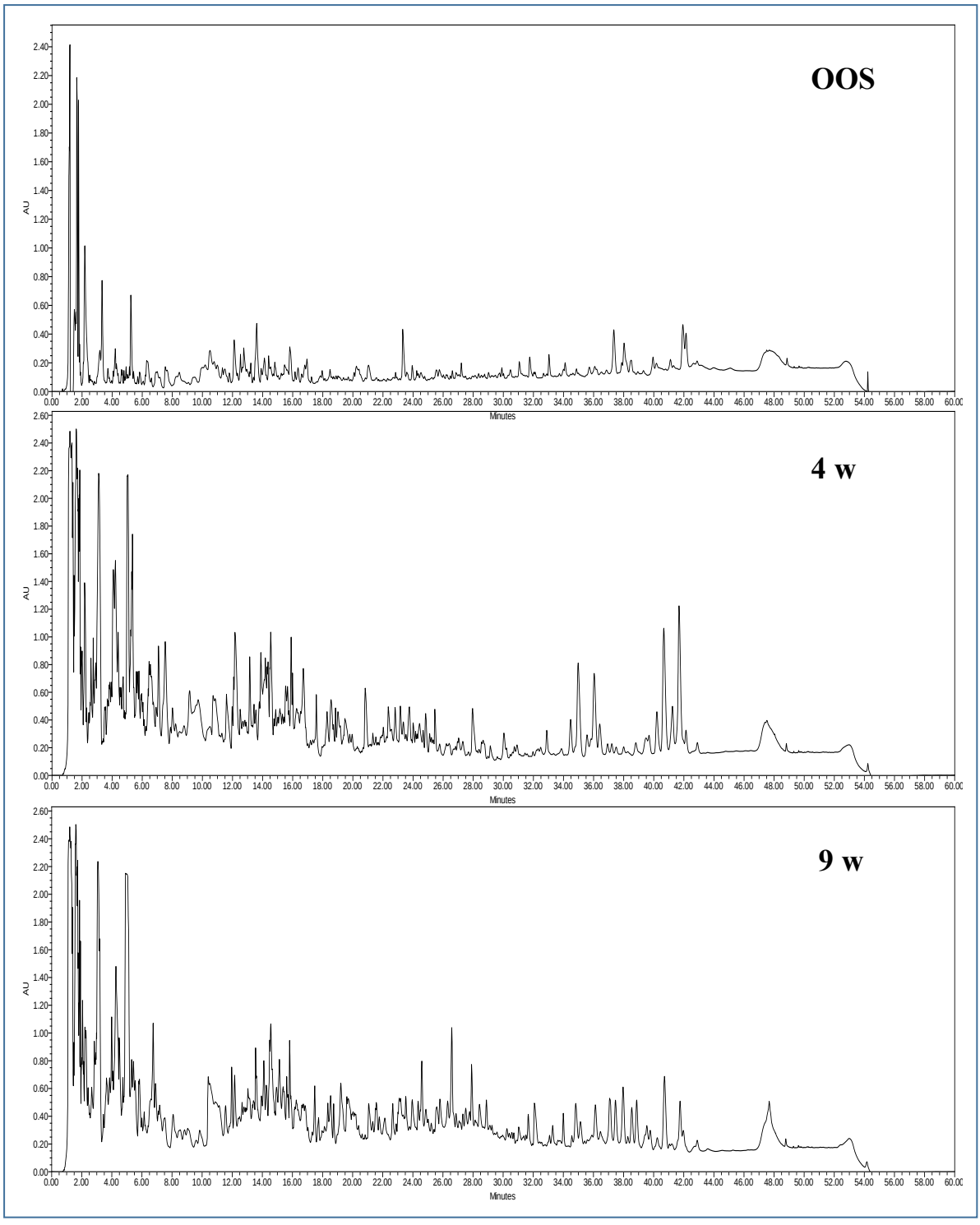

Fig. 2. Ultra performance liquid chromatograms (C_8 column) of pH 4.6-soluble extracts from Out of Salt (OOS), 4 weeks (4 w) 9 weeks (9 w) of ripening in Danish blue cheese, at wavelength of 214 nm.

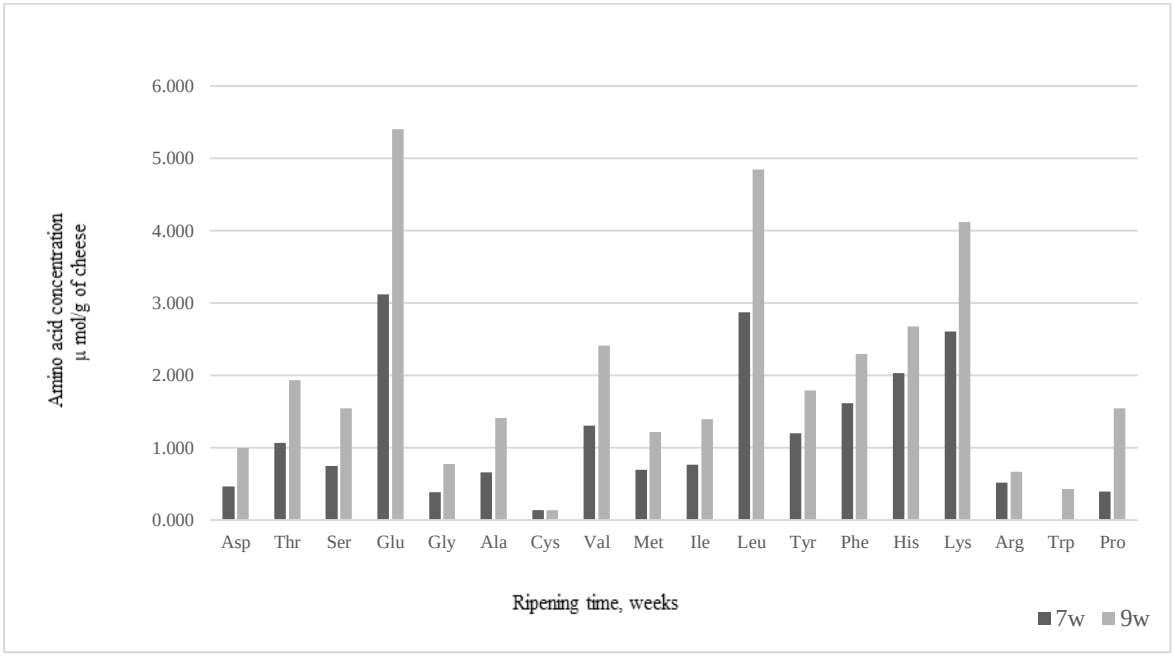

Fig. 3. Concentration (µ mol g^{-1} of cheese) of free amino acids in pH 4.6-soluble extracts from Danish blue cheese at 7 weeks (7 w) and 9 weeks (9 w) of ripening.

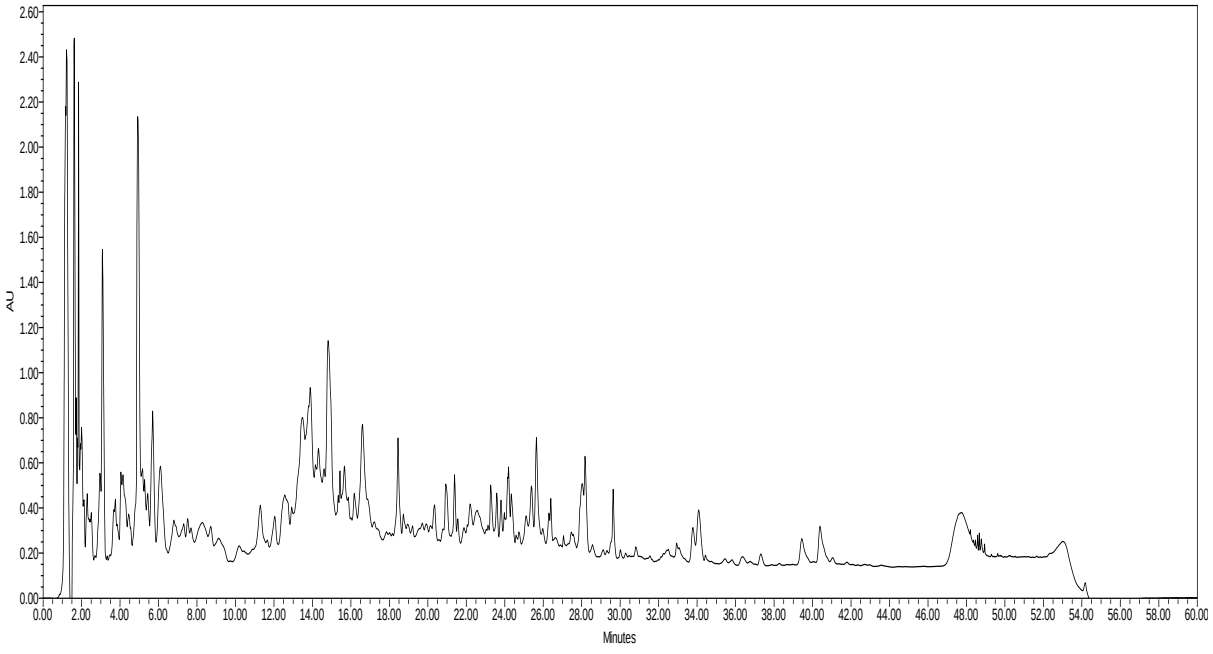

Fig. 4. Ultra performance liquid chromatograms (C_8 column) peptide profiles of cell free supernatants of *Penicillium roqueforti* strain PR-R in 1:1 ratio of 10% (low heat skimmed milk powder) LHSMP and potato dextrose broth for 7 d at 25ºC, at wavelength of 214 nm.

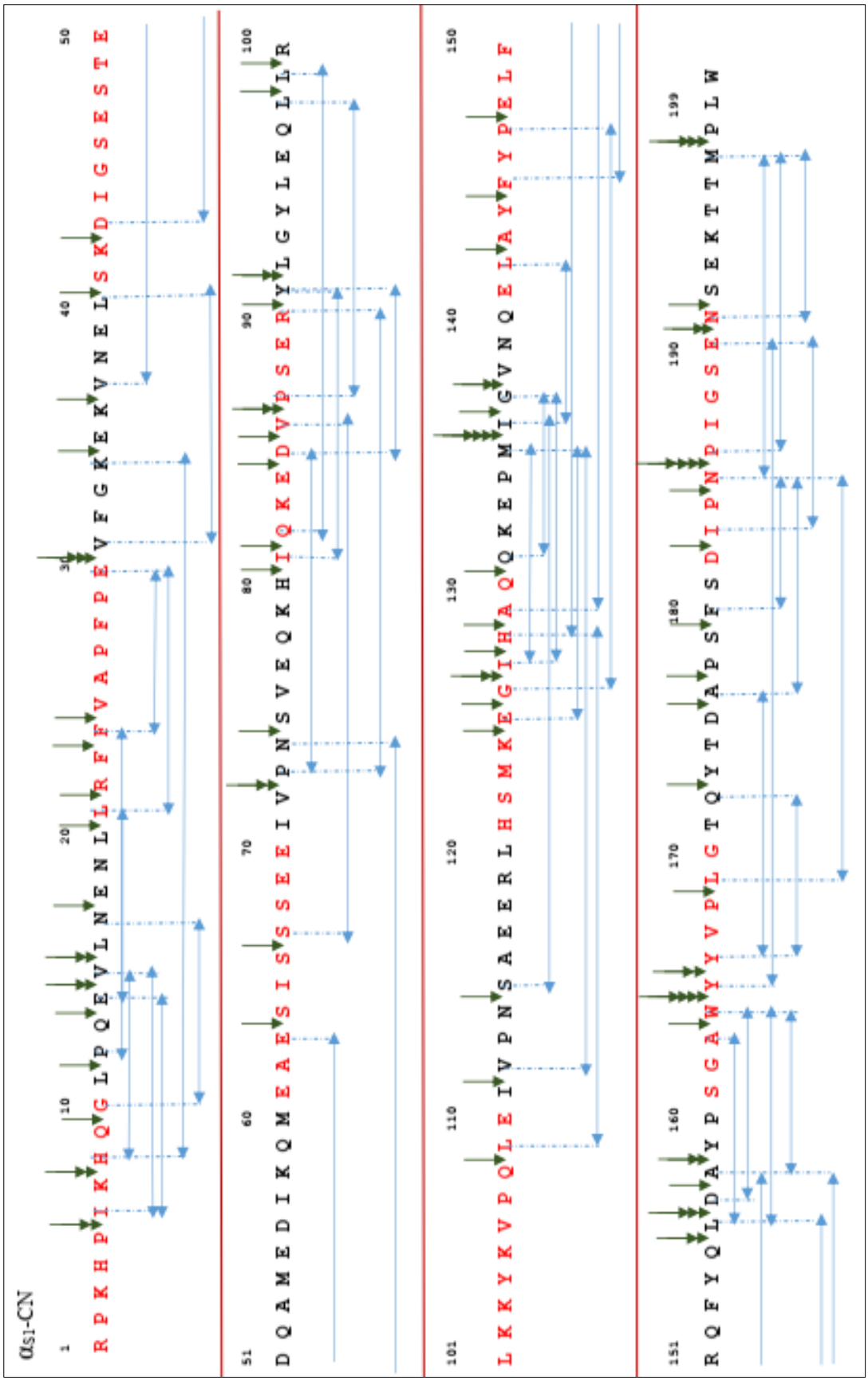

Fig 5. A]The primary structure of bovine α_{s1}-casein, , showing the peptides produced by proteinases from cell free supernatants of *Penicilium roqueforti*, PR-R incubated in 1:1 milk (10% low heat skimmed milk powder) and Potato Dextrose Broth suspension on shaking incubation for 7 days at 25°C.

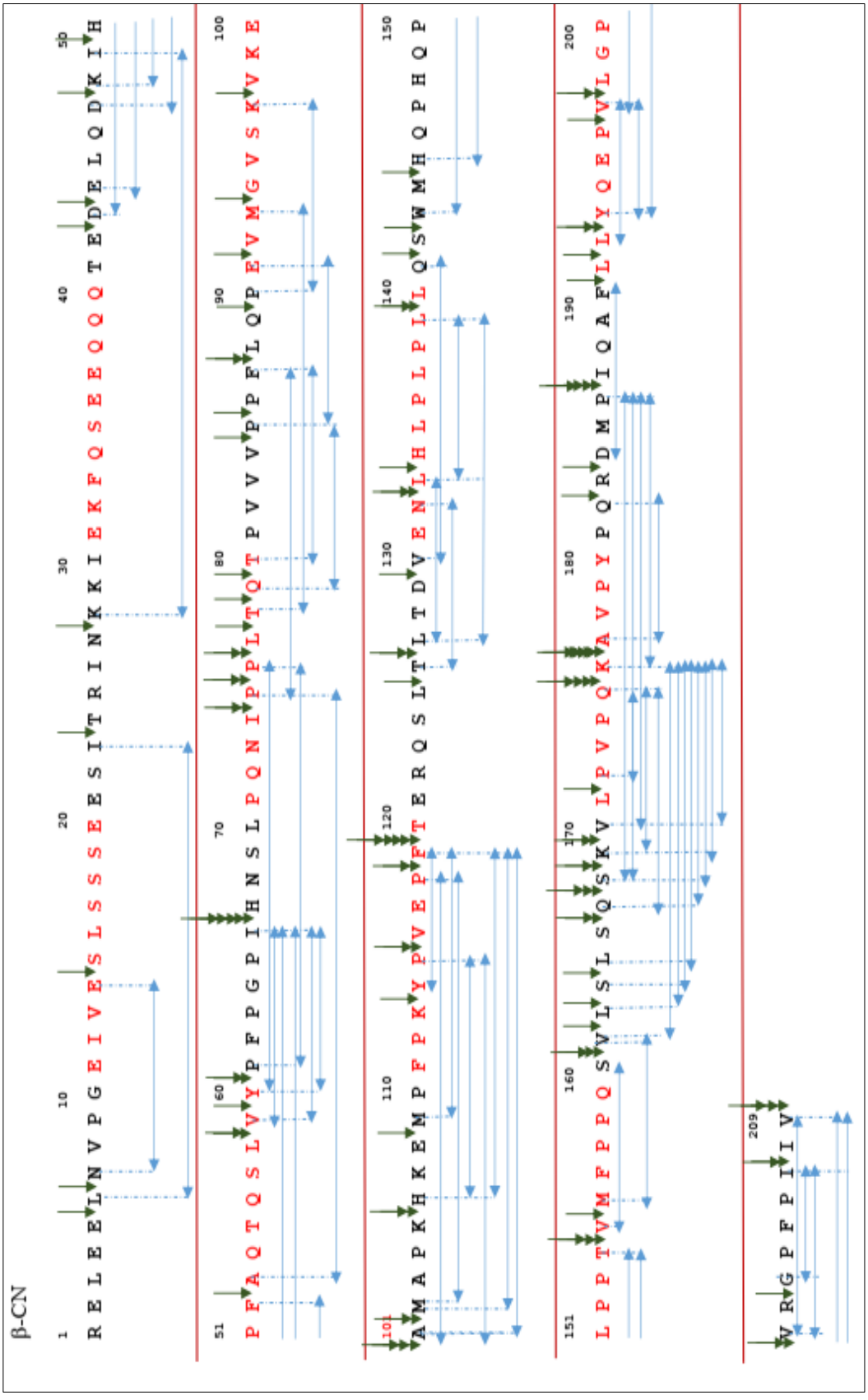

Fig 5. B] The primary structure of bovine β-casein, , showing the peptides produced by proteinases from cell free supernatants of *Penicilium roqueforti*, PR-R incubated in 1:1 milk (10% low heat skimmed milk powder) and Potato Dextrose Broth suspension on shaking incubation for 7 days at 25°C.

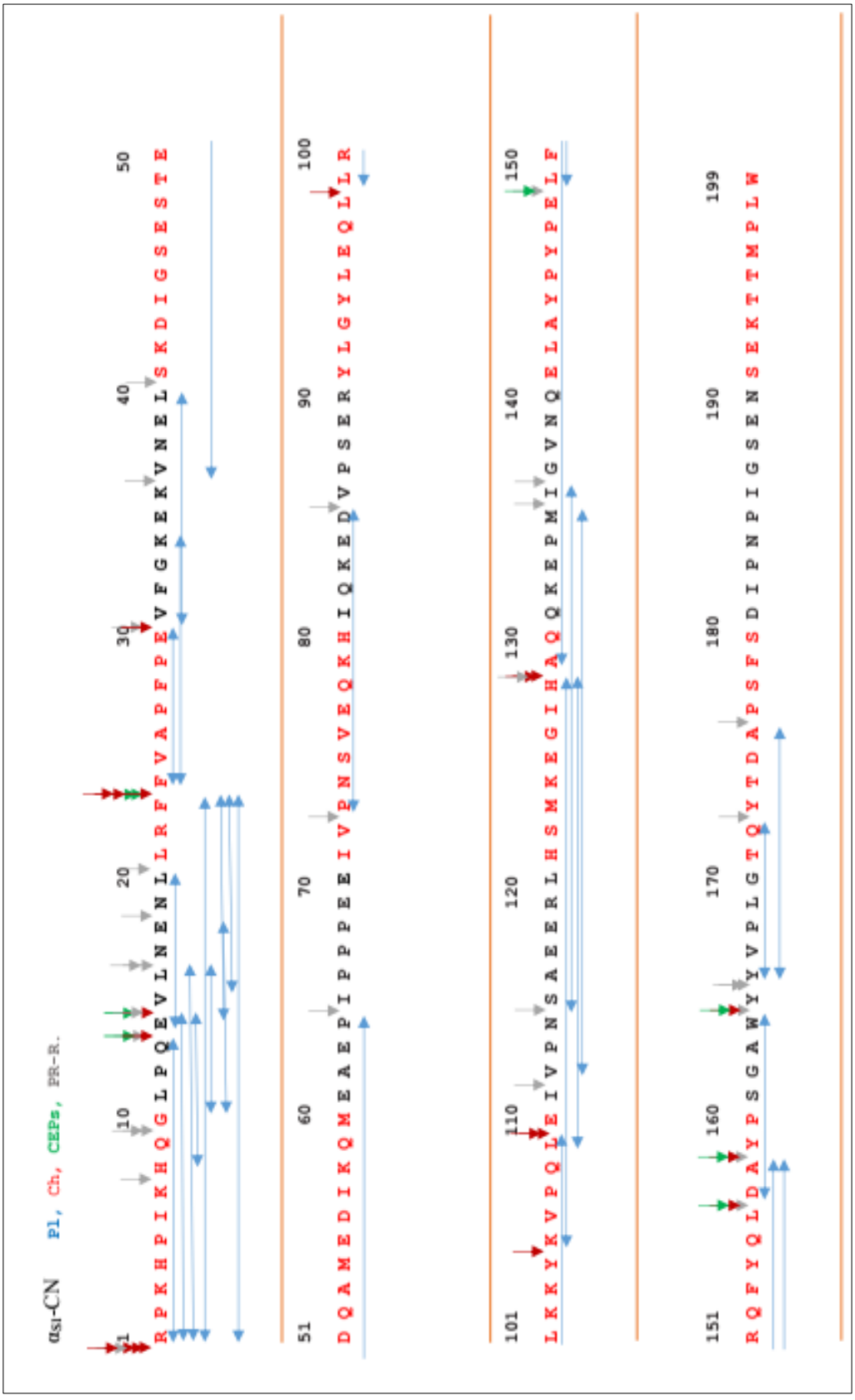

Fig.6. A] The primary structure of bovine α_{S1}-casein, , showing the peptides produced by proteinases from pH 4.6 soluble extracts of Danish blue cheeses, during 9 weeks of ripening.

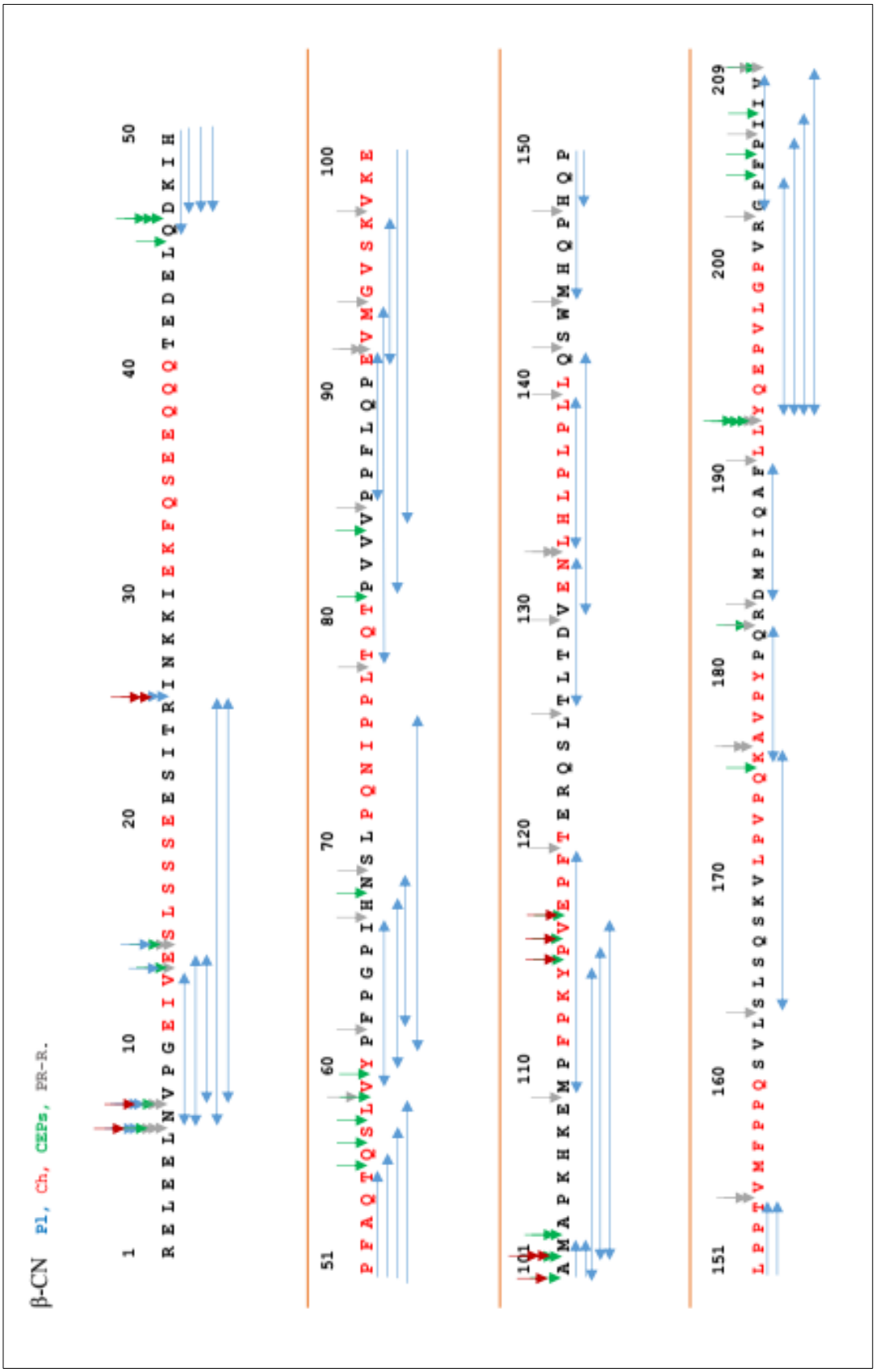

Fig.6. B] The primary structure of bovine β-casein, , showing peptides produced by proteinases from pH 4.6 soluble extracts of Danish blue cheeses, during 9 weeks of ripening.