Journal of Dairy Research (2015) **82** 491–498. doi:10.1017/S0022029915000254

Comparative study of the paracasein fraction of two ewe's milk cheese varieties

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Received 16 December 2014; accepted for publication 30 March 2015; first published online 19 June 2015

The aim of the present work was to assess the characteristics of the paracasein of two ewe's milk cheese varieties using various concentrations of urea and EDTA to solubilise caseins and calcium. The solubilised paracasein elements were evaluated by means of RP-HPLC and AAS. For this purpose cheeses with different physical and biochemical characteristics, i.e. Feta (53·1% moisture and pH 4·32) and Graviera Kritis (33·2% moisture and pH 5·54) were analysed. Soluble calcium of Feta was 71% of total calcium much higher than the 25% in Graviera. Treatment with 4 M urea fully solubilised Feta paracasein, whereas 6 M urea was needed to solubilise caseins from Graviera. Caseins were released from both cheeses by 100 mM EDTA. Solubilisation of paracasein induced by urea or EDTA was not significantly affected ($P < 0{\cdot}05$) by the type of cheese. Similarly to urea, EDTA induced significantly ($P < 0{\cdot}05$) lower solubilisation of αs1-casein in Graviera than in Feta, based on αs1-cn/β-cn ratio. A great part of calcium in both cheeses was solubilised by 50 mM EDTA while the release of casein was poor, confirming the important role of types of interactions other than protein-calcium bonds in the paracasein network. Hydrophobic interactions, hydrogen bonds and electrostatic attractions, contributed substantially to the paracasein stability of both cheese types. The interactions of αs1-casein with calcium played a more significant role in Graviera cheese than in Feta. Finally, the present study demonstrated that the profile of bonds and interactions within the cheese paracasein network was dynamicly configured by the conditions of cheese manufacture.

Keywords: Paracasein network, cheese calcium, urea, EDTA, Feta, Graviera Kritis.

Different cheesemaking conditions result in a great variety of cheeses with variable gross composition, appearance, sensory properties and shelf-life. The greatest part of them is made from rennet curds. This type of curd is a network resulting from the aggregation of paracasein micelles induced by the ionic calcium. Paracasein micelles are a complex of the enzymatically (rennet-) modified casein and inorganic milk components, mainly calcium and phosphorus. In this respect the common feature of the majority of the cheeses is the 3-dimentional paracasein network, in which fat, whey and microorganisms are embedded. Cheese making conditions determine the pH, the concentration of the minerals and the ratio of paracasein to other cheese components. Cheese ripening conditions induce changes in the paracasein network that affect all the characteristics of the final product e.g. through proteolysis (Lawrence et al. 1984; Johnson & Lucey, 2006).

The action of rennet on κ-casein destabilises casein micelles thus modifying the balance of electrostatic repulsions and attractive hydrophobic interactions (Horne, 1998) that configure bonding within the paracasein network. Lefebvre-Cases et al. (1998) concluded that the rennet milk gel at pH 6·7 is stabilised mainly through hydrophobic interactions and ion calcium bonds and to lesser extent through hydrogen bonds. On the other hand, Keim et al. (2006) suggest that 75% of the stabilising bonds in rennet-induced gels of UF concentrate of skim milk are calcium bonds, whereas the contribution of hydrophobic bonds is negative. The characteristics of cheese paracasein and especially the insoluble calcium content modulate cheese texture and determine cheese functionality (Lucey, 2008).

Dissociating agents like urea, SDS, EDTA mercaptoethanol and NaCl have been utilised in several studies as tools to investigate the paracasein of rennet curds, cheese curds and processed cheese or the effect of various treatments on these protein matrices. For example, Lefebvre-Cases et al. (1998) studied the structure of rennet gels by dispersing them in solutions containing from 2 to 20 g SDS/l, from 1 to 6 M urea or 2 mM EDTA and Alessi et al. (2007) assessed protein interactions in rennet curds made from reconstituted low-heat skim milk powder using

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treatments with 1–8 M urea, 2–50 mM EDTA and 0–2 M NaCl. Gagnaire et al. (2002) dissociated the curd of Emmental cheese by 0·5–6 M urea and 2–200 mM EDTA. Marchesseau & Cuq (1995) used SDS, urea, mercaptoethanol and EDTA to study the water-holding capacity and the protein interactions in processed cheese. The same approach was applied by Kim et al. (2011) to investigate protein interactions in reduced-fat and full-fat Cheddar cheeses during melting. Zamora et al. (2012) used 6 M urea, 1% SDS and 2 mM EDTA to study the effect of conventional and ultra high-pressure homogenisation of cheese milk on the protein-protein interactions in the curds made therefrom. Keim et al. (2006) and Hinrichs & Keim (2007) used different buffer systems with SDS, dithiothreitol, NaCl and EDTA/Na citrate to extract proteins from pressure-, heat- and rennet-induced gels of UF concentrate of skim milk and from cheeses.

The aim of the present work was to study the characteristics of the paracasein network of two ewe's milk cheese varieties, i.e. Feta and Graviera Kritis. Feta and Graviera were chosen because they differ widely in respect of cheesemaking conditions, physicochemical composition, biochemical features and textural properties. Feta is a white brined semi-hard cheese with low pH made from curds that have not been heat-treated. Dry salting is applied, draining is by gravity and ripening is carried out in brine with 7–8% salt for at least 2 months. Its mean gross physicochemical composition (w/w) is as follows: moisture 55%, fat-in-dry matter 51%, protein 17·5%, salt in moisture 5·2% and pH 4·60 (Moatsou & Govaris, 2011). Graviera Kritis is a hard cheese made from curds that drain intensively, scalded up to 50 °C at pH 6·2–6·3, pressed, salted in brine and ripened for at least three months. Mean moisture content of Graviera is about 33–35%, fat-in-dry matter 53–58%, protein 25–27%, salt-in-moisture 4 % and pH 5·5–5·8 (Kandarakis et al. 1998; Moatsou et al. 2004a; Nega & Moatsou, 2012). As a result of different cheesemaking technologies Feta cheese contain 318 ± 99·1 mg calcium/100 g and 248 ± 82·7 mg phosphorous/100 g much lower than respective Graviera contents 963 ± 104·8 and 623·9 ± 61·9 mg/100 g (Nega et al. 2011).

For the purpose of the present study various concentrations of urea and EDTA were used to dissociate caseins and calcium from the paracasein network, based mainly on the studies of Lefebvre-Cases et al. (1998) and Gagnaire et al. (2002). To our knowledge, there is only one previous comparative study of this type involving various ripened cheese varieties presented by Hinrichs & Keim (2007). They utilised destabilising buffers affecting protein-protein interactions and the assessment of the results was based on the nitrogen content of the supernatants.

Materials and methods

Materials

A Vydac C4 214 TP 5415 4·6 × 150 mm column was used for RP-HPLC analyses (Separation Group, Hesperia, CA, USA). Urea, EDTA (Ethylenediaminetetraacetic acid disodium salt), Tris (hydroxymethyl-aminomethane), trisodium hydrate, 1,4 dithiotheitol, lanthanum chloride, trichloroacetic acid (TCA) and acetonitrile (Lichrosolv grade) were purchased from Merck KgaA (Darmstadt Germany). Trifluroacetic acid (TFA) was from Sigma-Aldrich Co (St Louis, MO, USA) and nitric acid 65% was from Chem-Lab NV (Zedelgem, Belgium). Filter papers No 41 and 0·45 µm PVDF filters (Puradisc) were from Whatman International Ltd (Maidstone, England).

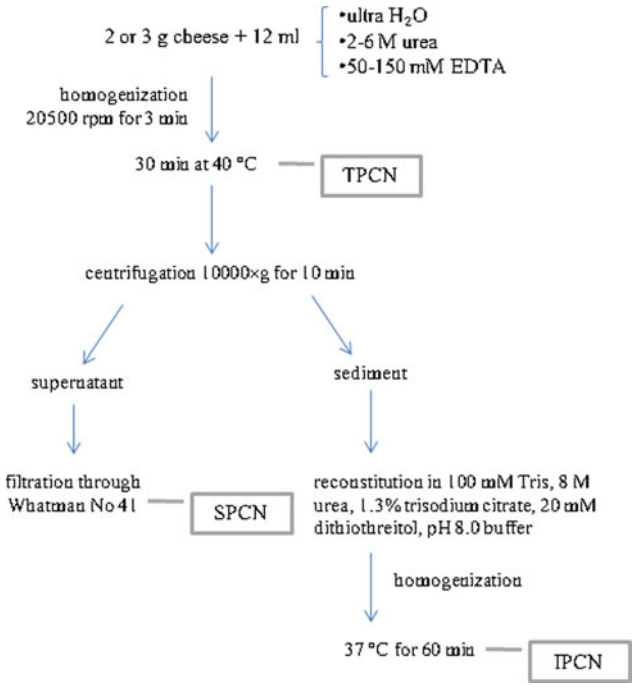

Fig. 1. Scheme applied for the solublisation of cheese paracasein. TPCN, cheese total paracasein; SPCN, solubilised cheese paracasein; IPCN, insoluble cheese paracasein.

Preparation of soluble casein fraction of cheeses

Three Feta and three Graviera Kritis cheese samples were analysed in respect of individual casein and calcium content of their paracasein fraction. The preparation was based on Gagnaire et al. (2002). Ultra pure water and various concentrations of urea and EDTA were used as extractants in the fractionation scheme of Fig. 1. During each extraction three fractions were collected, which were dispersions of cheese total paracasein (TPCN), soluble casein (SPCN) and insoluble paracasein (IPCN). Homogenisation was carried out using an Ultraturrax (Janke & Henkel, Bioblock, Illkirch, France).

Chromatographic analysis

RP-HPLC analysis of the above described fractions was according to Moatsou et al. (2004b). A Vydac C4, 5 µm, 300 Å, 4·6 × 150 mm column was used. The HPLC system consisted of a pump capable of mixing four solvents

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(Waters 600E, Waters, 34 Marple Street, Milford, MA, 01757, USA), a photodiode array detector (Waters 996), a helium degasser and an autosampler (Waters 717). Solvent A was 1060 µl TFA/L in ultra pure water and Solvent B was 1 ml TFA, 800 ml acetonitrile and 200 ml ultra pure water. Flow rate was 1 ml/min, and the eluent was monitored at 214 nm. A linear gradient from 350 to 620 ml/L solvent B within 54 min was applied. One ml TPCN or IPCN was mixed with 7 ml 100 mM Tris, 8-M urea–13 g trisodium citrate/l–20-mM dithiothreitol, pH 8·0 buffer. One ml SPCN was mixed with 7 ml 8·75 M urea–16 mM dithiotheitol solution. After incubation at 37 °C for 1 h, 150 µl 10% (v/v) TFA were added to sample preparations to achieve pH < 3·0. After filtration through 0·45 µm PVDF filter, 70 µl of each preparation were injected.

Calcium content of cheeses and SPCNs

Calcium in cheese ash was determined as described by Zoidou et al. (2015). In particular, the determination of Ca in the ash fraction of cheeses was carried out by means of the atomic absorption spectrometric (AAS) method (International Standard ISO/IDF, 2007) using a Shimadzu AA-6800 Atomic Absorption Spectrophotometer equipped with the autosampler Shimadzu ASC-6100. For the stock dilution 40 mg of cheese ash were diluted in 1 ml nitric acid 25% (v/v) and then the final volume was made up to 100 ml with ultra pure water. Various quantities of the stock ash dilution were further diluted in ultra pure water after the addition of 10 ml lanthanum chloride solution (1% in 25% nitric acid, v/v). Determinations were carried out in duplicate.

The SPCN filtrates of Fig. 1 were mixed with an equal quantity of 25% w/w TCA. After 2 h, the mixtures were centrifuged at 12500 ***g*** for 5 min at room temperature and the supernatants were filtered through Whatman No 41 filter paper (Brulé et al. 1974; Gagnaire et al. 2002). Six hundred µl were mixed with 1 ml 25% nitric acid (v/v) and the mixture was further diluted with ultra pure water up to 10 ml. Nine ml of this stock solution and 10 ml 1% (w/v) lanthanum chloride were mixed for the preparation of an 100 ml working dilution. Analyses were carried out in duplicate by means of the above mentioned AAS system and the estimations were based on reference curves constructed with and without urea and EDTA.

Statistical analysis

The software Statgraphics Centurion XVI (Manugistics, Inc., Rockville, MA 20852, USA) was used for the assessment of the results. The effect of cheese variety and urea or EDTA treatment on the solubilisation of paracasein elements was tested by two-way analysis of variance (ANOVA). One-way ANOVA was applied to test the effect of solubilising conditions, i.e. of various concentrations of urea or EDTA, on each cheese variety. Differences were tested using the least significance method (LSD) at $P < 0{\cdot}05$.

Results and discussion

The effect of various concentrations of urea and EDTA on the solubilisation of paracasein elements of Feta and Graviera Kritis cheese, was investigated by means of two-way ANOVA, considering as factors: the type of cheese and the level of the dissociating agent. The results are presented in Tables 1 & 2 respectively. In Fig. 2 are presented the RP-HPLC profiles of the soluble fractions prepared as described in Section 2.1 and Fig. 1. For the quantitative evaluation of the profiles only αs1-, para-κ- and β-casein were considered. The evaluation of low concentrations of αs2-casein was not reliable due to multiple overlapping peaks resulting from the phosphorylation pattern of this casein. The estimations of β-casein included also the co-eluted γ-caseins. Soluble paracasein, i.e. the sum of peaks of the above-mentioned profiles of the extracted fractions (Fig. 2) symbolised as SPCN was expressed as percentage of the sum of respective peaks of TPCN profiles before centrifugation (Fig. 1), that is 100× (SPCN/TPCN). No sediment coefficient was considered in the calculations; therefore percentages totalling more than 100% are presented. The detailed results of the present study, i.e. the solubilised total paracasein and calcium of cheeses and the ratios of individual caseins in the solublised fractions, are shown in Tables 3 & 4. In particular, Table 4 presents the ratios of solubilised αs1- and para-κ-casein on solubilised β-casein for each treatment.

Mean total calcium content of Feta samples was 436 ± 186 (108 mM) and that of Graviera was 1422 ± 106 mg/100 g (354 mM); the respective moisture contents were 53·1 ± 2·71% and 33·2 ± 4·21%. The treatment of cheese with water provided information about the partition of calcium in the paracasein and whey fractions of cheeses. High soluble calcium (SolCa) was determined in Feta, which was 71% of total Ca much higher than the 25% in Graviera (Table 3). Consequently, 583 mg calcium were soluble in 100 g moisture of Feta cheese and 1055 mg in 100 g moisture of Graviera on average. pH of Feta and Graviera were 4·32 ± 0·051 and 5·54 ± 0·051 respectively. The low acidification and the very low moisture content resulted in high insoluble calcium in Graviera Kritis paracasein matrix. The opposite was true for Feta cheese. In low pH cheeses more colloidal calcium phosphate is expected to be solubilised and the configuration of the paracasein matrix changes considerably as the pH decreases towards casein isoelectric point (Lawrence et al. 1984; Upreti & Metzger, 2007). In addition, there are two other factors that decrease insoluble calcium content of Feta. Firstly, there is migration of calcium into the keeping brine (Zoidou et al. 2015). Secondly, the higher Na content of Feta affects the calcium content and distribution, because Na can take the place of casein Ca, which is part of colloidal calcium phosphate linked to the organic P of casein (Kindstedt et al. 1992). With regard to individual caseins, the extraction with water induced a very limited solublisation in both cheeses. This water extract consisted mainly

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Table 1. Two way ANOVA results on the effect of various urea concentrations on the solubilisation of paracasein elements of Feta (F) and Graviera (G) cheeses

Factor	*n*†	STCN‡	SolCa§	αs1-cn/β-cn¶	p-κ-cn/β-cn¶
Cheese type					
Feta	*12*	62·1	$89·8^{b}$	$1·1^{b}$	$0·53^{b}$
Graviera	*12*	66·0	$47·8^{a}$	$0·6^{a}$	$0·41^{a}$
LSD††		*12·99*	*6·52*	*0·45*	*0·12*
Urea (M)					
0	*6*	$12·5^{a}$	$47·8^{a}$	$0·28^{a}$	$0·03^{a}$
2	*6*	$41·2^{b}$	$53·3^{a}$	$1·28^{c}$	$0·36^{b}$
4	*6*	$90·0^{c}$	$83·1^{b}$	$0·76^{b}$	$0·57^{c}$
6	*6*	$112·5^{d}$	$90·9^{b}$	$0·93^{b,c}$	$0·92^{d}$
LSD††		*20·20*	*9·23*	*0·32*	*0·17*

[a–d]Different superscript indicate statistically significant differences within columns of each factor, $P < 0·05$
†Number of observations
‡Soluble paracasein of Feta and Graviera Kritis, expressed as percentage of the respective total cheese paracasein
§Solubilised calcium expressed as percentage of total cheese Ca
¶Casein
††Least significance difference

Table 2. Two way ANOVA results on the effect of various EDTA concentrations on the solubilisation of paracasein elements of Feta (F) and Graviera (G) cheeses

Factor	*n*†	STCN‡	SolCa§	αs1-cn/β-cn¶	p-κ-cn/β-cn¶
Cheese type					
Feta	*12*	59·8	68·1	$0·71^{b}$	0·59
Graviera	*12*	58·1	65·4	$0·59^{a}$	0·59
LSD††		*2·96*	*13·67*	*0·13*	*0·12*
EDTA (mM)					
0	*6*	$12·5^{a}$	$47·8^{a}$	$0·28^{a}$	$0·03^{a}$
50	*6*	$10·8^{a}$	$74·5^{b}$	$0·43^{a}$	$0·28^{b}$
100	*6*	$104·8^{b}$	$74·1^{b}$	$0·97^{b}$	$1·05^{c}$
150	*6*	$107·8^{b}$	$70·6^{b}$	$0·93^{b}$	$0·99^{c}$
LSD††		*4·18*	*19·33*	*0·18*	*0·18*

[a–c]Different superscript indicate statistically significant differences within columns of each factor, $P < 0·05$
†Number of observations
‡Soluble paracasein of Feta and Graviera Kritis, expressed as percentage of the respective total cheese paracasein
§Solubilised calcium expressed as percentage of total cheese Ca
¶Casein
††Least significance difference

of β-casein, which is abundant in both milk casein and cheese paracasein and is less phosphorylated than αs1-casein. According to Holt et al. (1986) the loss of each casein from the micelle could be linked to the number of phosphorylated residues per protein molecule that interact directly with colloidal calcium phosphate (CCP).

Interestingly, the level of caseins extracted by water was similar for both cheese types, despite the great difference in their insoluble calcium percentage. The behaviour of paracasein elements under treatments with urea and EDTA was expected to provide information about the contribution of other mechanisms apart from calcium-casein interactions. Urea disorganises the paracasein complex as it forms hydrogen bonds with parts of the protein molecules, which disrupt intramolecular hydrogen bonds and weaken hydrophobic interactions (Nozaki & Tanford, 1963). EDTA chelates calcium thus depleting colloidal calcium phosphate from casein or paracasein complex.

According to Table 1, the solubilisation of paracasein (STCN) induced by urea was not significantly affected ($P <$ 0·05) by the type of cheese, indicating that the contribution of hydrogen bonds and hydrophobic interactions was not differentiated. The opposite was true for SolCa, which apparently was due to the initial differences in their SolCa percentage (Table 3). The profiles of solubilised caseins of Feta and Graviera were significantly different. The contribution of αs1-casein and para-κ-casein in the urea extractants of Feta were significantly higher than those of Graviera.

Treatment with 6 M urea was necessary to dissociate the paracasein of both cheese types (Tables 1 & 3), although a

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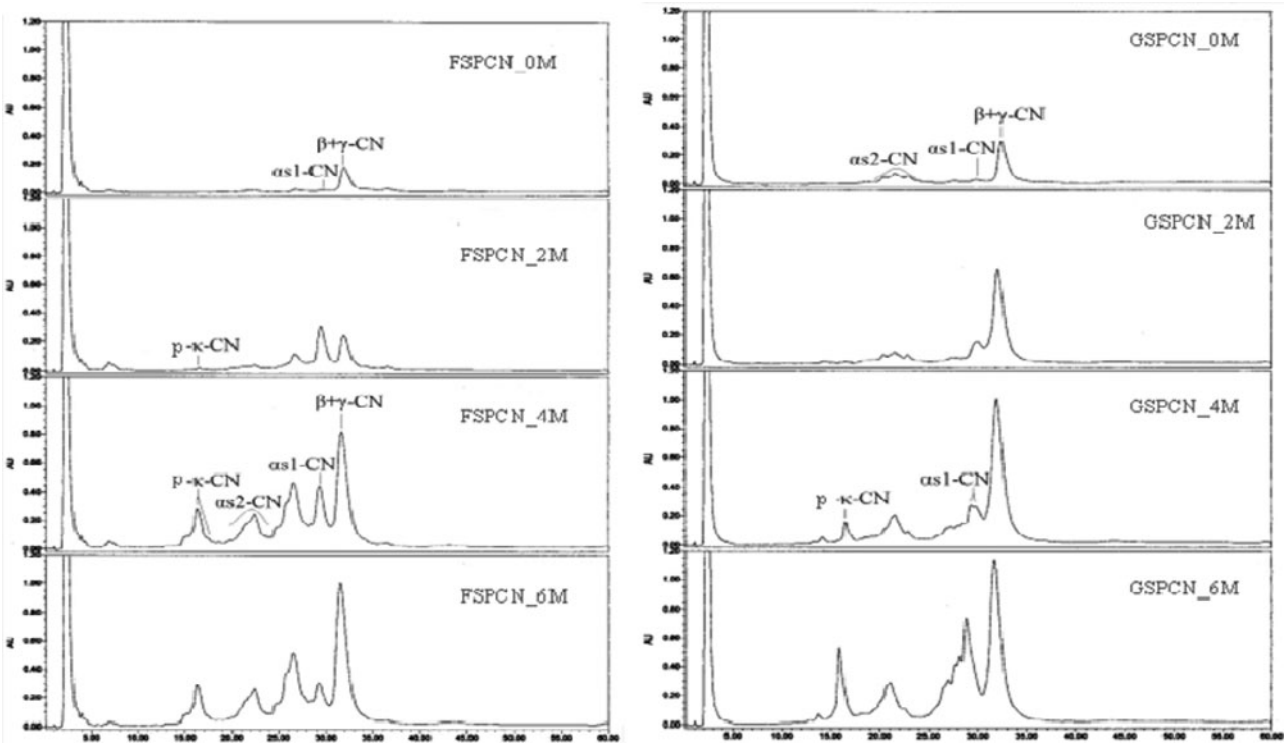

Fig. 2. RP-HPLC profiles of SPCNs of urea and EDTA cheese extracts prepared according to Fig. 1. FSPCN and GSPCN, soluble paracasein of Feta and Graviera Kritis respectively.

Table 3. Solubilisation of paracasein elements of Feta (F) and Graviera (G) cheeses by means of different urea and EDTA concentrations (average of three different cheese samples ± SD)

Solubilising agent	FSTCN† (% FTPCN)‡	GSTCN† (% GTPCN)‡	FSolCa (% total Ca)	GSolCa (% total Ca)
Urea (M)				
0	10·9 ± 2·15[a]	14·0 ± 3·19[a]	71·0 ± 13·94[a]	24·6 ± 1·05[a]
2	20·3 ± 5·20[a]	62·1 ± 13·40[b]	74·4 ± 10·55[a]	32·3 ± 2·31[b]
4	103·8 ± 13·10[b]	76·2 ± 5·06[b]	107·3 ± 6·82[b]	58·8 ± 4·57[b]
6	113·5 ± 5·55[b]	111·5 ± 0·93[c]	106·4 ± 2·69[b]	75·5 ± 5·49[c]
EDTA (mM)				
0	10·9 ± 2·15[a]	14·0 ± 3·19[a]	71·0 ± 13·94[a]	24·6 ± 1·05[a]
50	9·7 ± 2·52[a]	11·9 ± 2·00[a]	69·5 ± 11·74[a]	79·5 ± 5·77[b]
100	108·6 ± 1·72[b]	101·0 ± 4·46[b]	67·3 ± 5·92[a]	81·0 ± 4·19[b]
150	110·2 ± 1·65[b]	105·4 ± 1·14[b]	64·7 ± 9·59[a]	76·6 ± 2·82[b]

[a–c]Different superscript indicate statistically significant differences within columns, $P < 0{\cdot}05$
†FSPCN and GSPCN, soluble paracasein of Feta and Graviera Kritis respectively, expressed as percentage of TPCN
‡FTPCN and GTPCN, total paracasein (TPCN) of Feta and Graviera Kritis respectively
§ FSolCa and GSolCa, solubilised calcium of Feta and Graviera Kritis respectively, expressed as percentage of total cheese Ca

small amount of caseins remained in the sediment IPCN of Fig. 1 (data not shown). The increase of urea concentration to 4 M solubilised almost fully Feta paracasein, in contrast to 76% solubilisation of Graviera paracasein (Table 3). The dissimilar effect of urea concentrations <6 M was due to the behaviour of the most abundant components of the paracasein, i.e. αs1- and β-casein. In particular, low urea concentrations solubilised further the β-casein in Graviera but they affected more intensively the solubilisation of αs1-casein in Feta, as depicted in Table 4. Marchesseau et al. (2002) report that 4 M urea dissociates protein in acidified milk gel but in rennet milk gel 6 M urea is needed. On the other hand, Gagnaire et al. (2002) report that only β-casein was significantly affected by treatments with <6 M

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Table 4. Ratios of solubilised individual caseins of Feta and Graviera cheeses by means of different urea and EDTA concentrations (average of three different cheese samples ± SD)

	αs1-cn/β-cn†		p-κ-cn/β-cn†	
Solubilising agent	Feta	Graviera	Feta	Graviera
Urea (M)				
0	0·34 ± 0·041[a]	0·21 ± 0·059[a]	0·04 ± 0·032[a]	0·03 ± 0·025[a]
2	1·88 ± 0·803[b]	0·67 ± 0·136[b]	0·36 ± 0·142[b]	0·36 ± 0·087[b]
4	0·99 ± 0·040[a]	0·53 ± 0·127[b]	0·83 ± 0·035[c]	0·32 ± 0·087[b]
6	0·97 ± 0·055[a]	0·89 ± 0·035[c]	0·91 ± 0·056[c]	0·93 ± 0·075[c]
EDTA (mM)				
0	0·34 ± 0·041[a]	0·21 ± 0·059[a]	0·04 ± 0·032[a]	0·03 ± 0·025[a]
50	0·68 ± 0·035[b]	0·18 ± 0·026[a]	0·36 ± 0·180[b]	0·21 ± 0·274[b]
100	0·91 ± 0·052[c]	1·03 ± 0·0157[b]	0·98 ± 0·031[c]	1·13 ± 0·233[c]
150	0·91 ± 0·060[c]	0·95 ± 0·025[b]	0·98 ± 0·047[c]	0·99 ± 0·023[c]

[a–c]Different superscript indicate statistically significant differences within columns, $P < 0·05$
†Casein

urea in Emmental curds suggesting that β-casein is weakly bound to other caseins in Emmental paracasein similarly to its behaviour in casein micelles.

The behaviour of Feta paracasein can be explained by the findings of Lee et al. (2005) that in cheese with pH < 5·0, the electrostatic attractions, with the approach of the casein isoelectric point would dominate, even if there is a reduction in other attractive interactions such as insoluble Ca crosslinks. Low contribution of calcium-casein bonds has been found by Hinrichs & Keim (2007) who used various buffers to solubilise cheese protein. They report that the two thirds of bonds in cheese with pH 4·6 similar to that of Feta are hydrophobic interactions followed by electrostatic interactions and/or hydrogen bonds at 10% and calcium bonds at 5%, opposite to rennet curds, in which calcium bridges dominate being approximately 75% of the stabilising bonds. According to the same authors, electrostatic interactions and/or hydrogen bonds also dominate at 85% of the stabilising forces in Gouda cheese with pH 5·2. They suggest that the profile of stabilising bonds changes during cheese ripening because proteolysis affects parts of casein which stabilise the structure via calcium bonds. The pattern of Feta proteolysis during ripening is consistent with this suggestion. It is well-known that after the first 2 weeks of ripening, up to half αs1-casein is hydrolysed but hydrolysis of β-casein is limited (Moatsou & Govaris, 2011). Considering that, (i) αs-casein possesses phosphorylation clusters with high affinity for calcium ions enhancing attraction with other αs-casein and β-casein molecules (Marchesseau et al. (2002), (ii) the opposite is true for para-κ-casein (Gagnaire et al. 2002), and (iii) due to its proteolysis pattern the main Feta paracasein element is the hydrophobic β-casein, the strong effect of 4 M urea on the removal of individual caseins from Feta paracasein can be explained. Since individual caseins were solubilised, the insoluble Ca associated with them turned into SolCa (Table 3). The pattern of proteolysis is different in Graviera Kritis. Its main feature is the hydrolysis of β-casein and the concomitant accumulation of γ-caseins due to plasmin activity and non-inhibitory pH. Cheesemaking conditions do not favour the presence of active residual chymosin in this cheese type, which is the main proteolytic factor for αs1-casein. In particular, residual chymosin activity was 0·029 IMCU per g Graviera dry matter, that is very low compared with 0·164 IMCU per g Feta dry matter, whereas the respective plasmin plus plasminogen derived activities were 5·53 and 3·58 U per g cheese (Nega & Moatsou, 2012). Therefore, αs1-casein associated with insoluble calcium was expected to play a major role in the paracasein network of Graviera. Similarly, Gagnaire et al. (2002) concluded that among caseins, the αs1-casein seems to contribute more to the structure of the curd of Emmental curds than β-casein.

The soluble calcium of cheeses at various urea concentrations (Tables 1 & 3) paralleled the solubilisation of paracasein. But, the solubilisation of calcium was less pronounced in Graviera in which 25% of calcium remained insoluble after dissociation of casein at 6 M urea. In Emmental curds treated with 6 M urea 53% of calcium remained insoluble although about 90% of the caseins were released (Gagnaire et al. 2002). The authors suggest that either a very small amount of non-solubilised casein can react strongly with CCP or that CCP not interacting strongly with solubilised caseins may precipitate out in the pellet. These findings taken together indicate that in Graviera cheese, αs1-casein interactions with calcium play a more significant role than in Feta.

According to the results of Table 2 and Fig. 2, solubilisation of paracasein (STCN) and calcium (SolCa) by various EDTA concentrations was not significantly influenced by cheese type and the same was true for the ratio of solubilised p-κ-cn/β-cn. But similarly to the effect of urea, EDTA also induced significantly lower solubilisation of αs1-casein in Graviera than in Feta, according to αs1-cn/β-cn ratio. Moreover, the increase from 50 to 150 mM EDTA caused no significant effect on the SolCa percentage of both cheeses (Tables 2 & 3). Caseins were released from both

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cheeses by 100 mM EDTA, in contrast to 150 mM reported in the study of Gagnaire et al. (2002) for Emmental curd. On the other hand, SolCa in Table 3 was always below 100%, although 100 mM EDTA fully solubilised caseins. Since the available mols of EDTA were sufficient to chelate cheese calcium, it was possible that EDTA-Ca complexes remained in the sediment. In fact, in Feta cheese none of the treatments with EDTA caused any significant increase of SolCa percentage. Considering that, (i) in 100 ml of the extraction solution of Fig. 1, there were initially 87·2 (22 mM) and 203 mg (51 mM) calcium for Feta and Graviera respectively, and (ii) EDTA chelates calcium stoichiometrically (Udabage et al. 2000; Gagnaire et al. 2002), can be explained why the increase of EDTA concentration from 50 to 150 mM did not significantly increase the solublisation of paracasein calcium.

The finding that a great part of calcium of both cheeses was solubilised by 50 mM EDTA while the release of casein was poor, i.e. 9·7 and 11·9 (Table 3) confirms the important role of other types of interactions. Similar effect of calcium removal on pelleted casein of milk has been reported. Holt et al. (1986) have found that a 50% reduction of the concentration of calcium cations induced by dialysis of milk against calcium phosphate buffers does not reduce pelleted caseins. Similarly, Udabage et al. (2000) report that treatment of milk with EDTA at a given temperature causes redistribution of casein not directly proportional to the changes in CCP but in the same direction, e.g. 5 mM EDTA removes 20% of pelleted Ca and releases 5% of casein, 10 mM removes 44% of the former and 30% of the latter, whereas 50 mM results in the complete disintegration of the micelles. However, in the present study the increase of EDTA from 50 to 150 mM even though not resulting in more SolCa in the supernatants increased the solubilisation of milk casein micelles. Gagnaire et al. (2002) attributed a similar effect observed in Emmental curds to the increase of ionic strength which affects electrostatic interactions. As mentioned earlier, electrostatic interactions and/or hydrogen bonds are considered by Hinrichs & Keim (2007) as being by far the main stabilising forces in the protein network of Gouda cheese pH with 5·2 contributing at 85%, whereas in Gouda curd grains pH 6·5, 80% of the bonds are calcium bridges and only 10% electrostatic and/or hydrogen bonds.

There is scarce information about the paracasein calcium in cheeses like Feta, i.e. low pH cheeses not resulted from acid-coagulation but from the rennet action. The mechanism of CCP solubilisation in milk and its effect on casein micelles is well-known. Lucey (2008) discussed the findings that much CCP appears to remain undissolved in cheese even if pH is around 4·7 whereas in milk at pH $\leq$ 5·2 it is completely dissolved. He concluded that acid development in the hoop when the curd particles are lower in moisture content does not favour CCP solubilisation because a rapid increase in the serum calcium beyond a certain level can result in precipitation of calcium phosphate. He also suggested that apart from moisture also the pH of cheese influences the maximum serum calcium concentration possible before precipitation occurs. Lee et al. (2005) report that when the serum calcium content is ~700 mg/100 g in low pH~4·7 Cheddar cheeses, the solubilisation of insoluble calcium is retarded. O'Mahony et al. (2006) found that in Cheddar cheese with pH 5·12 the critical concentration is 850–900/100 g, suggesting that pH, temperature, presence of other ions, ionic strength etc affect the actual limit of solubility. Furthermore, according to Lee et al. (2010) insoluble calcium decreases during cheese ripening without pH decrease or additional lactic acid formation if the moisture content is favourable to solubilise insoluble calcium by increasing the concentration of the serum phase. These findings are confirmed by the present study. As mentioned above the mean soluble calcium content of the cheeses of the present study, was 583 and 1055 mg per 100 g moisture for Feta and Graviera respectively and the respective cheese pH was 4·32 and 5·54.

Conclusions

Types of interactions other than protein-calcium bonds, i.e. hydrophobic-hydrogen bonds and electrostatic attractions, contributed substantially to the paracasein stability of both cheese types. The present findings taken together indicated that hydrophobic interactions and hydrogen bonds contributed more in the structure of Feta paracasein than in Graviera due to changes in paracasein-calcium interactions induced by low pH and the proteolysis pattern of this cheese type, which decreases more extensively the calcium binding sites onto the paracasein elements. Cheesemaking conditions affecting the physicochemical composition and the residual activity of enzymes during ripening determined the insoluble calcium content and the protein profile of the paracasein of each cheese variety, which in turn determined the profile of stabilising bonds. In this respect, it is suggested that αs1-casein interactions with calcium play a more significant role in Graviera than in Feta. Finally, the present study demonstrated that the profile of bonds and interactions within the cheese paracasein network was dynamically configured by the conditions of cheese production.

The present study has been carried out in the framework of the Programme of Postgraduate Studies entitled 'Integrated Production Management of Milk and Dairy Products'

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