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Proteolysis in reduced sodium Kefalograviera cheese made by partial replacement of NaCl with KCl

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Abstract

Kefalograviera cheeses (five trials) of different sodium content were made from split lots of curd by varying the salting processes, i.e. brine — and dry — salting, with NaCl (control) or a mixture of NaCl/KCl (3:1 or 1:1, w/w basis). The extent and characteristics of proteolysis in the cheeses were monitored during aging by Kjeldahl determination of soluble nitrogen fractions (water-soluble nitrogen [WSN], trichloroacetic acid [TCA]-SN, phosphotungstic acid [PTA]-SN), the cadmium-ninhydrin method for the determination of total free amino acids (FAA), urea-polyacrylamide gel electrophoresis of cheese proteins, followed by densitometric analysis of the α_{s1}- and β-casein fractions, reverse-phase high-performance liquid chromatography (HPLC) analysis of the water-soluble extracts of cheeses, and ion-exchange HPLC analysis of FAA. The results showed that proteolysis was similar in control and experimental cheeses at all sampling ages, indicating that the partial substitution of NaCl by KCl in the manufacture of Kefalograviera cheese did not significantly influence the extent and characteristics of proteolysis during cheese aging.

Keywords: Kefalograviera cheese; Cheese; Proteolysis; Sodium

1. Introduction

As a result of the association of sodium intake primarily with hypertension (Abernethy, 1979; Dillon, 1987; Freis, 1976; Tobian, 1979;), but also with osteoporosis (Goulding, Gold, & Campbell, 1993) and the incidence of kidney stones (Goulding, 1997), consumer concern about sodium in processed foods has increased (Reddy & Marth, 1993). The most frequent estimate of the minimum adult daily requirement for sodium is 200 mg (0.5 g of NaCl), while the average total daily sodium intake by most persons in developed countries is 4–5 g (10–12 g of NaCl) (IFT, 1980; Dillon, 1987). These quantities, which are 10–35 times greater than the minimum adult requirement (NAS/NRC, 1980a; Shank, Youngmee, Harland, Vanderbeen, Forbes, & Prosky, 1982), are regarded as excessive or even dangerous, by many of those responsible for public health (Dillon, 1987). A sodium intake of 1100–3300 mg (2.8–8.3 g of NaCl) per day has been recommended as safe and adequate for adults (NAS/NRC, 1980b).

Various studies have indicated that an increased intake of potassium via the diet can exert a protective effect in individuals with sodium-induced hypertension (Fregly, 1981; Haddy 1991; Lecos, 1983; Linas 1991), reduces urinary calcium excretion and potentially protects skeletal mass (Lemann, Pleuss, Gray, & Hoffmann, 1993).

Some dairy foods, such as natural and processed cheeses, are high in sodium content and means to reduce this have therefore been sought by the dairy industry and scientific community (Reddy & Marth, 1991). When the salt concentration in cheese is simply reduced, proteolysis, water activity, acidity and bitterness all increase, while firmness and saltiness decrease (Editorial, 1993); abnormal fermentations may also occur (Olson, 1982; Petik, 1987). All of these factors make it difficult to reduce the sodium level in cheese substantially, without adversely affecting quality. However, replacing some of the NaCl by KCl helps to address some of the above problems (Editorial, 1993). KCl has been the most widely and successfully used partial replacement for NaCl in cheese (Reddy & Marth, 1991).

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Proteolysis is the principal and most complex biochemical event occurring during the maturation of the majority of ripened cheese varieties (Fox, McSweeney, & Singh, 1995b). In addition to softening the cheese body, proteolysis influences the development of cheese flavor via the formation of amino acids and peptides, which make a direct contribution to flavor (Fox, Singh, & McSweeney, 1995a). Salt influences the rate of proteolysis in cheese by changing the aggregation status of protein substrate molecules (Mulvihill & Fox, 1978), affecting and controlling the growth of the starter and non-starter bacteria (Turner & Thomas, 1980) and directly affecting the activity of the enzymes involved (Kelly, Fox, & McSweeney, 1996). Hence, when NaCl in cheese is replaced by another salt, it becomes necessary to investigate several changes in such cheese (Reddy & Marth, 1993; Zorrilla, Castelao, De Piante, & Rubiolo, 1996; Zorrilla & Rubiolo, 1997).

Fitzgerald and Buckley (1985) reported that, not partial but total, substitution of KCl for NaCl enhanced proteolysis rates in Cheddar cheese. However, Reddy and Marth (1993) found no significant ($P > 0.05$) differences in proteolysis at a given sampling time among cheeses made with NaCl, KCl or mixtures of the two salts. Seiber, Bosset, and Bican (1991) reported that NaCl reduction, with partial substitution by KCl, intensified protein breakdown in Appenzell type cheese. Finally, Aly (1995) and Zorrilla et al. (1996) found that partial replacement of NaCl by KCl did not significantly influence the proteolysis in UF Feta-type or Fynbo cheeses.

Kefalograviera cheese, a traditional Greek hard cheese of Controlled Denomination of Origin, has a high salt content of about 3.4% (Katsiari, Voutsinas, Alichanidis, & Roussis, 1998), which is equivalent to 1338 mg Na per 100 g of cheese, since salt consists of about 39% sodium (IFT, 1980). Recently, we reported (Katsiari et al., 1998) the feasibility of reducing up to 50% of the sodium content in Kefalograviera cheese by using mixtures of NaCl and KCl (3:1 or 1:1, w/w) in the salting processes, without adversely affecting the quality. Our results also indicated that the cheeses made with these NaCl/KCl mixtures exhibited no significant ($P > 0.05$) differences in compositional, physico-chemical, sensory or textural properties in comparison with the control cheese. To extend that previous work, our objective in this study was to determine and compare proteolysis in Kefalograviera cheeses made with the above mixtures of NaCl and KCl to that of cheese made with NaCl (control).

2. Materials and methods

2.1. Cheese manufacture

Kefalograviera cheese was manufactured from ewes' milk at the pilot plant of the Dairy Research Institute as described in detail by Katsiari et al. (1998). Cheese sampling was as in the above paper.

2.2. Proteolysis

The level of proteolysis was assessed in samples of 5, 25, 60, 90 and 180-day-old cheese. Five methods were used to monitor proteolysis during aging of Kefalograviera cheese.

2.2.1. Nitrogen fractionation

Water-soluble N(WSN) and N soluble in 12% trichloroacetic acid (TCA-SN) were determined in aliquots of the same water-soluble cheese extract (WSE) prepared as described by Kuchroo and Fox (1982), except that the cheese:water ratio was 1:5, a Sorvall Omni-mixer (Dupont Company, Newton, CT, USA) was used for homogenization and the supernatant obtained was filtered through Whatman No. 42 filter paper. The TCA-SN fraction was obtained by mixing 10 ml of WSE with 10 ml of 24% (w/v) aqueous solution of TCA, holding the mixture at room temperature for 1 h and then filtering it through Whatman No. 42 filter paper. Nitrogen soluble in 5% phosphotungstic acid (PTA-SN) was determined according to Stadhouders (1960), except that the WSE was prepared as described above. Total N (TN) of cheese and the above fractions was determined by the Kjeldahl method (IDF, 1986) using the Kjeldatherm digestion system KT 20S and a Vapodest 4 titramatic distillation system (C. Gerhardt GmbH & Co KG, Bonn, Germany) equipped with an end-point titration system ETS 822 (Radiometer Copenhagen, Denmark). The nitrogen content of each fraction was expressed as% of the TN of cheese.

2.2.2. Measurement of total free amino acids

The concentration of total free amino acids in the WSE of the cheeses was determined in duplicate by the Cd-ninhydrin method of Folkertsma and Fox (1992).

2.2.3. Urea-polyacrylamide gel electrophoresis (Urea-PAGE)

The PAGE method of Andrews (1983) was used to monitor the degradation of α_{s1}- and β-caseins during cheese aging. Electrophoresis was performed using a vertical slab unit (LKB 2001, Bromma Sweden) with 140×160×1.5-mm slabs, Tris–HCl pH 8.3 electrode buffer, constant current of 60 mA, a voltage limited to 500 V for 2 h, with a stacking gel of $T = 5\%$, $C = 4\%$ with buffer Tris–HC1 pH 7.6, and a separation gel of $T = 9\%$, $C = 5\%$ with buffer Tris–HC1, pH 8.9. The sample was prepared for electrophoresis as follows: 1 g of cheese was homogenized in a mixer at 7000 rpm for 3 min with 10 ml stacking gel buffer containing 6 M urea, 0.1 M β-mercaptoethanol and 0.5% bromophenol blue solution (0.5% w/v in 50% ethanol) as tracking dye.

The slurry was held at 40°C for 15 min, centrifuged (3000×*g*, 4°C, 15 min), and then the solidified fat layer was removed. A volume of 0.5 ml of this preparation was mixed with 3.5 ml of stacking gel buffer, and a sample of 12 μl was used for electrophoresis. The gels were stained by the method of Blakesley and Boezi (1977) using a Coomassie Brilliant Blue G-250 solution. Optical density of the gel bands was recorded at 590 nm as a profile using a model RTF scanning densitometer (Transidyne General Corp., Ann Arbor, MI, USA), which was linked to a data acquisition and processing system (Nelson Analytical Inc., Paramus, NJ, USA). Casein fractions of cheese samples were identified by reference to an isoelectric whole ovine casein standard. From the densitograms the levels of residual α_{s1}- and β-caseins in the aged cheeses were calculated in comparison with the level present in a reference sample from 1-day-old cheese.

2.2.4. Reverse-phase HPLC

Reverse-phase HPLC was performed using a binary system (LKB, Bromma, Sweden) fitted with a Nucleosil C_{18} wide pore analytical column (250×4 mm, 5 μm, 30 nm; Macherey-Nagel, Düren, Germany) and a guard column (30×4 mm) of the same material. Samples of WSE were applied using a Rheodyne injector (model 7125; Rheodyne Inc., Cotati, CA, USA), equipped with a 50 μl injection loop. Eluent A was 0.1% (v/v) trifluoroacetic acid (TFA) in deionized water, and eluent B was 0.09% (v/v) TFA in a mixture of 60:40 (v/v) acetonitrile (gradient grade; Merck, Darmstadt, Germany) and deionized water. Separations were conducted at room temperature (~22°C) at a flow rate of 0.8 ml min^{-1} with eluent A for 10 min and a linear gradient from 0 to 80% of eluent B for 80 min. The column was finally eluted with 100% eluent B for 10 min. The absorbance of the eluate was monitored at 214 nm, using a variable wavelength spectrophotometric detector (LKB, Bromma, Sweden), which was linked to a data acquisition and processing system (Nelson Analytical Inc., Paramus, NJ, USA). Solvents and samples were, respectively, filtered through 0.45 μm nylon 66 or cellulose acetate filters (Alltech Assoc., Inc., Deerfield, IL, USA). Peptide maps were visually compared to detect differences in the profiles.

2.2.5. Analysis of free amino acids

A ternary gradient HPLC system and a fluorescence spectophotometric detector set at λex: 330 nm and λem: 464 nm were used (Scientific System, State College, PA, USA). Free amino acids (FAA) were extracted from cheese samples according to Resmini, Hogenboom, Pazzaglia, and Pellegrino (1993), separated on an ion-exchange column (250×3mm Na^+ form) (Pickering Laboratories, Mountain View, CA, USA), set at 50°C and their *o*-phthalaldehyde derivatives were formed using a post-column reactor set at 40°C (Pickering Laboratories). Samples were injected through a Rheodyne injection port (model 9125; Rheodyne Inc.), equipped with a 100 μl injection loop. Gradient elution was performed using two sodium citrate buffers: A: 0.2 N Na^+, pH 3.15 and B: 1.0 N Na^+, pH 7.40. A mixture of 0.1 N NaOH and 0.1 N NaCl was used as the column regenerator. The column was eluted for 10 min with buffer A, then with a gradient from 100% A to 100% B for 26 min and finally with 100% B for 24 min. The column was regenerated for 2 min and then equilibrated with buffer A for 15 min. The flow rate of the buffers and OPA reagent was 0.3 ml min^{-1}. Quantitation of FAA was performed using a mixture of 27 amino acids at seven different concentrations and a data acquisition and processing system (Nelson Analytical Inc., Paramus, NJ, USA).

2.3. Statistical analysis

The data were analysed by one-way analysis of variance to test the differences among the three cheeses (at $P < 0.05$) at each sampling age using the software Statgraphics (Statistical Graphics Corp., Rockville, MD, USA).

3. Results and discussion

3.1. Nitrogen fractions

Table 1 shows the changes in the WSN, N soluble in 12% TCA (TCA-SN) and nitrogen soluble in 5% PTA (PTA-SN) during ripening and storage of Kefalograviera cheeses. Generally, these N fractions increased throughout aging in all cheeses. This trend in the change of N fractions was similar to that reported by Zerfiridis, Vafopoulou-Mastrogiannaki and Litopoulou-Tzanetaki (1984) for Greek Gruyere cheese salted with NaCl. The levels of the N fractions in the control and experimental cheeses were similar ($P > 0.05$) at all sampling ages (Table 1). These results agree with those of other workers (Aly, 1995; Fitzgerald & Buckley, 1985; Iwanczak, Reps, Wisniewska, Jarmul, & Kolakowski, 1995; Martens, Vanderpoorten, & Naudts, 1976; Reddy & Marth, 1993) for other cheese varieties. Thus, Martens et al. (1976) reported no differences in WSN between control Gouda cheese salted with NaCl and experimental cheeses salted with mixtures of NaCl and KCl. Fitzgerald and Buckley also found no significant differences in the WSN levels between control Cheddar cheese salted with NaCl and experimental cheeses salted with equivalent amounts of KCl or 1:1 mixture of NaCl/KCl. Aly (1995) reported that partial substitution (3:1, 1:1, 1:3) of NaCl by KCl in the salting of UF Feta-type cheese did not significantly ($P > 0.05$) influence the WSN of cheese throughout the storage period. Reddy

Table 1
Water-soluble nitrogen (WSN), nitrogen soluble in 12% trichloroacetic acid (TCA-SN), nitrogen soluble in 5% phosphotungstic acid (PTA-SN) and total free amino acids, measured by reaction with Cd-ninhydrin at absorbance at 507 nm (A_{507nm}) of Kefalograviera cheese[a,b] made with NaCl or mixtures of NaCl and KCl during aging

Age of cheese (days)	WSN (% TN)			TCA-SN (% TN)			PTA-SN (% TN)			Total free amino acids (A_{507nm})		
	A[c]	B[c]	C[c]	A	B	C	A	B	C	A	B	C
5	10.2	10.1	10.0	5.01	4.92	4.93	2.67	2.61	2.46	0.26	0.27	0.27
25	17.8	17.7	17.6	9.44	9.16	9.12	5.51	5.36	5.29	0.88	0.86	0.88
60	21.2	21.5	21.9	11.9	12.8	12.4	7.81	7.56	7.53	1.19	1.20	1.20
90	21.3	21.3	21.3	12.4	12.3	12.3	7.45	7.17	7.27	1.35	1.34	1.35
180	23.1	23.0	23.8	14.0	13.9	14.1	8.11	7.76	7.87	1.44	1.46	1.47

[a] Means of each parameter in the same row without a superscript did not differ significantly ($P > 0.05$).
[b] Means of five trials.
[c] Cheese: A, salted with NaCl (control); B, salted with 3:1 (w/w) mixture of NaCl and KCl; C, salted with 1:1 (w/w) mixture of NaCl and KCl.

Table 2
Residual α_{s1}- and β-caseins in Kefalograviera cheese[a,b] made with NaCl or mixtures of NaCl and KCl during aging

Age of cheese (days)	Residual α_{s1}-casein[d] (%)			Residual β-casein[d] (%)		
	A[c]	B[c]	C[c]	A	B	C
5	95±1	91±3	91±2	93±1	90±3	94±1
25	70±3	68±2	66±2	83±4	83±3	80±2
60	50±5	49±5	50±4	76±5	74±5	75±4
90	47±2	44±1	41±2	69±3	67±4	68±3
180	37±2	38±3	34±3	65±3	64±3	63±4

[a] Means of each parameter in the same row without a superscript did not differ significantly ($P > 0.05$).
[b] Mean values±S.E. of two trials.
[c] Cheese: A, salted with NaCl (control); B, salted with 3:1 (w/w) mixture of NaCl and KCl; C, salted with 1:1 (w/w) mixture of NaCl and KCl.
[d] Expressed as % of the α_{s1}- or β-casein content present in the 1-day-old cheese.

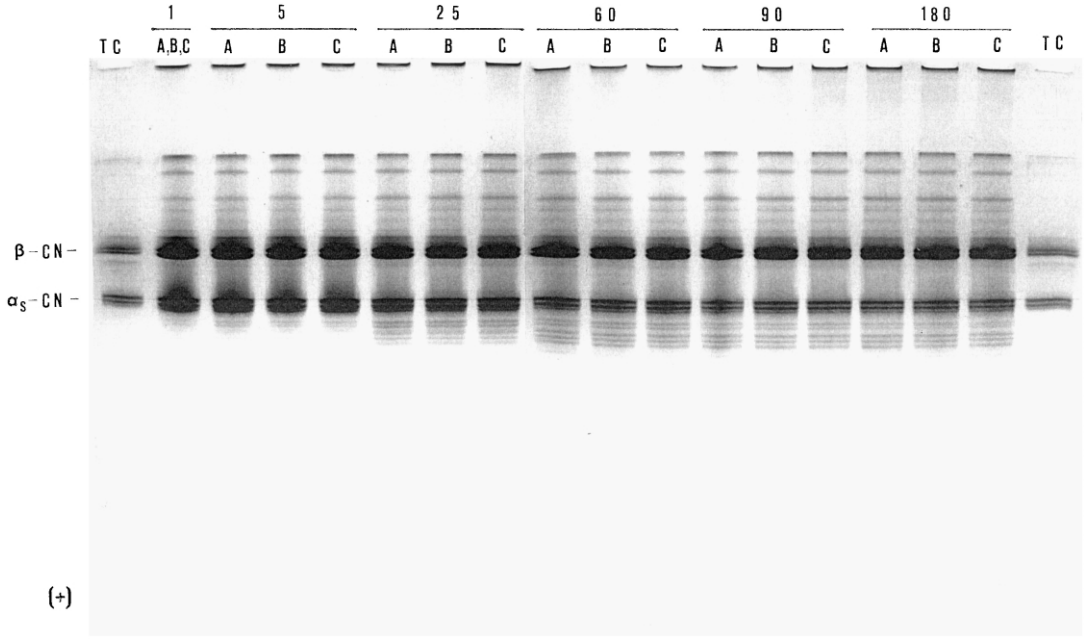

Fig. 1. Urea-polyacrylamide gel electrophoretograms of total ewe's casein (TC) and Kefalograviera cheese made with NaCl (A), 3:1 mixture of NaCl/KCl (B) or 1:1 mixture of NaCl/KCl (C) after aging for 1, 5, 25, 60, 90 and 180 days.

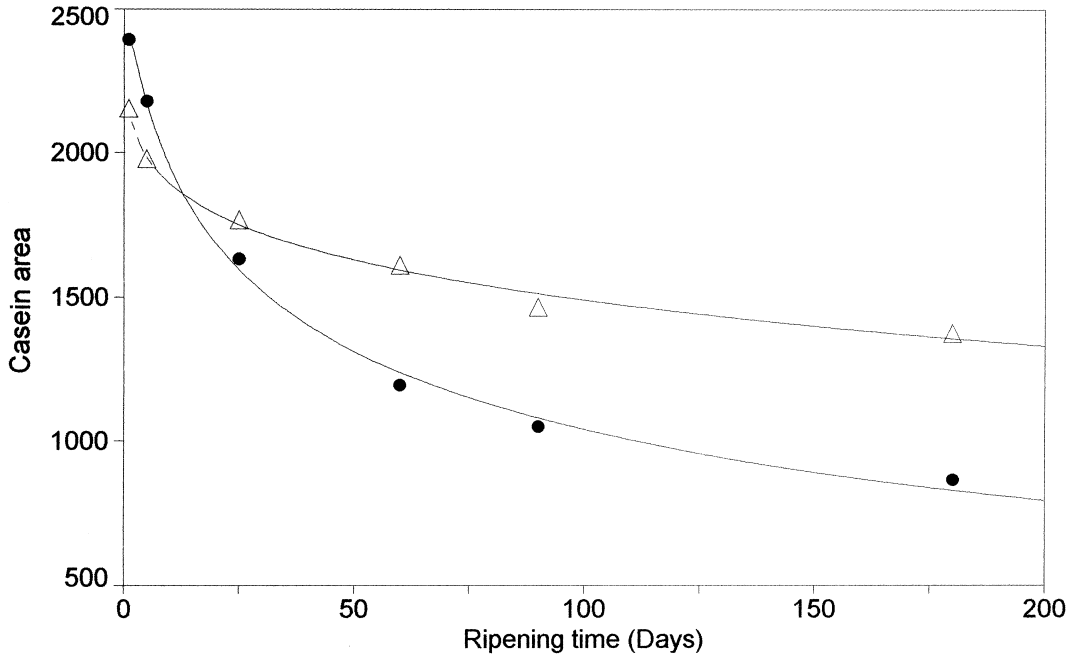

Fig. 2. Degradation of α_{S1}- (●) and β-caseins (△) of Kefalograviera cheese during aging.

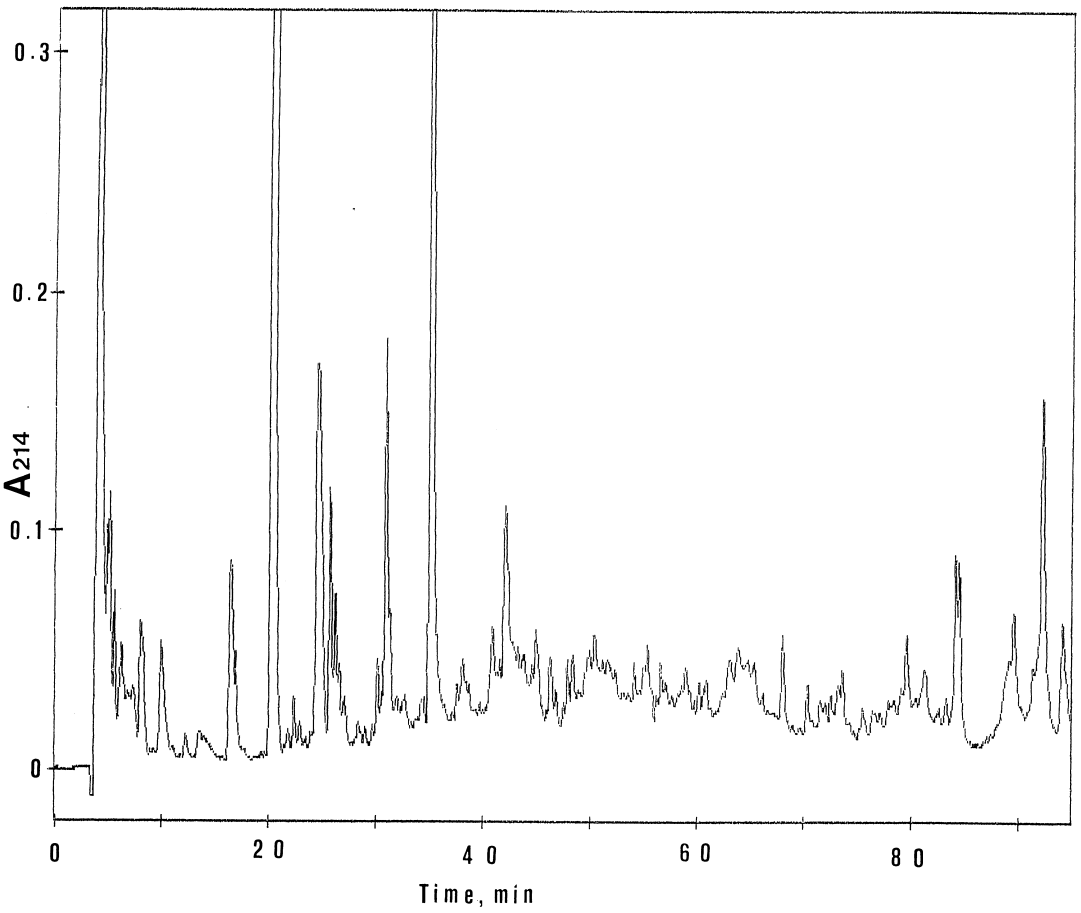

Fig. 3. Reversed-phase high-performance liquid chromatography profile of the water-soluble fraction of 90-day-old Kefalograviera cheese. The absorbance of the eluate was monitored at 214 nm.

and Marth (1993) found that the levels of TCA-SN and PTA-SN in Cheddar cheeses made with KCl or NaCl/KCl mixtures were similar ($P > 0.05$), within a sampling time, at 12, 24 and 36 weeks of age to those of control cheese made with NaCl. Furthermore, Iwanczak et al. found that partial (1:1) substitution of NaCl by KCl, in the salting of Camembert, Camping, Tilsit and Gouda type cheeses, did not affect their proteolysis and specifically the levels of pH 4.6-soluble nitrogen, non-protein nitrogen and peptide nitrogen.

3.2. Total free amino acids

The levels of total FAA in Kefalograviera cheeses during aging are given in Table 1. As can be seen, the total FAA concentration in all cheeses increased throughout aging, especially during ripening in the warm room (90 d). This finding agrees with the results of Folkertsma and Fox (1992) for Cheddar cheese salted with NaCl. The total FAA concentration in the control and experimental cheeses was similar ($P > 0.05$) at all sampling ages (Table 1), which indicates that the depth, as well as the level, of proteolysis was similar in all cheeses.

3.3. Urea-PAGE

Urea-PAGE electrophoretograms of Kefalograviera cheese samples of various ages are shown in Fig. 1. The main changes observed concerned the continuous decrease of the intensity of α_{s1}- and β-casein bands in all cheese with increasing cheese age. Even at 5 days the hydrolysis products of α_{s1}- casein were visible. At any sampling age, the PAGE patterns of the control and experimental cheeses were similar, suggesting that the mode and rate of casein breakdown were similar in all cheeses. It is also evident that the rates of hydrolysis of the two caseins were different. In all cases, α_{s1}-casein was hydrolysed much faster and to a greater extent than β-casein.

In order to quantify proteolysis in this study, the urea-PAGE was followed by densitometry. During aging, hydrolysis of α_{s1}- and β-casein fractions was expressed as a percentage relative to the concentration of the corresponding casein at 1 day. The relative proportions of residual α_{s1}- and β-caseins present in Kefalograviera cheese during aging are given in Table 2. As can be seen, the residual α_{s1}- and β-caseins in all cheeses

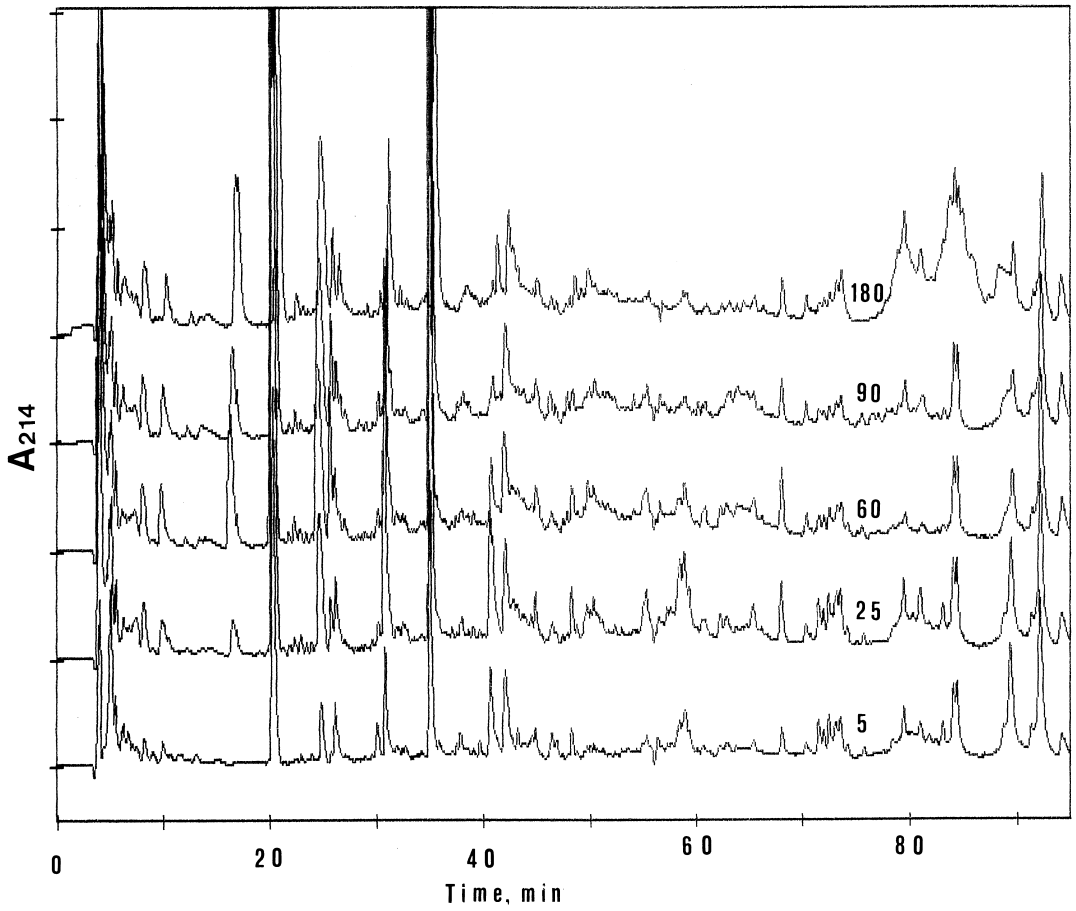

Fig. 4. Reversed-phase high-performance liquid chromatography profiles of the water-soluble fraction of Kefalograviera cheese made with NaCl (A) and aged for 5, 25, 60, 90 and 180 days. Detection at 214 nm.

continuously decreased during aging. There were no significant ($P > 0.05$) differences in the percentages of α_{s1}- and β-caseins between control and experimental cheeses at all sampling ages. Fig. 2, which was plotted using the means of residual α_{s1}- and β-caseins of the three cheeses at each sampling age, shows the rate of degradation of α_{s1}- and β-caseins in Kefalograviera cheese during aging. It is evident, that the rate and extent of α_{s1}-casein degradation were greater than those of β-casein. This finding is in agreement with the results of other workers (Basch, Farrell Jr., Walsh, Konstance, & Kumosinski, 1989; Lau, Barbano, & Rasmussen, 1991; Mayer, Rockenbauer, & Mlcak, 1998) for other cheese varieties. From Table 2 and Fig. 2, it can be seen that the half life of α_{s1}-casein in the cheeses was 60 days, while 64% of the α_{s1}-casein was degraded up to 180 days. On the other hand only 36% of β-casein was degraded at the same age. Lau et al. (1991) found that approximately 85% of the intact α_{s1}-caseins were degraded within 6 months in Cheddar cheeses made from pasteurized and raw milk. Moreover, Visser and de Groot-Mostert (1977) reported that the half life of β-casein in Gouda cheese was 6 months, while Basch et al. (1989) found that its half life in Cheddar cheese was 37 weeks.

The finding of the present study, that the degradation of α_{s1}- and β-caseins was similar in the control and experimental cheeses, agrees with the results of Zorilla et al. (1996), who found similar urea-PAGE electrophoretic patterns for a Fynbo cheese salted with 1:1 NaCl/KCl mixture and for a control cheese during ripening and concluded that the partial replacement of NaCl by KCl did not affect normal proteolysis in this cheese. Furthermore, Zorilla and Rubiolo (1997), using urea-PAGE, found that the partial (1:1) replacement of NaCl by KCl did not affect kinetic parameters of Fynbo cheese proteolysis. Rasmussen and Barbano (1987) studied the influence of KCl on Cheddar cheese proteolysis using KCl instead of NaCl in the salting of curd. Electrophoretic analysis of casein degradation products present in the NaCl and KCl cheeses indicated that there were no large changes in the amount and/or types of casein proteolysis products released by different milk coagulants during 4 months of cheese ripening.

3.4. RP-HPLC analysis of peptides

A typical RP-HPLC chromatogram of the WSF of a ripe (90-day-old) Kefalograviera cheese is shown in

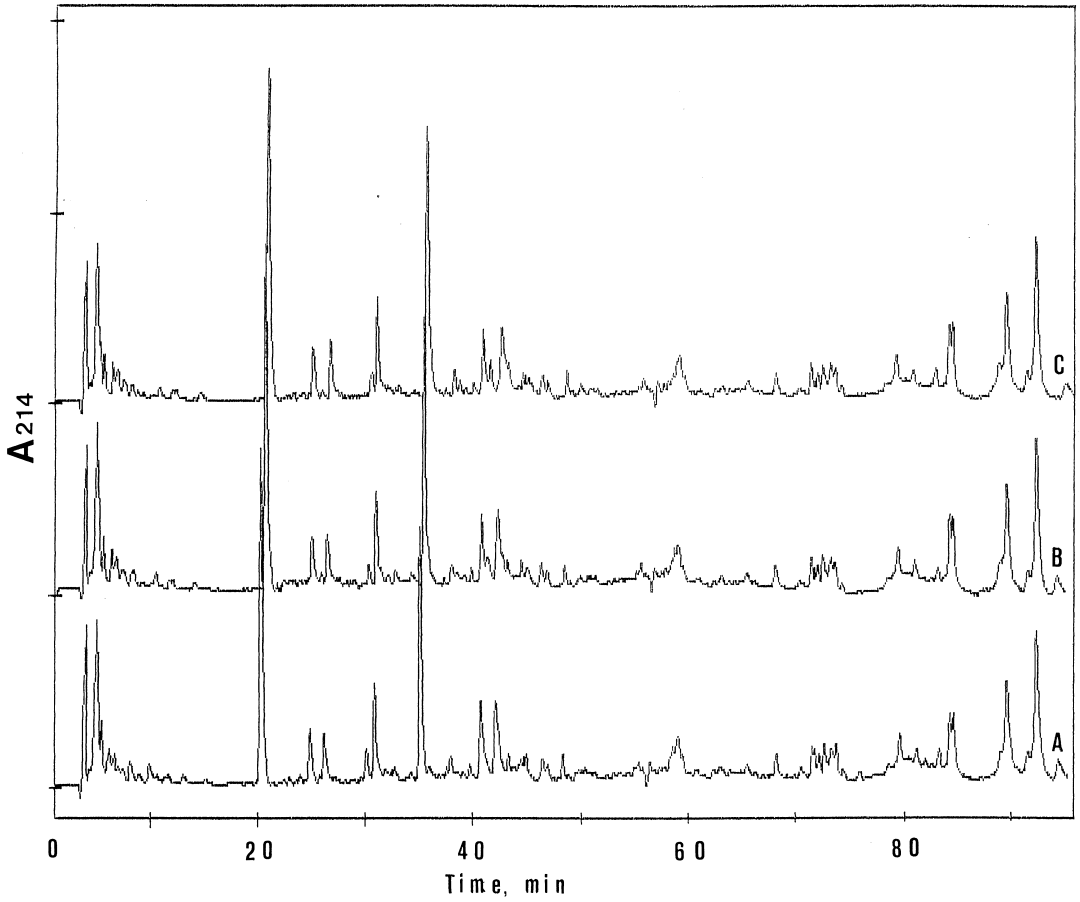

Fig. 5. Reversed-phase high-performance liquid chromatography profiles of the water-soluble fraction of Kefalograviera cheese made with NaCl (A), 3:1 mixture of NaCl/KCl (B) or 1:1 mixture of NaCl/KCl (C) and aged for 5 days. Detection at 214 nm.

Fig. 3. It is obvious that there are a great number of peaks in the hydrophilic region, some of which are at great concentrations, in contrast to a small number of peaks in the hydrophobic region, of which only 1–2 were at moderate levels.

Fig. 4 shows the RP-HPLC profiles of the WSF of control Kefalograviera cheese salted with NaCl (cheese A) and aged for 5, 25, 60, 90 and 180 d, using detection at 214 nm. It can be seen that, as the age of cheese increased, new peaks appeared while increased or decreased peaks existed at the initial stage of ripening. Thus, new peaks and increased existing peaks corresponded to amino acids and hydophilic products of casein degradation. In the region of hydrophobic peptides, the peak eluted at ~68 min increased in size up to 60 days, decreased at 90 day and then remained constant. On the other hand, two peaks, eluted at ~80 and 85 min, remained almost stable in size up to 90 day and increased at 180 day. Moreover, the peak eluted at ~89 min decreased in size throughout aging.

RP-HPLC profiles of the WSF of Kefalograviera cheese made with NaCl or mixtures of NaCl/KCl and aged for 5, 90 and 180 d are shown in Figs. 5–7, respectively. At any particular age, the peptide profiles of the control and experimental cheeses were similar, a fact indicating that peptidolysis in the cheeses was not influenced by the type of salt used in their manufacture. This finding agrees with the results of Laborda and Rubiolo (1999), who reported that the RP-HPLC profiles of the WSN extracts of Fynbo cheese salted with NaCl or with a NaCl/KCl mixture (1:1) were very similar in the number of peaks, retention times and areas, suggesting that the partial substitution of NaCl by KCl did not affect the specific activity of proteases and peptidases.

In order to make a quantitative comparison of the RP-HPLC peptide profiles of Kefalograviera cheeses at various ages, during ripening and storage, the chromatograms of their WSFs were divided in two regions with criterion the elution times of peaks. The first region of hydrophilic (HI) peptides contains the peaks eluted between 13 and 65 min. The second region of hydrophobic (HO) peptides contains the peaks eluted between 65 and 92 min. It should be noted that the region with elution times from 0 to 13 min contains mainly free amino acids and was not studied. The ratio of hydrophobic to hydrophilic (HO/HI) peptides was calculated by dividing the total area of the peaks in the region of

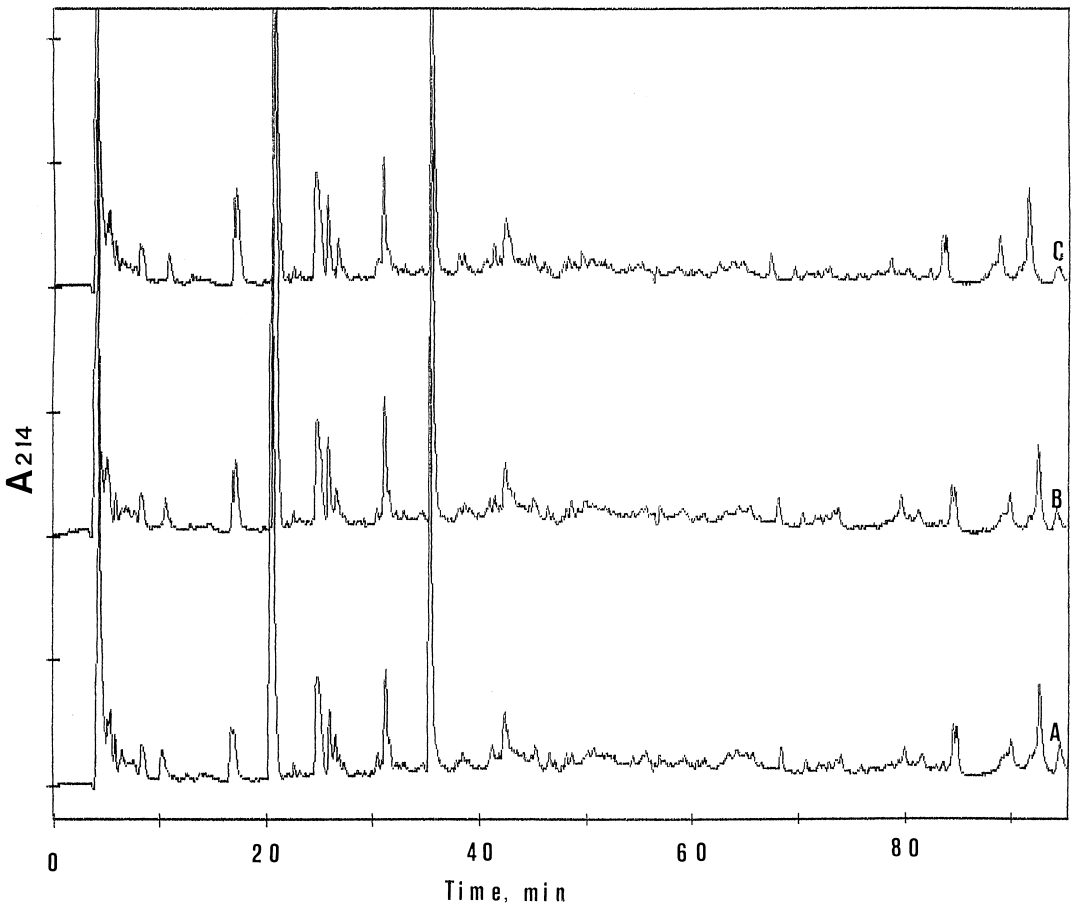

Fig. 6. Reversed-phase high-performance liquid chromatography profiles of the water-soluble fraction of Kefalograviera cheese made with NaCl (A), 3:1 mixture of NaCl/KCl (B) or 1:1 mixture of NaCl/KCl (C) and aged for 90 days. Detection at 214 nm.

HO peptides by the total area of peaks in the region of HI peptides. Table 3 shows the concentration of HO and HI peptides and their ratio (HO/HI) in the WSF of Kefalograviera cheese made with NaCl or mixtures of NaCl and KCl during aging. As can be seen, generally, the HO peptides in the WSF of all cheeses decreased rapidly between 5 and 25 days, and barely up to 90 day, while increased slightly at 180 day. On the contrary, the HI peptides increased rapidly between 5 and 25 days and then decreased gradually up to the end of aging. The decrease of HO peptides and the simultaneous increase of HI peptides observed between 5 and 25 days of aging should be attributed to the degradation of HO peptides and the formation of HI peptides (Cliffe, Revell, & Law, 1989; Engels & Visser, 1994) as well as highly HO peptides which were no longer water soluble

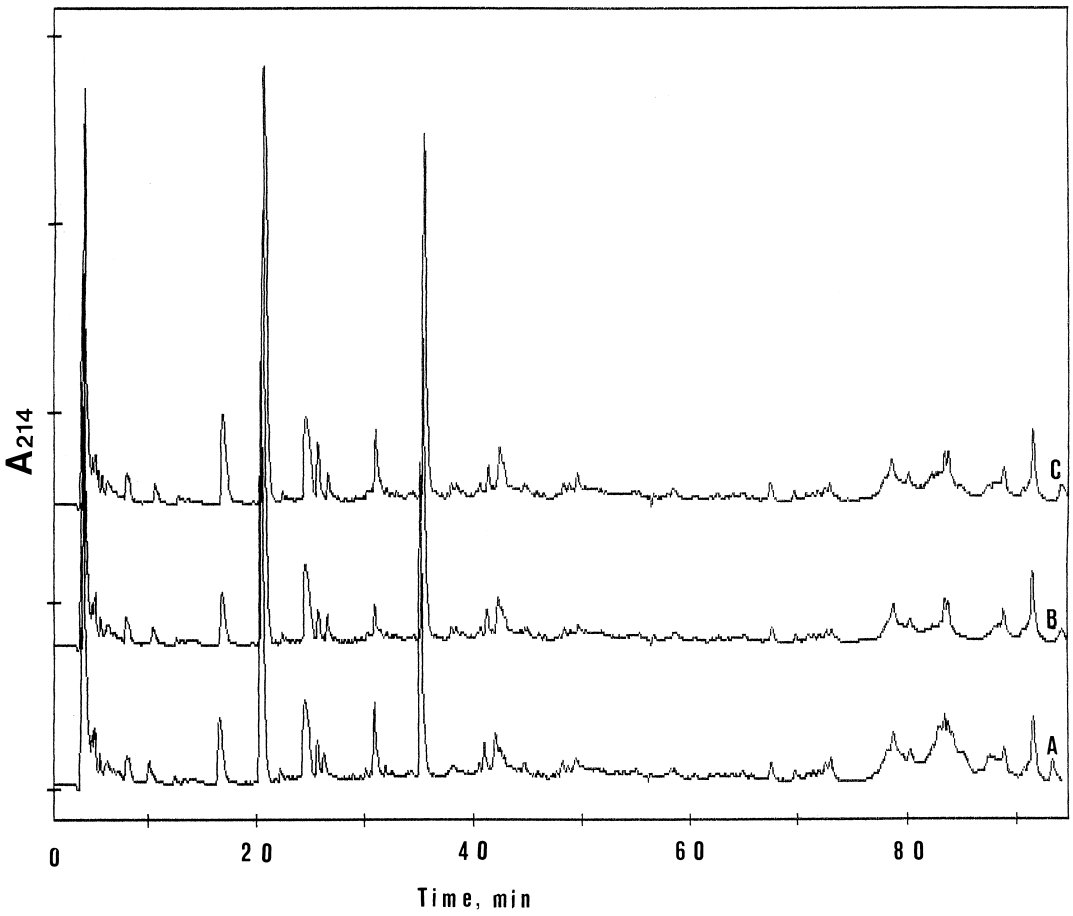

Fig. 7. Reversed-phase high-performance liquid chromatography profiles of the water-soluble fraction of Kefalograviera cheese made with NaCl (A), 3:1 mixture of NaCl/KCl (B) or 1:1 mixture of NaCl/KCl (C) and aged for 180 days. Detection at 214 nm.

Table 3
Hydrophobic (HO) and hydrophilic (HI) peptides and their ratio (HO/HI) in the water-soluble fraction of Kefalograviera cheese[a,b] made with NaCl or mixtures of NaCl and KCl during aging

Age of cheese (days)	HO (% TA)[d]			HI (% TA)[d]			HO/HI		
	A[c]	B[c]	C[c]	A	B	C	A	B	C
5	38.6	37.6	38.1	44.9	47.5	46.5	0.86	0.79	0.82
25	24.3	26.8	27.4	59.1	59.3	58.8	0.41	0.45	0.47
60	23.1	25.5	20.9	58.8	58.3	63.1	0.39	0.44	0.33
90	24.1	23.2	20.4	57.0	58.3	59.9	0.42	0.40	0.34
180	28.4	26.6	23.7	54.1	54.7	56.5	0.52	0.49	0.42

[a] Means of each parameter in the same row without a superscript did not differ significantly ($P > 0.05$).
[b] Means of two trials.
[c] Cheese: A, salted with NaCl (control); B, salted with 3:1 (w/w) mixture of NaCl and KCl; C, salted with 1:1 (w/w) mixture of NaCl and KCl.
[d] Expressed as % of the total area (TA) of the chromatograms.

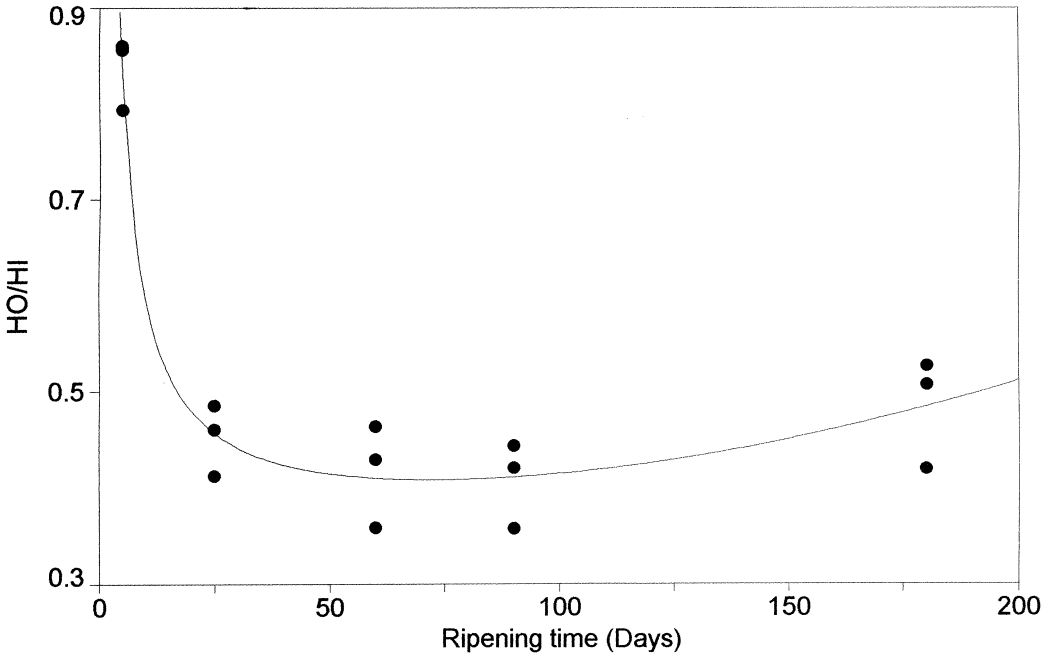

Fig. 8. Change of the ratio of hydrophobic to hydrophilic (HO/HI) peptides of the reversed-phase high-performance liquid chromatography chromatogram of the water-soluble fraction of Kefalograviera cheese during aging.

Table 4
Free amino acids (μmol/g) of Kefalograviera cheese[ab] made with NaCl or mixtures of NaCl and KCl during aging

Amino acid	Age of cheese (days)														
	5			25			60			90			180		
	A[c]	B[c]	C[c]	A	B	C	A	B	C	A	B	C	A	B	C
Asp	0.39	0.39	0.41	1.16	1.12	1.14	1.77	1.64	1.74	2.14	1.91	2.08	2.40	2.03	2.17
Thr	0.60	0.57	0.59	1.59	1.57	1.52	2.35	2.16	2.16	2.78	2.36	2.46	2.91	2.41	2.46
Ser + Gln + Asn	2.74	2.56	2.80	5.35	6.00	5.79	9.50	9.02	8.97	11.8	10.6	10.8	12.3	11.0	11.2
Glu	1.52	1.40	1.51	4.28	4.43	4.39	7.70	7.05	7.47	9.78	8.46	9.25	10.9	9.53	10.2
Cit	0.00	0.00	0.00	0.15	0.14	0.15	0.31	0.26	0.29	0.34	0.30	0.35	0.59	0.58	0.59
Gly	0.30	0.28	0.30	1.02	0.94	0.97	1.69	1.67	1.68	2.09	2.03	2.26	2.39	2.28	2.43
Ala	0.96	0.91	0.96	2.07	1.99	2.01	2.92	2.83	2.71	3.29	3.03	3.25	3.66	3.21	3.38
α-Aba + Cys	0.03	0.01	0.02	0.05	0.03	0.05	0.17	0.13	0.11	0.13	0.13	0.13	0.15	0.08	0.05
Val	1.25	1.18	1.32	3.05	3.20	3.19	5.85	5.40	5.07	6.91	6.02	6.41	7.31	6.08	6.28
Met	0.48	0.46	0.50	1.23	1.21	1.21	1.85	1.79	1.74	2.09	1.92	2.02	2.28	1.98	2.07
Ile	0.69	0.65	0.72	1.93	1.94	1.93	3.44	3.11	3.13	4.28	3.75	4.02	4.78	4.18	4.31
Leu	1.86	1.71	1.93	3.82	4.33	4.19	6.86	6.96	6.83	8.24	7.79	8.14	8.70	7.95	8.32
Tyr + β-Ala	0.37	0.36	0.39	0.51	0.52	0.50	0.59	0.56	0.50	0.77	0.75	0.77	0.83	0.84	0.81
Phe	0.74	0.67	0.73	2.25	2.22	2.17	3.84	3.54	3.59	4.47	4.03	4.18	5.00	4.30	4.49
γ-Aba	0.04	0.03	0.11	0.44	0.44	0.36	1.14	1.18	0.82	1.28	1.49	1.31	1.37	1.27	1.25
Orn	0.10	0.09	0.06	1.06	1.05	0.97	1.78	1.81	1.86	2.00	1.87	1.92	1.92	1.69	1.89
Lys	1.05	0.99	1.05	2.75	2.87	2.87	5.16	4.85	4.80	6.51	5.92	6.28	6.97	6.52	6.76
His	0.39	0.40	0.39	1.16	1.13	1.12	2.21	1.97	1.90	3.11	2.53	2.55	3.15	2.96	2.92
Trp	0.06	0.06	0.12	0.17	0.27	0.24	0.09	0.36	0.21	0.36	0.07	0.24	0.21	0.22	0.16
Arg	0.66	0.64	0.66	0.63	0.67	0.69	0.76	0.58	0.54	1.34	1.01	1.07	1.24	1.10	1.05
Total	14.24	13.35	14.59	34.65	36.04	35.45	59.96	57.04	56.11	73.66	65.94	69.47	79.00	70.21	72.69

[a] Means in each row and at the same age without a superscript did not differ significantly ($P > 0.05$).
[b] Means of three trials.
[c] Cheese: A, salted with NaCl (control); B, salted with 3:1 (w/w) mixture of NaCl and KCl; C, salted with 1:1 (w/w) mixture of NaCl and KCl.

(Lau et al., 1991; Picon, Gaya, Medina, & Nunez, 1994). Table 3 shows that there were no significant ($P > 0.05$) differences in the concentrations of HO and HI peptides and their ratio (HO/HI) between control and experimental cheeses at all sampling ages. It is also evident that the ratio of HO/HI peptides of cheeses decreased sharply between 5 and 25 day, barely up to 90 day and increased slightly at 180 day. This trend can also be seen in Fig. 8, which was plotted using the mean HO/HI peptides ratio of the three cheeses at each sampling age, and shows the change of the ratio of HO/HI peptides in Kefalograviera cheeses as a function of aging. A continuous decrease of the ratio of HO/HI peptides, during aging, in the WSF of Feta cheese, Cheddar cheese made from raw or pasteurized milk, and of white and red Afuega'l Pitu cheeses was reported by Michaelidou-Koniordou (1997), Lau et al. (1991), and Gonzalez de Llano, Polo and Ramos (1995), respectively.

Table 3 shows, that the ratio of HO/HI peptides was less than 1 at all sampling ages, which means that the amount of HI peptides present in the cheeses was greater than the amount of HO peptides throughout aging. The ratio of HO/HI peptides (0.39) found in the control Kefalograviera cheese at 60 day was almost comparable to those reported by Gonzalez de Llano et al. (1995) for white (0.48) and red (0.61) 60-day-old Afuega'l Pitu cheeses. Moreover, the ratio of HO/HI peptides (0.52) found in the control Kefalograviera cheese at 180 day was lower than those (1.18 and 1.40) reported by Lau et al. (1991) for 180-day-old Cheddar cheese made from raw or pasteurized milk, respectively.

3.5. Free amino acids

The concentrations of individual FAA in Kefalograviera cheeses during aging are given in Table 4. Glutamic acid, leucine and valine were the major FAA in all cheeses throughout aging. Moreover, lysine, phenylalanine, isoleucine and alanine were found at relatively high concentrations in all cheeses during aging. Of special interest during cheese ripening are the amino acids α- and γ-aminobutyric acid, ornithine and citrulline, which do not originate from casein, but accumulate as metabolic products of microorganisms (Bütikofer, 1996). Thus, glutamic acid is the precursor for α- and γ-aminobutyric acids and arginine for ornithine and citrulline (Bütikofer & Fuchs, 1997). In the present study, γ-aminobutyric acid and ornithine were found at appreciable concentrations in ripe cheeses with citrulline in low amounts. Generally, the concentrations of individual FAA in the cheeses increased throughout aging (Table 4). Moreover, the concentrations of individual FAA in the control and experimental cheeses were similar ($P > 0.05$) at all sampling ages.

The depth of proteolysis in Kefalograviera cheeses was ascertained by measuring the total amount of FAA produced. Table 4 shows that the total FAA content in all cheeses increased throughout aging. No significant ($P > 0.05$) differences were found in the total FAA content between control and experimental cheeses at all sampling ages (Table 4). This finding indicates that the depth of proteolysis was similar in all cheeses. It has been reported that starter peptidases are mainly responsible for the level of FAA in cheese (Lane & Fox, 1996; O'Keeffe, Fox, & Daly, 1978; Visser, 1997). Furthermore, Reddy and Marth (1995a, b) found that the use of KCl to replace some of the NaCl had no significant ($P > 0.05$) effect on the counts and the types of lactic acid bacteria developed during Cheddar cheese ripening. Therefore, the finding in the present study, that control and experimental cheeses had similar total FAA contents, could be explained on the basis of the results of the above studies.

4. Conclusions

The partial substitution of NaCl by KCl in the manufacture of Kefalograviera cheese, using mixtures of NaCl/KCl (3:1 or 1:1), did not influence the extent and characteristics of proteolysis (as shown by Kjeldahl determination of soluble N fractions, determination of total FAA by the Cd-ninhydrin method, urea-PAGE of caseins, RP-HPLC analysis of peptides, and ion-exchange HPLC analysis of free amino acids) during aging.

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