Kinetics of Proteolysis of β-Casein During Ripening of Fynbo Cheese Salted with NaCl or NaCl/KCl and Ripened at Different Temperatures

GUILLERMO A. SIHUFE, SUSANA E. ZORRILLA, AND AMELIA C. RUBIOLO

ABSTRACT: The proteolysis of β-casein during ripening of low-fat Fynbo cheese was studied using 1st-order kinetics to improve the knowledge of the β-casein hydrolysis in Fynbo cheeses salted with NaCl or NaCl/KCl and ripened at different temperatures. Effects of ripening temperature, partial replacement of NaCl by KCl during cheese salting, and total salt concentration were evaluated. Central and external zones from cheeses at 1, 5, 10, 20, 30, 60, and 90 ripening days were analyzed by polyacrylamide gel electrophoresis. No significant differences in the kinetic parameters were observed between cheeses salted with NaCl and those salted with a NaCl/KCl brine. Kinetic constants were significantly affected by region within cheese and ripening temperature. Kinetic constant values were in the range of 0.004/d to 0.018/d, and the activation energy of the reaction was approximately 19 kcal/gmol.

Keywords: β-casein, Fynbo cheese, ripening, temperature, NaCl/KCl

Introduction

Proteolysis is one of the main events during cheese ripening and plays a vital role in the development of the typical organoleptic characteristics (Sousa and others 2001). Particularly, the breakdown of β-casein affects the yield, texture, and flavor of the cheese (Jolivet and others 2000). The primary structure of β-casein has the highest hydrophobicity among caseins, and the sequence includes many hydrophobic fragments that can be involved in the development of bitterness during cheese ripening (Habibi-Najafi and Lee 1996). Rennet enzymes and plasmin are primarily responsible for the proteolytic activity on β-casein. In solution, chymosin hydrolyzes β-casein to β-I-CN, β-II-CN, β-IIIa-CN, and β-IIIb-CN, and its activity is dependent on pH and NaCl concentration (Grappin and others 1985). In cheese, β-I-CN is the fraction most frequently identified (Noomen 1978; Marcos and others 1979; Exterkate and others 1997; Lane and others 1997). Plasmin cleaves β-casein in solution to give the γ-caseins, and its activity depends more on pH than on NaCl concentration (Grappin and others 1985). The importance of plasmin in cheese ripening is still being debated and probably depends on the cheese variety (Bastian and Brown 1996). As a result, the overall breakdown of β-casein could be studied to improve the knowledge of the biochemical events that occur during the ripening of different cheese varieties. Furthermore, kinetic studies should include the determination of kinetic parameters and its temperature dependence to improve the knowledge of the effect of ripening or shelf-life temperature on casein proteolysis and to provide useful information when different temperature conditions need to be tested (for example, in simulation or optimization processes).

Fynbo is a semihard cheese of either regular or low fat content, commonly salted for 10 h at 12 °C in a 20% NaCl brine solution, and ripened for 30 d. In a previous work, Sihufe and others (2003) studied the effect of temperature and salt substitution on casein degradation during Fynbo cheese ripening. Ripening temperature influences the rate of proteolysis, composition of the cheese microflora, texture, and quality of cheese (Folkertsma and others 1996). Replacement of NaCl by KCl in cheese manufacture helps in fulfilling the requirement of sodium intake reduction (Katsiari and others 1997) but may affect cheese quality. Sihufe and others (2003) reported that the replacement of NaCl by KCl did not affect any of the parameters studied, whereas total salt concentration and ripening temperature affected proteolysis significantly. Kinetic parameters for proteolysis of α_{s1}-casein were calculated. First-order kinetic constants were in the range of 0.002/d to 0.016/d, and the activation energy of the reaction was approximately 26 kcal/gmol. The proteolysis of β-casein was significantly affected by total salt concentration, ripening temperature, and aging time. Therefore, our objective in this work was to determine characteristic kinetic parameters of proteolysis of β-casein, using data published by Sihufe and others (2003), to complete the kinetic study and to improve the knowledge of the β-casein hydrolysis in Fynbo cheeses salted with NaCl or NaCl/KCl and ripened at different temperatures.

Materials and Methods

The cheeses used in this study correspond to the cheeses used by Sihufe and others (2003). Unsalted low-fat Fynbo cheeses (782.9 ± 27.4 g weight, 11.5 ± 0.3 cm dia, 6.1 ± 0.2 cm height) were brought from a local factory to our laboratory. Their initial composition was as follows: 49.34% ± 0.29% (w/w) moisture, 29.72% ± 1.75% (w/w) protein, 12.56% ± 0.15% (w/w) fat, and pH 5.15 to 5.35. Zorrilla (1993) and Zorrilla and Rubiolo (1997) did not observe significant differences in data obtained from cheeses made from different cheese vats. Therefore, cheeses manufactured on the same date from the same cheese vat were used in this study to reduce the number of cheeses assayed. Twenty-one cheeses were salted for 10 h at 12 °C in a solution of 190 g NaCl/L (cheese S) and 21 cheeses in a solution of 100 g NaCl/L and 100 g KCl/L (cheese K). Both brines

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also contained 0.55% Ca^{2+} to prevent softening of cheese rind (Geurts and others 1972). After brining, each cheese was wiped and packed under vacuum in a heat-shrinkable plastic bag. During ripening, batches of 7 cheeses S and 7 cheeses K were stored at 5 °C, 12 °C, or 16 °C. Different cheeses were sampled at 1, 5, 10, 20, 30, 60, and 90 d. Slices (1 cm thick) were cut parallel to the flat surface from the surface. Concentric rings of 9.9-cm minor dia and approximately 12-cm major dia were cut from those slices (external zone). Slices (1.5 cm thick) were cut parallel to the flat surface from the center. Cylindrical cores of 4.8-cm dia were cut from those slices (central zone). Samples were analyzed for moisture and chloride contents (Zorrilla and Rubiolo 1994) and electrophoresis. All determinations were carried out in duplicate.

Electrophoretic analysis

Grated cheese (3 g) was dissolved in 25 mL of 8.66 *M* urea. Fat was removed by cold filtration and centrifugation before electrophoretic analysis. The zone electrophoretic method for whole casein or γ-casein fractions described by McKenzie (1971) was used to prepare the polyacrylamide gel (7.5% acrylamide). Vertical anodic discontinuous buffers were used in LKB-2001 electrophoresis equipment (LKB Produkter AB, Bromma, Sweden). The constant current was set at 50 mA, and Coomassie blue R 250 was used to stain the gels. Quantitative analysis of electrophoretic separations was performed by scanning the photograph at 632.8 nm using an LKB -2202 Ultroscan laser densitometer (LKB Produkter AB, Bromma, Sweden). Samples obtained from the same cheese were run in the same gel. Electrophoretic bands were scanned at 3 different tracks. A relative area for each band was calculated on the basis of the entire band area. Quantification of relative band densities was performed because information related to all casein fractions selected can be evaluated simultaneously. The relationship between area and concentration was considered linear (Lesage and others 1993). Standards of α_{s1}-casein and β-casein (Sigma Chemical Co., St Louis, Mo., U.S.A.) were run in 2 lanes of each gel for identification.

Kinetic study

Zorrilla and Rubiolo (1997) showed that the proteolysis of α_{s1}-casein could be adequately described by 1st-order reaction kinetics. In this work, we hypothesized that the proteolysis of β-casein also followed 1st-order kinetics with respect to the substrate concentration:

$$C/C_0 = e^{-kt} \quad (1)$$

where C is the β-casein concentration at any ripening time t; C_0 is the initial β-casein concentration; and k is the kinetic constant of the reaction at a given temperature. The effect of temperature on the kinetic constant was expressed by an Arrhenius-type equation:

$$k = A\, e^{-Ea/RT} \quad (2)$$

where R is the gas constant; A is a constant called the frequency factor; Ea is the activation energy; and T is the ripening temperature in K.

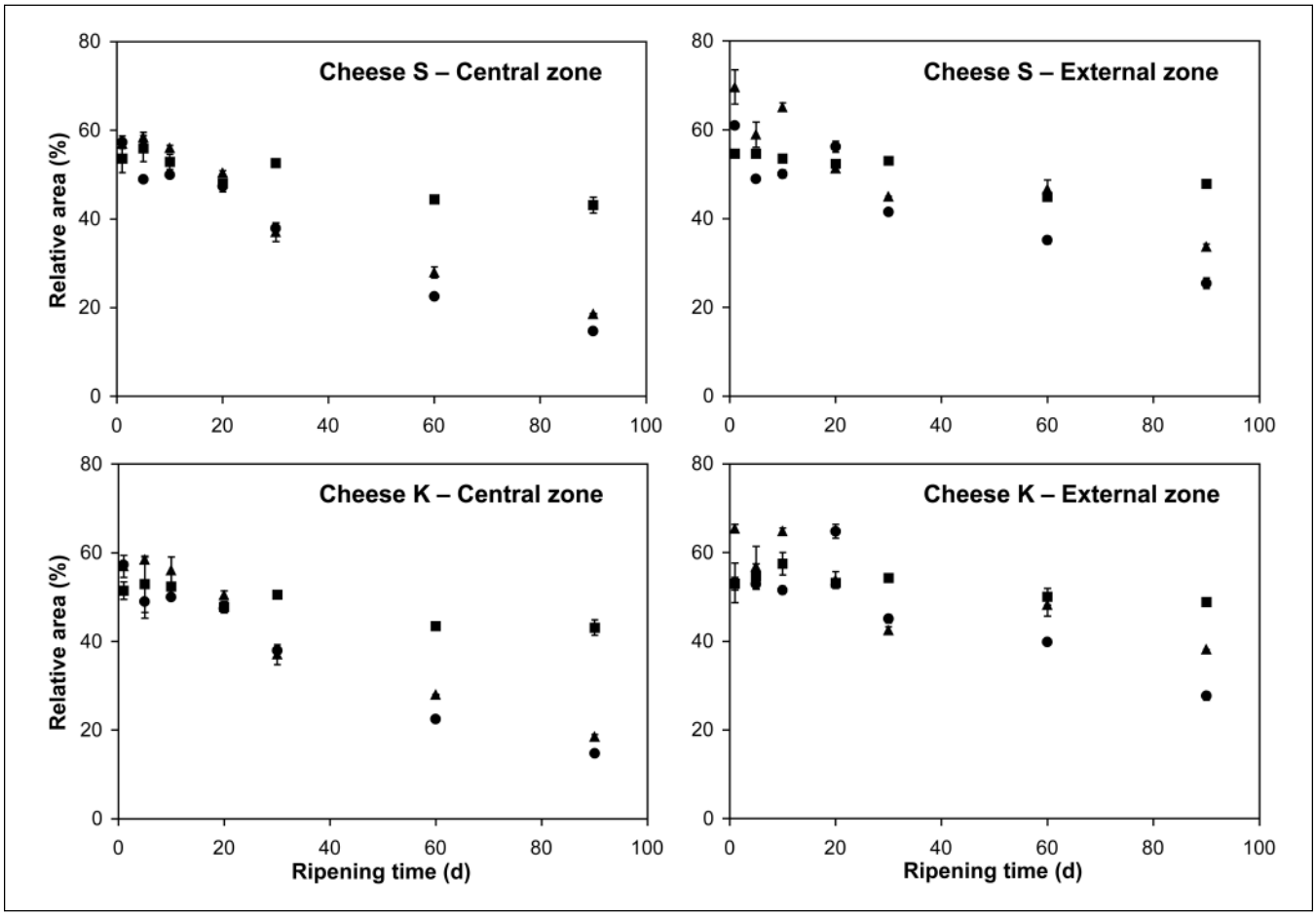

Figure 1—Relative areas corresponding to α_{s1}-casein during the ripening of Fynbo cheeses at 5 °C (■), 12 °C (▲), and 16 °C (●). Bars indicate the standard deviation for the analysis of 2 samples.

Statistical analysis

Data were analyzed using ANOVA. When differences between treatment effects were significant ($P < 0.05$), a multiple comparison of means was performed using the method of least significant differences (LSD). The comparison method of 2 or more sets of data given by Green and Margerison (1978) was used to test whether each set provided different values for k. The sample variances S_i^2's of each set were tested with the Cochran's test (Walpole and Myers 1991) and the Levene's test (Green and Margerison 1978) at $P \leq 0.05$.

Results and Discussion

Sihufe and others (2003) studied 6 different fractions from a characteristic electrophoretogram of Fynbo cheese: γ-CN, β-CN, α_{s1}-CN, α_{s1}-I-CN, and 2 unidentified fractions F1 and F2. The authors studied the effect of salt substitution, region within cheese,

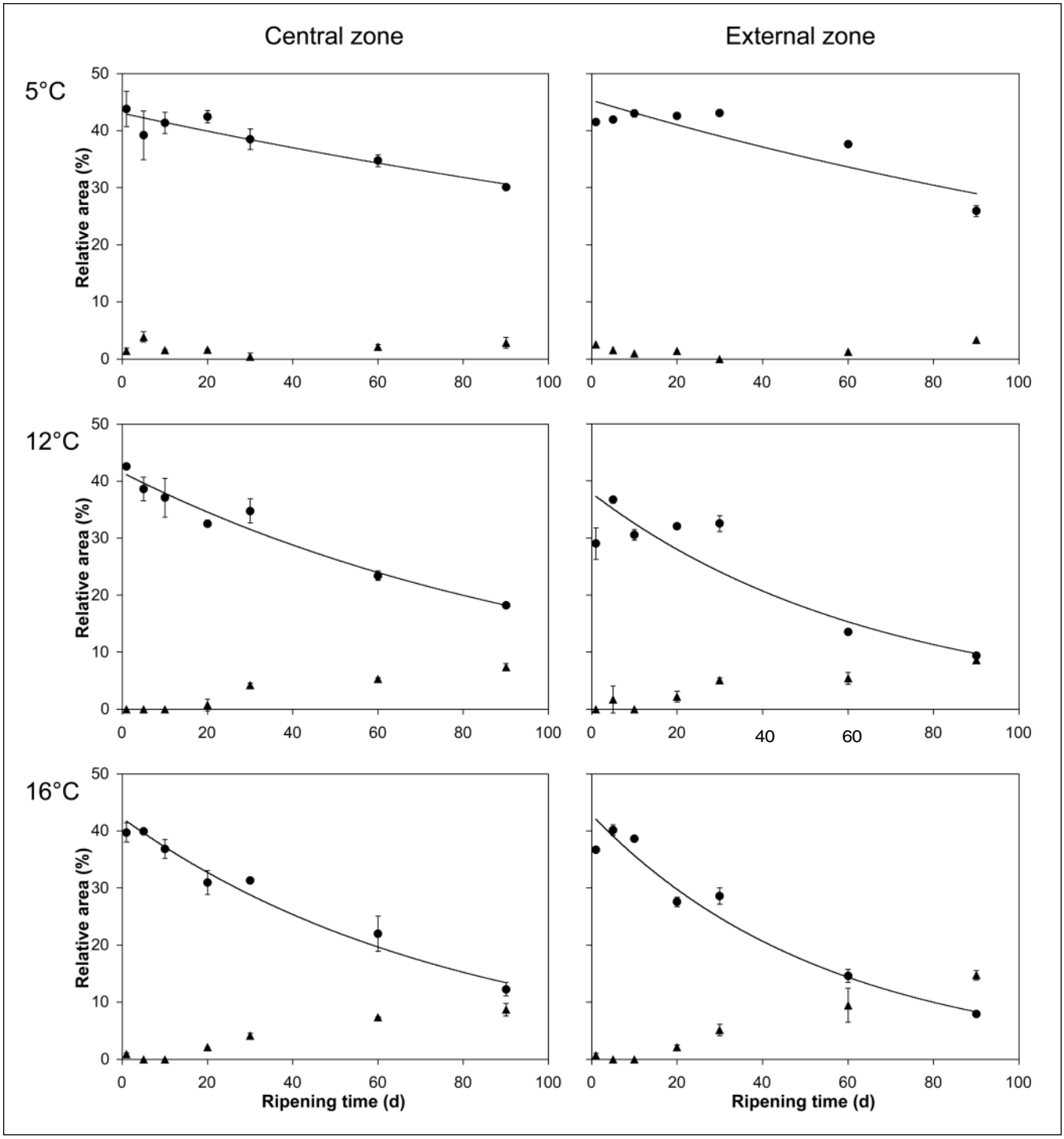

Figure 2—Relative areas (●) and regression curves (–) corresponding to β-casein and relative areas corresponding to γ-casein (▲) during the ripening of Fynbo cheeses salted with NaCl. Bars indicate the standard deviation for the analysis of 2 samples.

and ripening temperature on casein hydrolysis in general and on the kinetic parameters associated with the proteolysis of α_{s1}-casein. In this case, the results related to β-casein proteolysis were used for further discussion.

Figure 1 shows the relative areas of α_{s1}-casein during ripening of cheeses S and K considering central and external zones and different temperatures. Figure 2 and 3 shows the relative areas and the regression curves corresponding to the 1st-order kinetics for proteolysis of β-casein and the relative areas for γ-casein, during ripening of cheeses S and K, respectively. The quantitative analysis showed that β-casein decreased at a slower rate than α_{s1}-casein during the first 30 d of ripening (Sihufe and others 2003), which is observed in most cheese varieties (Marcos and others 1979). The preponderant action of the residual coagulant on α_{s1}-casein during the 1st stage of proteolysis explains that behavior. After 30 d of ripening, the residual coagulant and plasmin may be the main agents

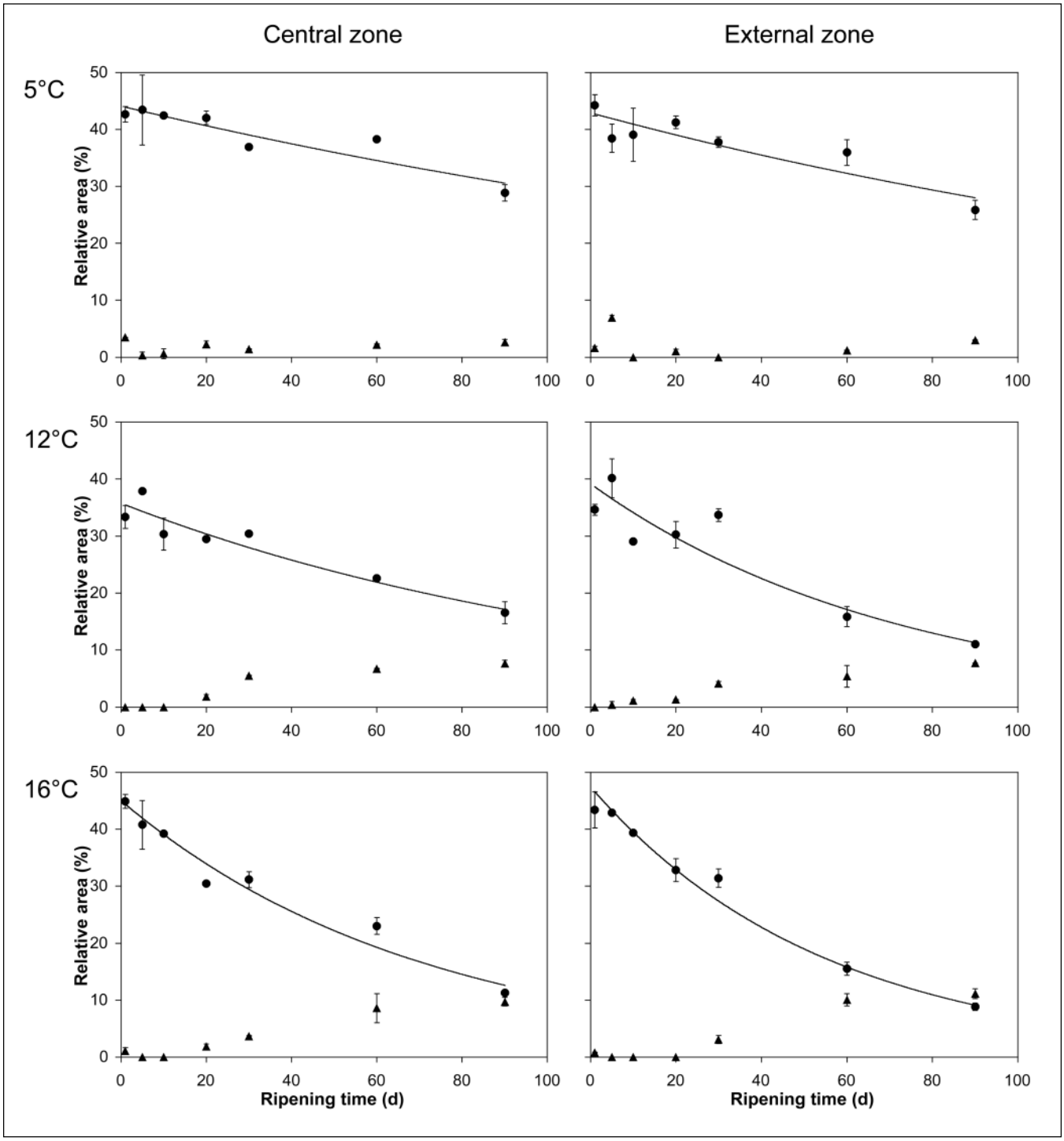

Figure 3—Relative areas (●) and regression curves (–) corresponding to β-casein and relative areas corresponding to γ-casein (▲) during the ripening of Fynbo cheeses salted with NaCl/KCl. Bars indicate the standard deviation for the analysis of 2 samples.

responsible for the primary proteolysis of β-casein. Plasmin activity in Fynbo cheese was evident by the proteolysis of β-casein with the concomitant formation of γ-caseins (Figure 2 and 3).

Results obtained for β-casein for cheeses S and K during ripening were not significantly different, suggesting that the hydrolysis of β-casein was not influenced by the type of salt used (Figure 2 and 3). Katsiari and others (2001) compared the extent and the characteristics of proteolysis during ripening of Kefalograviera cheeses salted with NaCl (control) or with a mixture of NaCl/KCl (3:1 or 1:1, w/w basis). The authors showed that the rate and extent of proteolysis of α_{s1}-casein were greater than those of β-casein. While 64% of α_{s1}-casein was degraded within 180 d, only 36% of β-casein was degraded at the same period. Furthermore, there were no significant differences in the percentage of α_{s1}- and β-casein between control cheeses and those salted with NaCl/KCl brines at all sampling ages.

On the other hand, significant differences in proteolysis of β-casein between central and external zones were observed, proteolysis being slightly higher at the external zone but only at the end of the ripening period studied (Figure 2 and 3). Figure 4 shows the salt in moisture concentrations during ripening of cheeses S and K considering central and external zones and different temperatures. Although a pronounced salt gradient between cheese zones studied at the beginning of the maturation was observed, the proteolysis of β-casein was quite similar for central and external zones. However, both zones reached a uniform salt concentration at approximately 30 d, when the proteolysis of β-casein started being more extensive, which may explain the slight effect of region within cheese on proteolysis of β-casein.

Temperature is an important factor for biochemical processes during ripening (Folkertsma and others 1996; Innocente and Corradini 1996; Shakeel-Ur-Rehman and others 2000). In this case, the rate of hydrolysis of β-casein depended strongly on the ripening temperature for the temperature range studied, this fraction being more extensively hydrolyzed at higher ripening temperature (Figure 2 and 3; Table 1).

Table 1 shows the kinetic constants and correlation coefficients for the regressions of levels of β-casein over ripening time. Correlation coefficients for cheeses ripened at 5 °C were lower than those ripened at 12 °C or 16 °C because an initial lag period of 20 to 30 d can be observed (Figure 2 and 3). The temperature effect on the enzymatic systems may be the main cause of that lag time.

No significant differences in k values were detected between cheeses S and K at different temperatures and zones assayed (Table 1). Significant differences in k values between central and external zones were only observed in cheeses ripened at 12 °C and 16 °C. As expected, k values increased with ripening temperature. Statistical analysis indicated significant differences between cheeses ripened at the 3 temperatures studied, which showed the influence of this variable on the different enzymatic systems present in cheese.

Table 2 shows the values of Ea for the different cheese zones and type of salt used, the results obtained being not significantly dif-

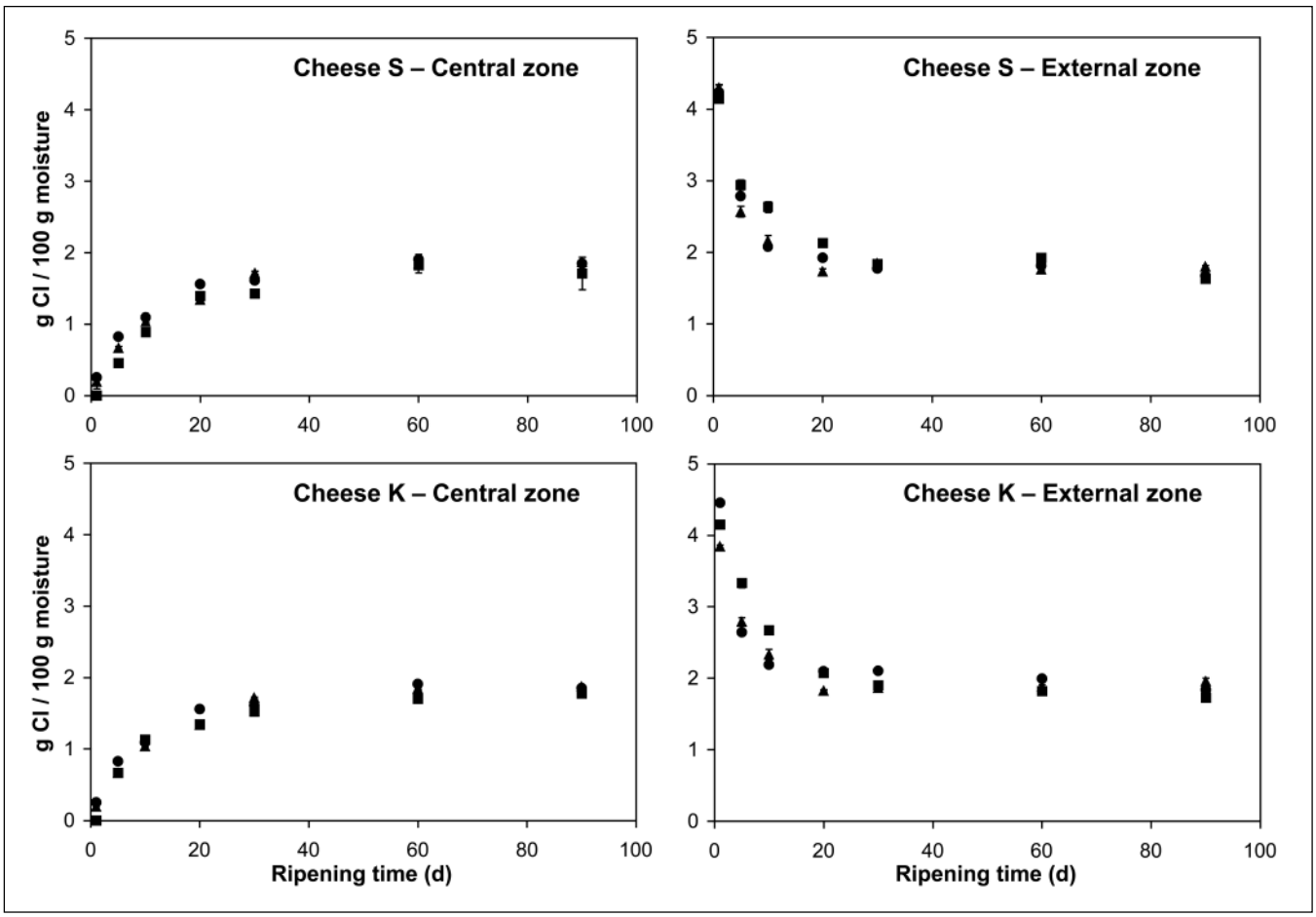

Figure 4—Salt in moisture concentration during the ripening of Fynbo cheeses at 5 °C (■), 12 °C (▲), and 16 °C (●). Bars indicate the standard deviation for the analysis of 2 samples.

Table 1—Kinetic constants of proteolysis of β-casein, correlation coefficients (*r*), and standard errors (SE) using Eq. 1[a]

		Cheese S			Cheese K		
Zone	Temperature (°C)	k (10^{-2}/d)	*r*	SE (10^{-2})	k (10^{-2}/d)	*r*	SE (10^{-2})
Central	5	0.378a	0.948	0.056	0.407a	0.921	0.077
	12	0.915b	0.985	0.070	0.817b	0.966	0.097
	16	1.276c	0.984	0.101	1.415c	0.978	0.014
External	5	0.497a	0.881	0.119	0.474a	0.906	0.099
	12	1.508d	0.940	0.245	1.379d	0.953	0.195
	16	1.821d	0.988	0.129	1.834e	0.994	0.088

[a]Different letters in a same column represent significant differences ($P < 0.05$).

Table 2—Values for activation energy (Ea) of proteolysis of β-casein, correlation coefficients (*r*), and standard errors (SE) using Eq. 2.

	Cheese S			Cheese K		
Zone	Ea (kcal/gmol)	R	SE	Ea (kcal/gmol)	*r*	SE
Central	17.93	0.996	1.62	17.79	0.995	1.74
External	19.59	0.975	4.44	20.17	0.988	3.18

ferent taking into account the different variables analyzed. The approach used for calculating kinetic parameters may be useful not only for carrying out simulation or optimization processes but also for analysis of proteolysis in other cheese systems.

Conclusions

Temperature affected significantly the proteolysis of β-casein during the ripening of Fynbo cheese. The proteolysis of β-casein was significantly affected by region within cheese but only at the end of the ripening period studied, whereas it was not affected by the replacement of NaCl by KCl. Characteristic kinetic parameters of proteolysis of β-casein at different zones corresponding to Fynbo cheeses salted with NaCl or NaCl/KCl and ripened at different temperatures were calculated. This phenomenon could be adequately described by 1st-order rate kinetics. Kinetic constants were in the range of 0.004/d to 0.018/d, and the activation energy of the reaction was approximately 19 kcal/gmol in the range of 5 °C to 16 °C.

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