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International Dairy Journal 15 (2005) 363–370
INTERNATIONAL DAIRY JOURNAL
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Effects of the freezing process on proteolysis during the ripening of Port Salut Argentino cheeses

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Received 23 March 2004; accepted 1 August 2004

Abstract

The effect of freezing at −30 °C, frozen storage at −22 °C for 30 days and thawing at 5 °C on proteolysis during ripening of Port Salut Argentino cheese was studied. Cheeses were sampled at different ripening times (1, 6, 13, 27 and 56 days) and two sampling zones (central and external). Moisture content, salt concentration and RP-HPLC of the nitrogenous fractions (water-insoluble fraction, water-soluble fraction and free amino acids in the sulfosalicylic acid-soluble fraction) were analysed. The freezing process did not affect moisture and salt contents at the beginning of the ripening period nor moisture and salt redistribution during the ripening period studied. However, the freezing process affected proteolysis during ripening of Port Salut Argentino cheeses that had been frozen prior to ripening. There was increased breakdown of α_{s1}-casein and α_{s1}-I-casein, and increased breakdown of peptides of the water-soluble fraction (including α_{s1}-CN (f1-23)) along with an early development of free amino acids.

Keywords: Cheese proteolysis; Freezing; HPLC

1. Introduction

Although freezing of dairy products is an appropriate preservation technique, freezing of cheese has generally been avoided because of the tendency towards physical breakdown in body and structural characteristics caused by ice crystal formation (Webb & Arbuckle, 1977). The freezing process includes freezing, frozen storage and thawing. The changes produced during cheese freezing may lead to protein and fat destabilisation (Lück, 1977) and also affects microorganisms (Fennema, Powrie, & Marth, 1973). When cheeses are frozen prior to ripening, the freezing process may influence some of the several transformations that occur during cheese maturation.

Throughout manufacture and ripening, a series of microbiological, biochemical, chemical and physical changes occur, resulting in characteristic flavour, aroma and texture (Fox & McSweeney, 1998). Protein breakdown is the most important event during the ripening of most cheese varieties (Law, 1987). Several enzymes are involved in the sequential hydrolysis of casein: the coagulant, indigenous milk proteinases, proteinases and peptidases from starter cultures and non-starter bacteria, enzymes from secondary cultures, and exogenous enzymes (Sousa, Ardö, & McSweeney, 2001).

Several authors have investigated cheese proteolysis after freezing, although the literature on proteolysis in frozen cheeses provides conflicting information (Alonso, Juarez, Ramos, & Martín-Alvarez, 1987). For example, some researchers have reported that the freezing process did not affect cheese proteolysis. Mozzarella cheeses that underwent the freezing process before ripening

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doi:10.1016/j.idairyj.2004.08.002

showed no significant differences in primary proteolysis due to the coagulant (Chaves, Viotto, & Grosso, 1999). Sheep milk cheeses, frozen up to 6 months prior to ripening, showed no significant differences in the water-soluble, non-protein and amino acid nitrogen contents compared to the controls (Tejada, Sanchez, Gomez, Vioque, & Fernandez-Salguero, 2002). However, other studies have reported that the freezing process significantly affected proteolysis in cheeses frozen prior to ripening. Mould-ripened Cabrales cheeses had lower water-soluble, non-protein and amino acid nitrogen contents than control cheeses (Alonso et al., 1987; Ramos, Cáceres, & Polo, 1987). Therefore, the effects of the freezing process on proteolysis of cheeses frozen prior to ripening differ between cheese varieties.

Port Salut Argentino cheese is one of the most popular soft cheese varieties in Argentina (Zalazar, Meinardi, & Hynes, 1999). Port Salut Argentino is a semi-cooked cheese, produced from pasteurised milk, acidified by lactic bacteria, coagulated by rennet and/or other specific enzymes, salted by immersion in brine, and ripened for a short period (Código Alimentario Argentino, 1981). We have recently characterised the proteolysis of Port Salut Argentino cheese using chromatographic profiles of different nitrogenous fractions that allowed the separation of several components including α_{s1}-casein, α_{s1}-I-casein, water-soluble fragments (including α_{s1}-CN (f1-23)), and free amino acids (Verdini, Zorrilla, & Rubiolo, 2004). Earlier studies (Verdini, Zorrilla, & Rubiolo, 2002) found that the freezing process increased the content of water-soluble nitrogen and total free amino acids during the ripening of Port Salut Argentino cheeses frozen prior to ripening. Moreover, the first-order kinetics constant for α_{s1}-casein hydrolysis increased significantly and differences due to sampling zone were found in those cheeses (Verdini, Zorrilla, & Rubiolo, 2003). However, the effects of the freezing process on different steps of Port Salut Argentino cheese proteolysis have not yet been studied.

In the present study, chromatographic profiles of the nitrogenous fractions were used to characterise the effects of the freezing process, prior to ripening, on proteolysis of Port Salut Argentino cheeses.

2. Materials and methods

2.1. Cheese samples

Commercial Port Salut Argentino cheeses (3.55±0.11 kg weight, 23.2±0.3 cm diameter, 7.7±0.3 cm height, 28.7±0.7% w/w fat, 20.4±0.9% w/w total protein, 48.8±2.6% w/w moisture, and 5.2±0.1 pH) were used for this study. Cheeses were manufactured at a local factory, salted in a brine solution for 3 h at 3 °C, stored for 20 h and packed in heat-shrinkable plastic bags.

Thirty cheeses were transported in insulated boxes with ice from the factory to our laboratory and randomly separated into two groups. Fifteen cheeses were frozen in a Tabai Comstar PR 4GM chamber (Tabai Espec Corp., Osaka, Japan) at −30 °C until the centre reached −22 °C, held in frozen storage at −22 °C for 30 days, and then thawed at −5 °C. After thawing, cheeses were held at 5 °C for ripening (cheeses F). Fifteen cheeses were held at 5 °C for ripening and were used as a control (cheeses R).

Cheeses R and F were sampled at different ripening times (1, 6, 13, 27, and 56 days) in triplicate. Cubic pieces of 25 mm were cut as described by Creamer and Olson (1982) from two different cheese zones, the central zone (C) and the external zone (E). The geometric centre of the cubic pieces of the central and external zones were separated approximately 5.5 cm in the radial direction and 2.0 cm in the axial direction (Verdini & Rubiolo, 2002a).

2.2. Moisture and chloride analysis

Moisture content was measured using a microwave oven method (CEM AVC 80, CEM, Mattheus, NC, USA). Chloride concentration was determined using a potentiometric method with an Automatic Titrator model DL40RC (Mettler Instrumente AG, Greifensee, Switzerland) as described by Fox (1963).

2.3. Extraction and chromatographic analysis of nitrogenous compounds

Grated cheese (10 g) mixed with three times the sample weight of water was homogenised using an Ultra-Turrax® T25 (IKA® Werke, Janke & Kunkel GmbH & Co KG, Staufen, Germany) for 2 min (Kuchroo & Fox, 1982). The fractionation method described by Verdini et al. (2004) was followed for the separation of three nitrogenous fractions: the water-insoluble fraction, the water-soluble fraction, and the sulfosalicylic acid-soluble fraction (SSA-soluble fraction). The nitrogenous fractions were stored in a freezer at −22 °C until analysed.

The chromatographic analysis of the three nitrogenous fractions was performed as described in Verdini et al. (2004). Two peaks were identified in the water-insoluble fraction of Port Salut Argentino cheese samples: the α_{s1}-casein peak was identified using a standard of α_s-casein (Sigma-Aldrich, St. Louis, MO, USA) and the α_{s1}-CN (f24-199) fragment, known as α_{s1}-I-casein, was identified as previously described by Verdini et al. (2004). Sixteen peaks of the water-soluble fraction that characterised Port Salut Argentino cheese ripening (Verdini et al., 2004) were selected for further analysis (Fig. 1). Peak 13 of the water-soluble fraction chromatogram was assigned to the fragment α_{s1}-CN (f1-

23) as described by Verdini et al. (2004). Fifteen free amino acids were detected and quantified in the SSA-soluble fraction of Port Salut Argentino cheese samples (Verdini et al., 2002, 2004).

2.4. Statistical analysis

The experimental design was a full factorial design with three cheeses per treatment. Data were analysed using ANOVA with Minitab 13.20 (Minitab Inc., State College, PA, USA). When differences between treatment effects were significant ($p<0.05$), a multiple comparison of means was performed using Tukey's test. Principal component analysis (PCA) was used to reduce the dimensionality of the data obtained from the water-soluble fraction and free amino acid profiles in order to assess the effects of the freezing process, the ripening time, and the sampling zone on the formation of water-soluble peptides and free amino acids. PCA was applied to the mean centred data matrix as described by Verdini and Rubiolo (2002b).

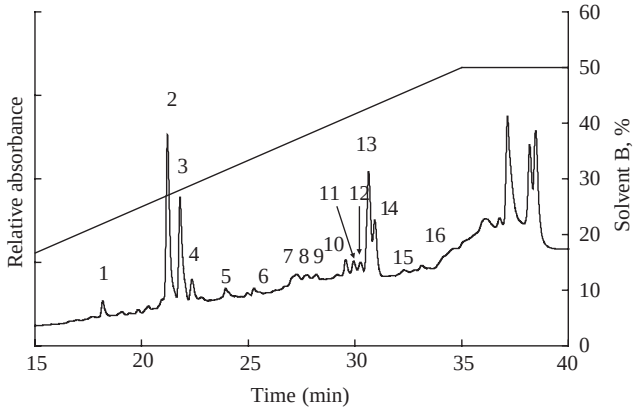

Fig. 1. Chromatogram of the water-soluble fraction of a cheese sample (cheese R, zone C, 6 days of ripening). Peaks that were identified and used as variables in the multivariate statistical analysis are indicated by numbers.

3. Results and discussion

3.1. Moisture and salt content

Moisture and salt in moisture content of cheese samples are shown in Tables 1 and 2, respectively. Salt and moisture contents of F cheeses were similar to R cheeses (Verdini et al., 2004). Moisture and salt gradients were observed at the beginning of the ripening time (1 day). Cheeses reached a uniform salt concentration at the end of the ripening period but did not reach a uniform moisture content during the studied ripening period (56 days). Consequently, the freezing process did not affect moisture and salt content at the beginning of

Table 1
Moisture content of cheese samples

Cheese	Zone	Ripening time (days)				
		1	6	13	27	56
R	C	46.33 ± 0.89^{a}	45.22 ± 1.97^{a}	47.06 ± 1.56^{a}	45.24 ± 0.39^{a}	48.64 ± 0.95^{b}
	E	50.64 ± 0.48^{c}	50.51 ± 1.61^{c}	51.17 ± 0.54^{c}	50.65 ± 0.23^{c}	52.48 ± 0.75^{d}
F	C	44.82 ± 1.93^{a}	47.53 ± 1.98^{a}	45.71 ± 1.13^{a}	46.00 ± 2.39^{a}	48.64 ± 0.95^{b}
	E	50.45 ± 1.01^{c}	51.45 ± 0.43^{c}	50.71 ± 0.86^{c}	50.29 ± 0.83^{c}	52.48 ± 0.75^{d}

Mean values and standard deviations of three samples.
Values with the same letters are not significantly different ($p<0.05$) from each other.
See Materials and Methods section for explanation of codes.

Table 2
Salt in moisture content of cheese samples

Cheese	Zone	Ripening time (days)				
		1	6	13	27	56
R	C	0.54 ± 0.11^{a}	0.86 ± 0.20^{ab}	1.39 ± 0.27^{bc}	1.44 ± 0.27^{c}	2.27 ± 0.20^{d}
	E	1.88 ± 0.12^{d}	1.93 ± 0.07^{d}	2.53 ± 0.60^{d}	1.88 ± 0.15^{d}	2.25 ± 0.02^{d}
F	C	0.55 ± 0.16^{a}	0.85 ± 0.15^{ab}	1.36 ± 0.27^{bc}	1.81 ± 0.21^{c}	1.86 ± 0.27^{d}
	E	2.10 ± 0.13^{d}	1.99 ± 0.13^{d}	2.08 ± 0.03^{d}	2.08 ± 0.34^{d}	1.84 ± 0.14^{d}

Mean values and standard deviations of three samples.
Values with the same letters are not significantly different ($p<0.05$) from each other.
See Materials and Methods section for explanation of codes.

the ripening period or moisture and salt redistribution during the studied ripening period.

3.2. Primary proteolysis of α_{s1}-casein

Peak areas of α_{s1}-casein, α_{s1}-I-casein, and α_{s1}-CN (f1-23) per 100 g cheese during the ripening of Port Salut Argentino cheeses are shown in Figs. 2, 3a and 3b, respectively.

The freezing process affected the rate of α_{s1}-casein hydrolysis (Verdini et al., 2003). There was an earlier decrease in the α_{s1}-casein content during the ripening of F cheeses (Fig. 2). The decrease was observed between 13 and 27 days in R cheeses, while it was detected between 6 and 13 days in F cheeses. Moreover, Verdini et al. (2003) showed that the first-order kinetics constant of α_{s1}-casein breakdown during the ripening of Port Salut Argentino cheeses frozen prior to ripening, was significantly affected by the freezing process. The freezing process also affected the subsequent degradation of α_{s1}-I-casein and α_{s1}-CN (f1-23), but the effects of the freezing process were not similar for both the water-insoluble and the water-soluble fragments (Fig. 3a and b). During the ripening of F cheeses, the content of α_{s1}-I-casein rapidly increased between 6 and 13 days and then dramatically decreased (Fig. 3a). On the other hand, the α_{s1}-CN (f1-23) content increased until 13 days and reached a plateau between 13 and 56 days (Fig. 3b). Both α_{s1}-I-casein and α_{s1}-CN (f1-23) contents increased between 13 and 56 days during the ripening of R cheeses (Verdini et al., 2004).

Alichanidis, Polychrooniadou, Tzanetakis, and Vafopoulou (1981) have reported an increase in the rate of

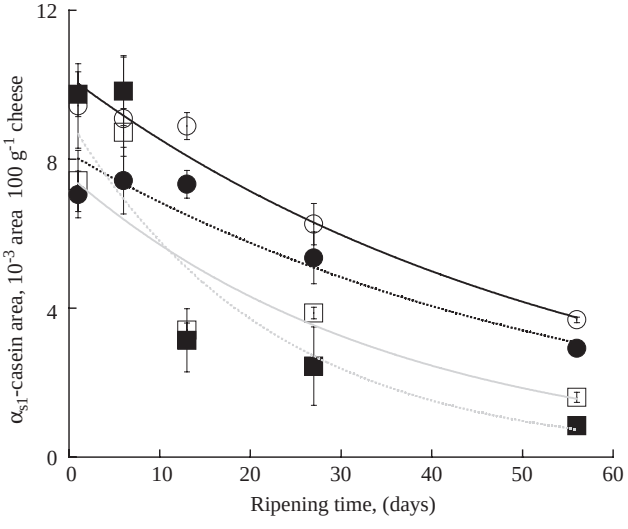

Fig. 2. Peak areas of α_{s1}-casein during Port Salut Argentino cheese ripening: (○) Cheeses R—Zone C, (●) Cheeses R—Zone E, (□) Cheeses F—Zone C, (■) Cheeses F—Zone E. Fitted curves for (—) Cheeses R—Zone C, (———) Cheeses F—Zone C, (- - - - -) Cheeses R—Zone E, (– – – –) Cheeses F—Zone E. Bars show standard deviations.

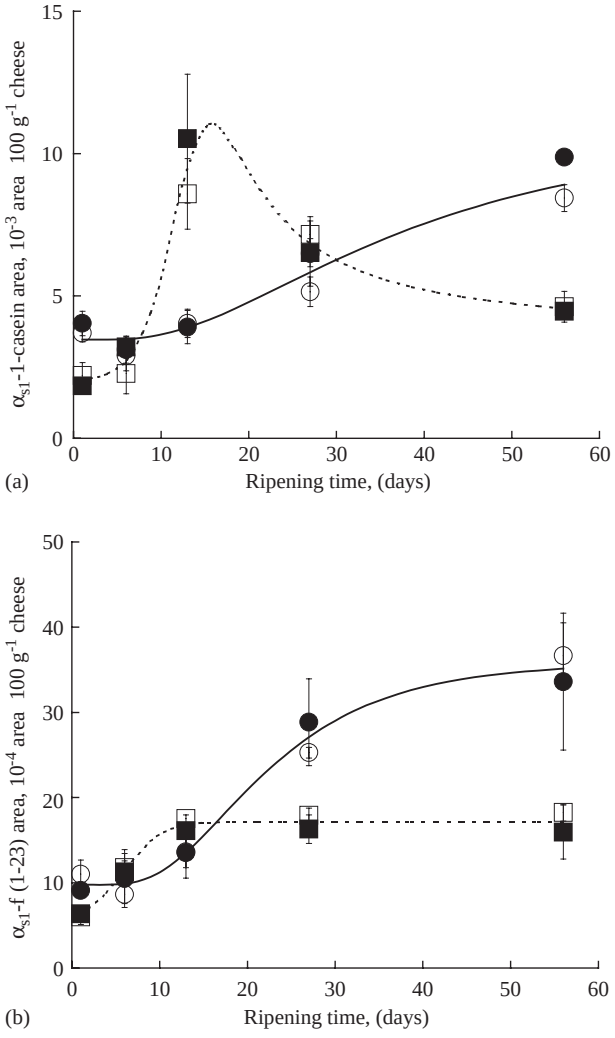

Fig. 3. Peaks areas of products of primary α_{s1}-casein proteolysis during Port Salut Argentino cheese ripening: (a) α_{s1}-I-casein, and (b) α_{s1}-CN (f1-23); (○) Cheeses R—Zone C, (●) Cheeses R—Zone E, (□) Cheeses F—Zone C, (■) Cheeses F—Zone E. Trend lines for (—) Cheeses R, (- - - - - -) Cheeses F. Bars show standard deviations.

casein hydrolysis during the ripening of Teleme cheese made from frozen curd. The authors suggested that the curd structure would have undergone changes during the freezing process, which may have enabled hydrolysis of proteins to proceed at a faster rate. Several factors may contribute to proteolysis in Port Salut Argentino cheeses frozen prior to ripening. The changes resulting from cheese freezing may lead to protein destabilisation (Lück, 1977), but proteins are not destabilised instantaneously during freezing, rather they are altered gradually during frozen storage (Fennema et al., 1973). In particular, the conformation of α_{s1}-casein in the cheese matrix restrained rennet action (Exterkate, Lagerwerf, Haverkamp, & Van Schalkwijk, 1997). These authors suggested that complex interactions between forces regulating enzyme-substrate binding might change when the surrounding environment is changed. Accordingly,

the freezing process might produce conformational changes in α_{s1}-casein that may increase its susceptibility to chymosin attack. Conformational changes in α_{s1}-casein may also affect the stability and consequently the reactivity of the water-insoluble fragment, α_{s1}-1-casein, while, further hydrolysis of α_{s1}-CN (f1-23) may be related to an increasing availability of microbial enzymes as described below.

There were no significant differences in the first-order kinetics constant for α_{s1}-casein hydrolysis of R cheeses; but in contrast, the first-order kinetics constant of F cheeses was higher in the external zone than in the central zone (Verdini et al., 2003). No significant difference in α_{s1}-I-casein and α_{s1}-CN (f1-23) were observed in relation to sampling zone for either F cheeses or R cheeses during the studied ripening period.

3.3. Water-soluble fraction

Maturation was determined as the percentage of water-soluble nitrogen of the total cheese nitrogen. However this method does not measure the hydrolysis of the water-soluble peptides into smaller water-soluble peptides that still remain in the water-soluble fraction. Maturation of Port Salut Argentino cheeses, frozen for 1 month, increased, between 13 and 56 days (Verdini et al., 2002). The freezing process significantly affected maturation, however a trend due to factor interaction was not observed.

The formation of water-soluble peptides was analysed via chromatograms of the water-soluble fraction. Sixteen selected peaks were examined (Fig. 1) and peak areas per 100 g cheese were measured. PCA was applied to the 60 samples and 16 mean centred variables yielding three principal components (PC) that explained 95.5% of the data set variation (PC1 86.5%, PC2 4.6% and PC3 4.4%). Plots of PC2 vs. PC1 scores, and PC2 vs. PC1 loadings are shown in Figs. 4a and b, respectively.

F cheeses were concentrated in two groups along the PC1 axis (Fig. 4a), samples from 1 to 6 days, and samples from 13 to 56 days; while cheeses R were distributed along PC1 scores axis in three groups, samples from 1 to 13 days, samples of 27 days, and samples of 56 days. All PC1 loadings were positive and peaks with higher PC1 loadings were 2, 3, 5, 7, and 13 (Fig. 4b). PC2 loadings were both positive and negative. Among peaks with higher PC2 loadings, peaks 7, 13, and 4 had negative PC2 loadings while peaks 5 and 8 had positive PC2 loadings (Fig. 4b).

Fig. 5 shows the area profiles of selected peaks with higher PC loadings during the ripening of Port Salut Argentino cheeses. There were significant differences in the behaviour of those peaks during the ripening of F and R cheeses. The area of peaks 2 and 3 (Fig. 5a and b) increased until 13 days during the ripening of F cheeses and reached a plateau between 13 and 56 days.

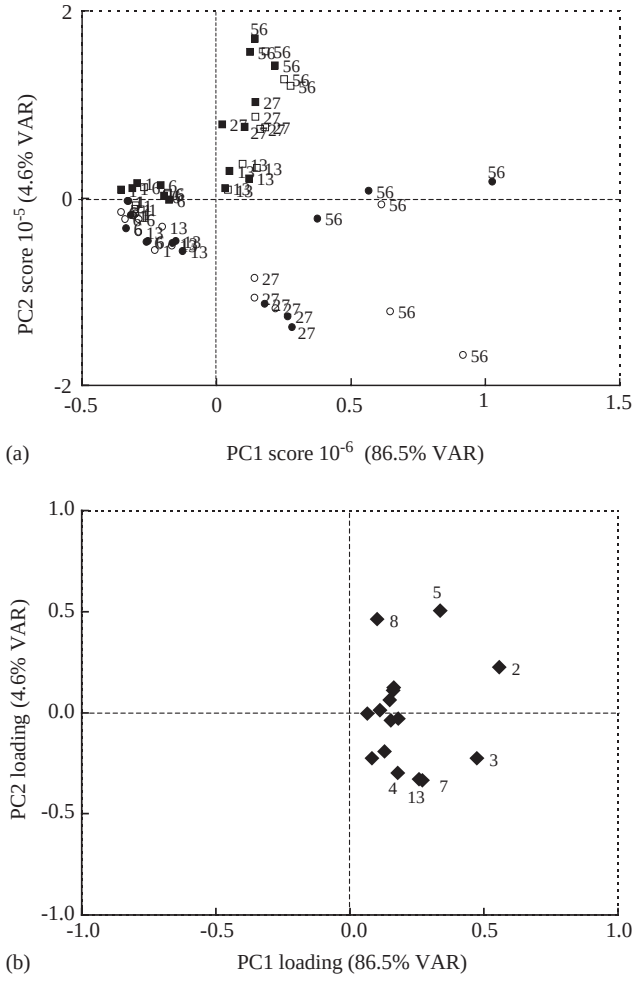

Fig. 4. Plots of the PC when data for the water-soluble fraction of Port Salut Argentino cheeses were analysed: (a) PC scores plot; (○) Cheeses R—Zone C, (●) Cheeses R—Zone E, (□) Cheeses F—Zone C, (■) Cheeses F—Zone E. Numbers indicate the ripening time in days of the samples. (b) PC loadings plot. Numbers indicate peaks with higher PC1 loadings.

However, in the case of R cheeses, the area of peaks 2 and 3 increased between 13 and 56 days. These observations are in agreement with the previously discussed behaviour of peak 13 (assigned to α_{s1}-CN (f1-23)). The area of peak 5 increased during the ripening of F and R cheeses between 13 and 56 days, but at slightly different rates (Fig. 5c). Finally, the area of peak 7 (Fig. 5d) remained almost constant during the ripening of F cheeses, but increased between 13 and 56 days during the ripening of R cheeses.

The freezing process led to an early production of peaks 2, 3, 5, and 7 but also to an increment in their degradation rate (Fig. 5) in agreement with the described behaviour of α_{s1}-CN (f1-23). The early production of the water-soluble fragments is a consequence of hydrolysis of α_{s1}-casein. However, further hydrolysis might be related to the fact that starter microorganisms that are injured by the freezing process

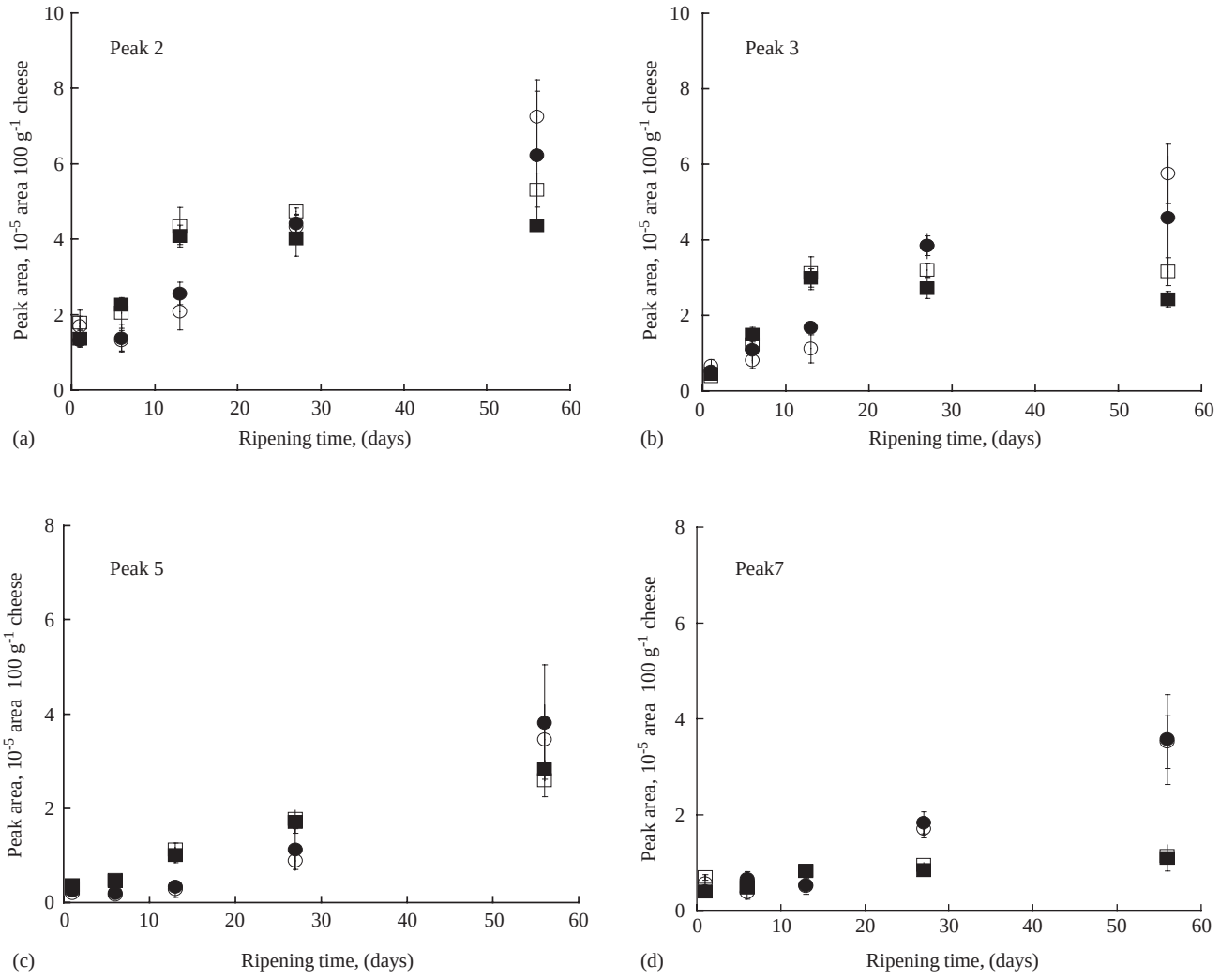

Fig. 5. Areas of peaks of the water-soluble fraction during Port Salut Argentino cheese ripening: (a) peak 2, (b) peak 3, (c) peak 5, and (d) peak 7; (○) Cheeses R—Zone C, (●) Cheeses R—Zone E, (□) Cheeses F—Zone C, (■) Cheeses F—Zone E. Bars show standard deviations.

might suffer damage to membranes ranging from changes in permeability to loss of viability (Fennema et al., 1973). As a result, damaged microorganisms might release more proteinases and peptidases. The fact that the formation of most water-soluble peptides was affected by the freezing process, might relate increased proteolysis of small fragments to increased levels of enzymes released by damaged microorganisms.

Previous studies of the water-soluble fraction during the ripening of Port Salut Argentino cheese showed that peaks with higher PC1 loadings had increased peak areas during ripening. The first PC was related to an increasing production of most of the water-soluble peptides during ripening (Verdini et al., 2004). In this study, areas of peaks with higher PC1 loadings of R cheeses increased at different rates between 13 and 56 days of ripening and areas of peaks with higher PC1 loadings of F cheeses showed a plateau between 13 and 56 days of ripening (Fig. 5). Nevertheless, the first PC can still be related to the production of water-soluble peptides during ripening. The fact that F cheeses are compacted in the PC1 score axis between 13 and 56 days of ripening can be related to an increased hydrolysis of most of the water-soluble peptides due to the freezing process. As described in a previous study, the second PC was related to some differences observed in the rate of peptide formation (Verdini et al., 2004). In this study, peaks with higher PC2 loadings showed different profiles for F and R cheeses according to its PC2 loading sign. Consequently, the second PC may be related to different rates of water-soluble peptide formation and hydrolysis both related to the freezing process.

No significant differences in the areas of selected peaks were observed between sampling zones, for either F or R cheeses during the studied ripening period.

3.4. Free amino acids

Total free amino acid content (determined as the sum of the detected amino acid concentrations) of Port Salut Argentino cheeses, frozen for 1 month, was higher than the controls (Verdini et al., 2002). PCA was applied to data obtained from free amino acids in the SSA-soluble fraction (mg amino acid 100 g^{-1} cheese) of both cheeses R and F (Verdini et al., 2002). The 60 samples and 15 mean centred variables yielded two PC that explained 97.5% of the data set variation (PC1 93.7% and PC2 3.8%). Plots of PC scores and PC loadings are shown in Fig. 6a and b, respectively.

F cheeses were dispersed in four groups along the PC1 score axis (Fig. 6a), samples from 1 to 6 days, samples of 13 days, samples of 27 days, and samples of 56 days; while, R cheeses were distributed along the PC1 score axis in three groups, samples from 1 to 13 days, samples of 27 days, and samples of 56 days. All PC1 loadings were positive while most PC2 loadings were negative except for lysine, tyrosine, and glutamine. Amino acids with higher PC1 loadings were leucine, lysine, asparagine and phenylalanine; while amino acids with higher PC2 loadings were lysine and leucine (Fig. 6b).

Leucine and lysine contents during the ripening of Port Salut Argentino cheeses are shown in Fig. 7. There was a gradual increase of leucine content (Fig. 7a) during the whole ripening period of F cheeses. However, in the case of R cheeses the increase took place between 13 and 56 days of ripening. Finally, there was an increase of lysine content (Fig. 7b) during the ripening of F cheeses between 13 and 27 days as well as for R cheeses.

The early development of free amino acids in cheeses that underwent frozen storage before ripening compared to the controls might be related to increasing levels of

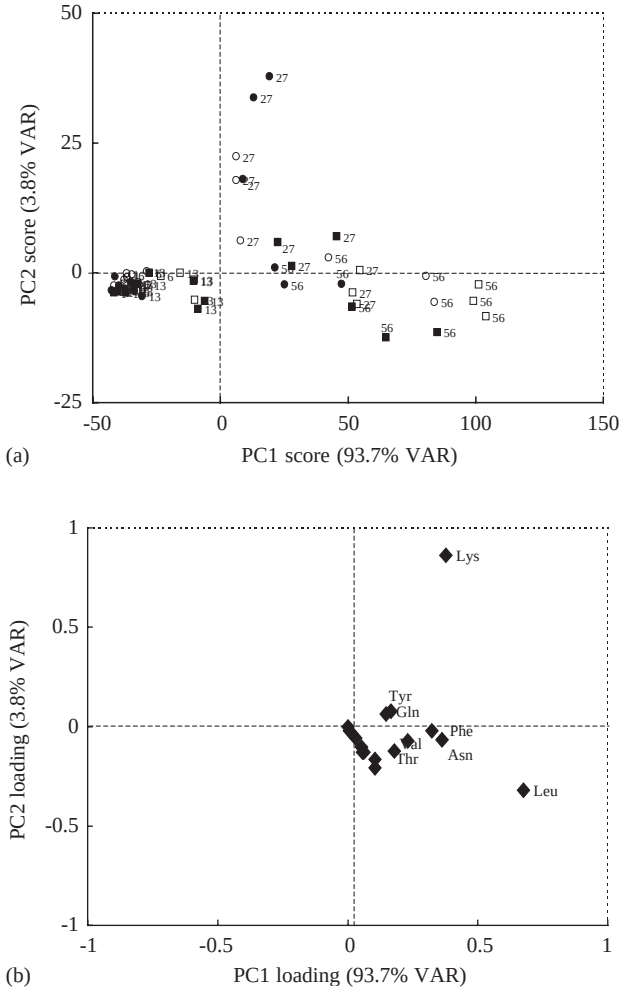

Fig. 6. Plot of the first two PC when data for the free amino acid content in the SSA-soluble fraction of Port Salut Argentino cheeses were analysed: (a) PC scores plot. (○) Cheeses R—Zone C, (●) Cheeses R—Zone E, (□) Cheeses F—Zone C, (■) Cheeses F—Zone E. Numbers indicate the ripening time in days of the samples. (b) PC loadings plot. Numbers indicate peaks with higher PC1 loadings.

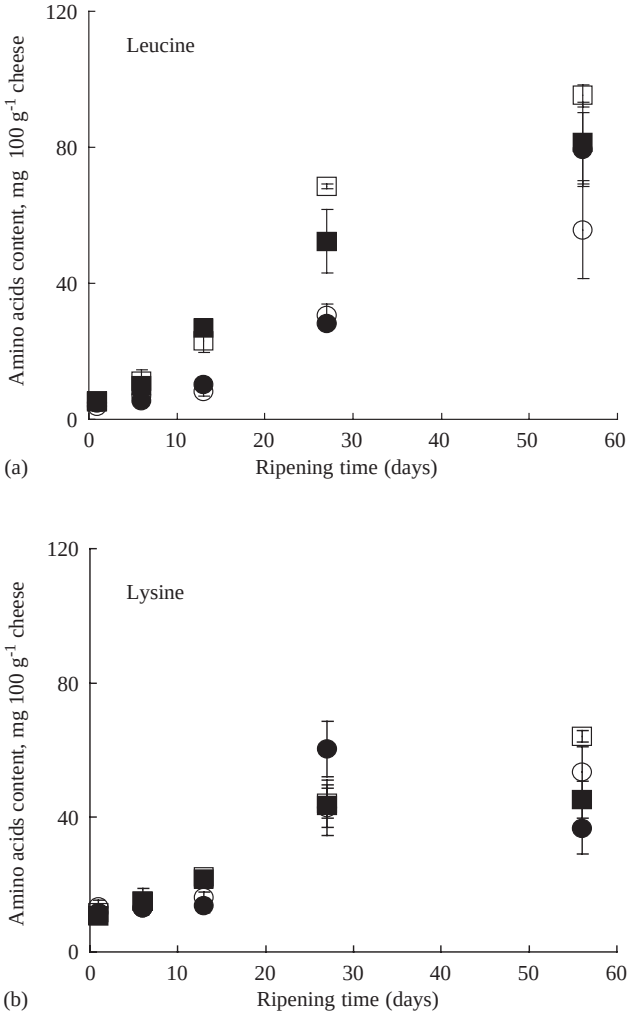

Fig. 7. Free amino acid content in the SSA-soluble fraction during Port Salut Argentino cheese ripening: (a) leucine, and (b) lysine; (○) Cheeses R—Zone C, (●) Cheeses R—Zone E, (□) Cheeses F—Zone C, (■) Cheeses F—Zone E. Bars show standard deviations.

enzymes released by damaged microorganisms, as previously discussed for the small peptides present in the water-soluble fraction. Previous studies of free amino acids during the ripening of Port Salut Argentino cheese showed that amino acids with higher PC1 loadings increased their areas during ripening and the first PC was related to an increasing production of most free amino acids as ripening time increased (Verdini et al., 2004). In this study, the second PC may be related to the different rates of amino acid formation that may depend on the different enzyme activities as discussed in previous studies (Verdini et al., 2002).

Some differences were observed between the sampling zones at 27 and/or 56 days of ripening for both F and R cheeses.

4. Conclusions

The freezing process affected casein proteolysis during the ripening of Port Salut Argentino cheeses frozen prior to ripening. Hydrolysis of α_{s1}-casein and α_{s1}-I-casein increased that may be related to conformational changes affecting their susceptibility to chymosin attack. There was an increased hydrolysis of water-soluble peptides, particularly α_{s1}-CN (f1-23) and an early development of free amino acids that may be related to increased levels of enzymes released from microorganisms damaged by the freezing process. The freezing process therefore influenced the rates of the occurring chemical reactions but not the sequence of the different stages of the proteolytic pathways.

Acknowledgements

The authors acknowledge SanCor Cooperativas Unidas Ltd. for the supply of cheeses. This work was done with the financial support of Universidad Nacional del Litoral (Santa Fe, Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), and Agencia Nacional de Promoción Científica y Tecnológica (Argentina).

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