Dairy Sci. & Technol. (2013) 93:691–698
DOI 10.1007/s13594-013-0133-6

NOTE

Effect of freezing prior to ripening on the peptide profile present in the water-soluble fraction at pH 4.6 of a commercial low-fat soft cheese

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Received: 6 December 2012 / Revised: 23 April 2013 / Accepted: 29 April 2013 /
Published online: 30 May 2013

Abstract Freezing was proposed as an alternative preservation technique to extend the shelf life of soft cheeses. However, the freezing process (freezing, frozen storage, and thawing) can affect a number of cheese quality parameters, such as the water-soluble nitrogen content at pH 4.6, which is related to the maturation index of cheeses. The objective of this work was to assess the effect of the freezing process prior to ripening of low-fat soft cheese containing microparticulated whey proteins as fat replacer. The freezing process consisted of freezing at −25 °C, frozen storage at −25 °C during 33 days, and thawing at 6 °C. Refrigerated cheeses, stored at 6 °C, were selected as control samples. High-performance liquid chromatography was used for analyzing the evolution of peptides present in the water-soluble fraction at pH 4.6 at different ripening times. Principal component analysis was applied to reduce the dimensionality of the data obtained from chromatograms. Results indicated that peptide profile was affected by the freezing process. Areas of peaks with hydrophilic characteristics increased during the ripening time, from 1 to 48 days, in both refrigerated and frozen cheeses. Furthermore, areas of hydrophilic peaks were higher in frozen cheeses than in refrigerated cheeses at 21 and 48 days of maturation. However, areas of peaks with hydrophobic characteristics increased in refrigerated cheeses but stayed invariable in frozen cheeses during all the studied ripening periods. In this case, areas of hydrophobic peaks were lower in frozen cheeses than in refrigerated cheeses at the same day of ripening time.

Keywords Low-fat soft cheese · Water-soluble fraction · Freezing · RP-HPLC

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1 Introduction

Low-fat cheeses made with functional ingredients or additives, such as fat replacers, are being consumed by people who are interested in healthy and tasty foods. Fat replacers (for instance, microparticulated whey proteins) are used in the low-fat cheese-making process because those particles improve the texture and the nutritional value of low-fat food products (Singer 1996).

Fresh and soft cheeses are distinguished by their high moisture content and short shelf life period. This type of cheeses should be preserved at low temperatures (4–6 °C). Taking into account this concern, freezing was proposed as an alternative preservation technique to prolong the shelf life of several types of soft cheeses, such as caprine milk cheese (Van Hekken et al. 2005), Port Salut Argentino cheese (Verdini et al. 2005), and Mozzarella cheese (Kuo and Gunasekaran 2009).

However, the freezing process can affect a number of cheese quality parameters. For example, freezing can produce an increase in the water-soluble nitrogen content at pH 4.6 which is related to the maturation index (MI) of cheeses (Graiver et al. 2004). A quality defect of low-fat cheeses is the low extent of protein breakdown during ripening (Mistry 2001). With regard to this phenomenon, freezing was proposed as a strategy for improving the quality of low-fat soft cheeses, because their MI was increased by frozen storage (Meza et al. 2011).

The water-soluble fraction at pH 4.6 (WSF) contains intermediate-sized peptides with different hydrophobicities and is used to characterize the degree of cheese proteolysis during ripening (Sousa et al. 2001). Nevertheless, the WSF does not consider the possible hydrolysis of the intermediate-sized peptides into other small peptides that remain in the WSF. Several studies have been carried out in order to analyze the peptide profile in the WSF of numerous varieties of full-fat cheeses (Hayaloglu et al. 2010; Pripp et al. 2006) and low-fat cheeses (Fenelon et al. 2000), using techniques such as reverse-phase high-performance liquid chromatography (RP-HPLC). However, information about the effect of freezing on the peptide profile present in the WSF of a low-fat soft cheese has not been found in literature. This study is necessary to obtain a more detailed characterization of cheese proteolysis that complements previous published results (Meza et al. 2011).

The objective of this work was to assess the effect of the freezing process prior to ripening of low-fat soft cheese containing microparticulated whey proteins as fat replacer. RP-HPLC was used for analyzing the evolution of peptides present in the WSF at different ripening times.

2 Materials and methods

2.1 Low-fat soft cheeses and treatments

Commercial low-fat soft cheeses containing microparticulated whey proteins as fat replacer (Simplesse® D100, NutraSweet Co., Deerfield, IL, USA) were used for this study. Low-fat cheeses were manufactured at a local factory by rennet coagulation of pasteurized skim milk according to regional legislation, salted in brine, and packed in heat-shrinkable plastic bags. Samples presented a rectangular shape (28.7±0.3 by

11.6±0.3 cm side and 7.4±0.2 cm height) and 3.0±0.1 kg of weight. The initial composition was: 52.8±0.5% moisture, 33.6±0.0% proteins, 5.75±0.04% fat, and 0.41±0.01% salt.

Nineteen low-fat cheeses from the same lot of cheese-making process were carried on ice containers from the local factory to the testing laboratory. Samples were randomly divided in two groups. Nine cheeses were held at 6 °C for ripening and used as control samples (refrigerated cheeses). This temperature was selected according to manufacturers' recommendation for this type of cheese.

The other ten low-fat cheeses were frozen at −25 °C (freezing rate of 1.46 °C/h) until the center reached −25 °C in a Tabai Comstar PR 4GM chamber (Tabai Espec Corp., Osaka, Japan). Subsequently, cheeses were held in frozen storage at −25 °C for 33 days and then thawed at 6 °C. The selected freezing temperature corresponds to the minimum temperature reached by the chamber. For practical purposes, the time of frozen storage was selected to ensure approximately 1 month of frozen storage.

The samples' temperature during freezing and thawing was measured in the center of one cheese using a thermocouple and a data acquisition system Tabai Comstar THP-18 (Tabai Espec Corp., Osaka, Japan). After thawing, cheeses were held at 6 °C for ripening (frozen cheeses).

Representative samples were taken from both refrigerated and frozen cheeses at different ripening times (1, 21, and 48 days) in triplicate, using a sampling procedure already reported (Meza et al. 2011). For example, in refrigerated cheeses, 21 days of ripening imply 21 days from the time cheeses were placed in storage at 6 °C. In frozen cheeses, 21 days of ripening imply 21 days from the time cheeses were thawed and placed in storage at 6 °C.

Moisture content was determined by standard procedure (AOAC 1990). Protein content was estimated from total nitrogen content determined by the Kjeldahl method, using a Büchi 430 automatic digestor (Büchi, Flawil, Switzerland), a Büchi 322 distillation unit, and a Mettler DL40RC automatic titrator (Mettler Instrument AG, Greifensee, Switzerland). Fat content was determined using a standard protocol (IDF 1969). Salt concentration was calculated using a potentiometric method (Fox 1963). All the analyses were made in duplicate.

2.2 Analysis of water-soluble fraction at pH 4.6

The WSF was extracted with a protocol developed by Kuchroo and Fox (1982) with modifications. An aliquot of WSF (100 mL), filtered through a disposable 0.2-μm filter, was injected in a Waters chromatography system (Waters Corporation, Mildford, MA, USA). The system consisted of Waters 1500 Series HPLC pump, Waters 717 plus autosampler, Waters 2487 dual λ absorbance detector, and Waters Breeze System software. A Chrompack (250×4.6 mm) C_{18}, 300 Å column (Varian Inc., Palo Alto, CA, USA) at 30 °C was used for chromatographic separations. The separations were made at a flow rate of 1 mL min^{-1} using solvent A (1 mL trifluoracetic acid in 1 L water) for 10 min, linear gradient from 0 to 80% of solvent B (1 mL trifluoracetic acid in 1 L acetonitrile/water, 60:40) over 80 min and isocratic step at 80% B for 15 min. Detection was performed at 214 nm, and all determinations were made in triplicate.

2.3 Statistical analysis

Principal component analysis (PCA) was applied to reduce the dimensionality of the data obtained from chromatograms of the WSF, using Minitab 13.20 (Minitab Inc., State College, PA, USA).

3 Results and discussion

Interpretation of results obtained in this work applies only to low-fat soft cheeses made with microparticulated whey proteins (Simplesse®) in the amounts used by the manufacturer at the moisture level used for this type of cheese.

In order to analyze the evolution of the peptides present in the WSF, 22 peaks were selected from chromatograms of refrigerated and frozen cheeses (Fig. 1). Hydrophobic peaks eluting at the end of the chromatograms (90–100 min) correspond to serum proteins (α-lactalbumin and β-lactoglobulin A and B). Consequently, they were not included in the water-soluble peptide analysis (Verdini et al. 2004). In agreement, the area of those peaks did not change during the studied ripening period.

The high number of peaks observed in the chromatograms indicates the presence of a complex mixture of casein hydrolysis products in the WSF (Fig. 1). PCA was applied to the 22 selected peaks in order to assess the effects of the freezing process on the formation of water-soluble peptides and to identify the peaks that characterize low-fat soft cheese ripening.

The first two principal components (PC1 and PC2) contained the meaningful variance in the data set, explaining the 94% of the total variance (PC1 71.6% and PC2 22.4%). The PC1 and PC2 scores plot is shown in Fig. 2a. According to the results, PC1 and PC2 illustrate the separation of samples in five groups (Fig. 2a). The first principal component (71.6% VAR) can be related to the ripening time, due to the

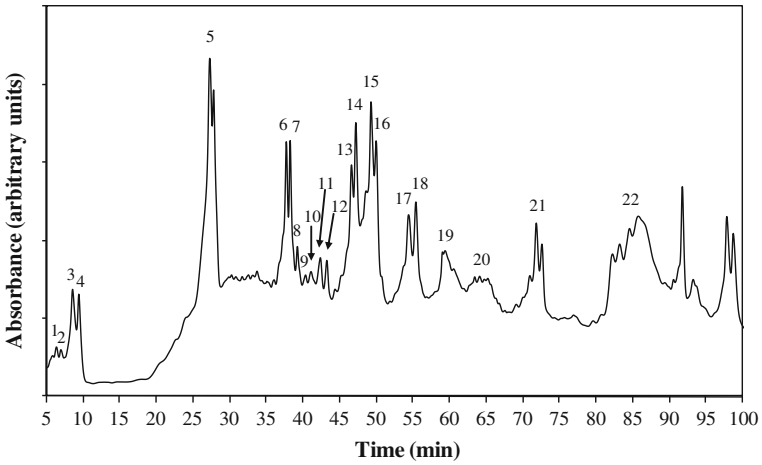

Fig. 1 Example of a chromatogram of the water-soluble fraction at pH 4.6 of a commercial low-fat soft cheese containing microparticulated whey proteins as fat replacer. *Numbers* indicate the selected peaks

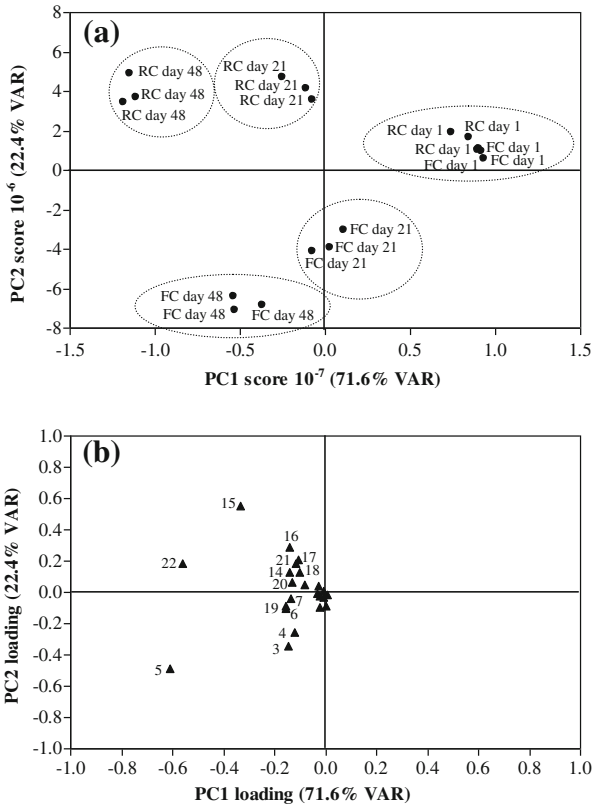

Fig. 2 Plots of scores (**a**) and loadings (**b**) of the two first principal components of data obtained from chromatograms of the water-soluble fraction at pH 4.6 for refrigerated (*RC*) and frozen (*FC*) commercial low-fat soft cheeses containing microparticulated whey proteins as fat replacer

separation of samples from right to left according to ripening time (1, 21, and 48 days). The second principal component (22.4% VAR) can be related to the effect of freezing process, due to the separation of samples from top to bottom according to the treatment (refrigerated and frozen cheeses) in samples with 21 and 48 days of ripening.

The PC1 and PC2 loadings plot, representing the selected peaks in the bidimensional PC space, is shown in Fig. 2b. Peaks with the highest PC1 loading values were: 3, 5, 6, 7, 14, 15, 16, 19, and 22, while peaks with the highest PC2 loadings values were: 3, 4, 5, 15, 16, 17, 21, and 22. All peaks had negative values of PC1 loadings, but some peaks presented positive and others negative values of PC2 loadings. According to the results, the evolution of peaks 3, 4, and 5 (negative values of PC2 loadings) and 15, 16, 17, 21, and 22 (positive values of PC2 loadings) was different depending on the ripening time and treatment.

Analyzing the effect of ripening time, areas of peaks 3, 4, and 5 of both refrigerated and frozen cheeses increased during all the studied ripening periods, from 1 to 48 days. The evolution of peaks 3 and 4 is shown in Fig. 3a and b. The area of peak 5 presented the same behavior (data not shown). This result

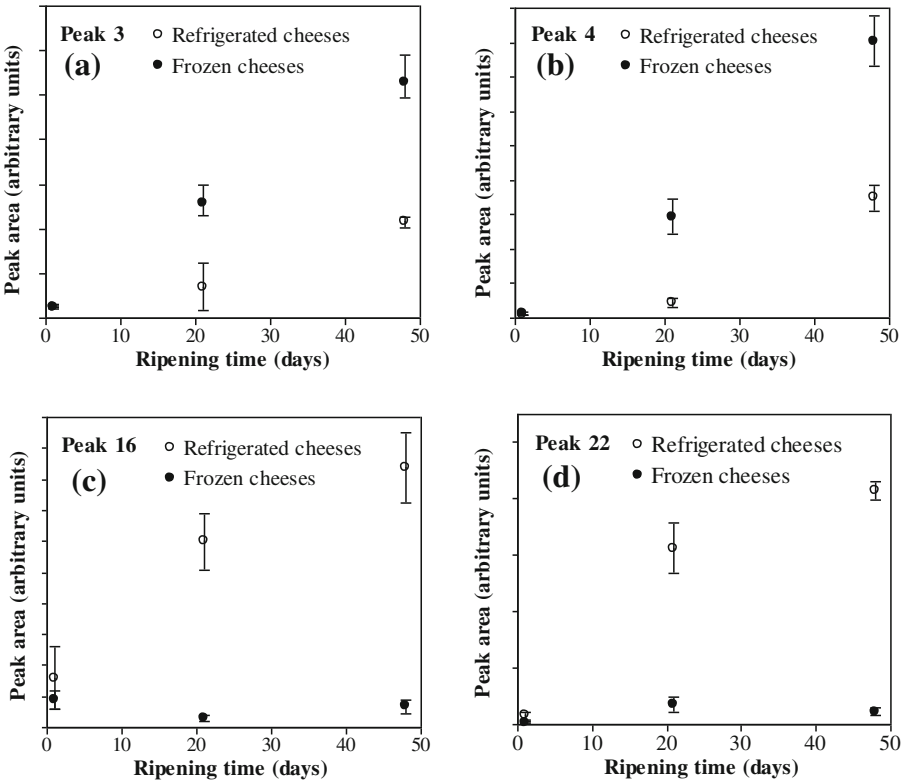

Fig. 3 Areas of peaks of the water-soluble fraction at pH 4.6 during the ripening of refrigerated and frozen low-fat soft cheese containing microparticulated whey proteins as fat replacer: (**a**) peak 3, (**b**) peak 4, (**c**) peak 16, (**d**) peak 22. *Points* represent the mean value of three replicates, and *bars* are based on standard deviations

could indicate that peptides with low molecular weight and hydrophilic characteristics increased during low-fat soft cheese maturation. According to Fenelon et al. (2000), this behavior is expected because the concentration of peptides increases in the WSF due to a progressive breakdown of para-casein by the coagulant and microbial enzymes during cheese maturation. However, areas of peaks 15, 16, 17, 21, and 22 increased in refrigerated cheeses but stayed invariable in frozen cheeses during the studied ripening period. Because all of these peaks have shown the same behavior, two representative peaks (16 and 22) were selected in order to illustrate their evolution (Fig. 3c and d). This result could be related to the degradation of hydrophobic peptides with the subsequent formation of hydrophilic ones in frozen cheeses (Sousa et al. 2001). Similar results were obtained by Romeih et al. (2002), who studied the proteolysis of a low-fat white-brined cheese made with a carbohydrate-based and a whey protein-based fat replacer (Simplesse®), using full-fat and low-fat cheeses (without fat replacer) as control samples. A transition in the molecular weight distribution and hydrophobicity of peptides in full-fat and low-fat cheese chromatograms during maturation was observed. The total amount of hydrophobic peptides decreased

(eluting region between 65 and 92 min), and the amount of hydrophilic peptides increased (eluting region between 12 to 65 min) from 4 to 90 days of ripening. According to those authors, changes in amount of eluting peptides during cheese ripening can be attributed, for instance, to the degradation of the hydrophobic peptides with the subsequent formation of hydrophilic ones and to the progressive insolubilization of the highly hydrophobic peptides (Cliffe et al. 1989; Lau et al. 1991).

On the other hand, analyzing the effect of the freezing process prior to ripening, areas of peaks 3, 4, and 5 were higher in frozen cheeses than in refrigerated cheeses at 21 and 48 days of ripening (Fig. 3a and b). This behavior is in agreement with the previous MI and rheological results obtained for low-fat soft cheeses made with microparticulated whey proteins (Meza et al. 2011). In this case, MI increased from 3.6 to 10.24% in refrigerated cheeses and from 4.09 to 12.97% in frozen cheeses during 76 days of ripening. MI values were higher in frozen cheeses than in refrigerated cheeses at the same day of ripening time. In addition, values of a rheological parameter obtained with a power law equation for elastic modulus data decreased significantly during the studied ripening period, those values in frozen cheeses being lower than in refrigerated cheeses. The decrease in the elastic modulus parameter was attributed to the loss of the protein cheese network by proteolysis. Similar behavior was observed by Verdini et al. (2005), who have analyzed the effect of freezing on the proteolysis during the ripening of full-fat Port Salut Argentino cheese. Freezing led to an early production of hydrophilic peaks (2, 3, 5, and 7) and also to an increment in their rate of degradation. This phenomenon was attributed to the freezing process, which can produce damage to the cell membrane, generating changes in permeability and loss of viability of starter lactic bacteria (Fenema et al. 1973). Consequently, microorganisms could liberate proteases and peptidases into the media, producing an increase in the proteolysis with the formation of a high amount of hydrophilic peptides in frozen cheeses. However, areas of peaks 15, 16, 17, 21, and 22 were lower in frozen cheeses than in refrigerated cheeses at the same day of ripening time (21 and 48 days) (Fig. 3c and d). As discussed before, this result could be related to the degradation of hydrophobic peptides with the subsequent formation of hydrophilic ones in frozen cheeses.

4 Conclusions

The peptide profile present in the water-soluble fraction at pH 4.6 was affected by the freezing process. Results indicate that areas of peaks with hydrophilic characteristics increased during the ripening time, from 1 to 48 days, in both refrigerated and frozen cheeses. Furthermore, areas of hydrophilic peaks were higher in frozen cheeses than in refrigerated cheeses at 21 and 48 days of maturation. However, areas of peaks with hydrophobic characteristics increased in refrigerated cheeses but stayed invariable in frozen cheeses during all the studied ripening periods. In this case, areas of hydrophobic peaks were lower in frozen cheeses than in refrigerated cheeses at the same day of ripening time (21 and 48 days).

Acknowledgments This work was done with the financial support of Universidad Nacional del Litoral, Consejo Nacional de Investigaciones Científicas y Técnicas, and the Agencia Nacional de Promoción Científica y Tecnológica of Argentina. The authors acknowledge Dr. Guillermo A. Sihufe for technical assistance in RP-HPLC tests.

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