Effects of High Pressure on Proteolytic Enzymes in Cheese: Relationship with the Proteolysis of Ewe Milk Cheese

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ABSTRACT

Ewe milk cheeses were submitted to 200, 300, 400, and 500 MPa (2P to 5P) at 2 stages of ripening (after 1 and 15 d of manufacturing; P1 and P15). The high-pressure-treated cheeses showed a more important hydrolysis of β -casein than control and 2P1 cheeses. Degradation of α_{s1} -casein was more important in 3P1, 4P1, and P15 cheeses than control and 2P1 cheeses. The 5P1 cheeses exhibited the lowest degradation of α_s -caseins, probably as a consequence of the inactivation of residual chymosin. Treatment at 300 MPa applied on the first day of ripening increased the peptidolytic activity, accelerating the secondary proteolysis of cheeses. The 3P1 cheeses had extensive peptide degradation and the highest content of free amino acids. Treatments at 500 MPa, however, decelerated the proteolysis of cheeses due to a reduction of microbial population and inactivation of enzymes.

Key words: high-pressure treatment, ewe milk cheese, proteolysis

INTRODUCTION

Proteolysis is the most complex and important biochemical event that occurs during ripening (Fox, 1989), and it plays a direct role on cheese flavor and texture development in most cheese varieties (Sousa et al., 2001). The rate and extent of proteolysis that occur during ripening are determined by the types and activities of the proteolytic enzymes present (i.e., residual coagulants such as chymosin, plasmin, and proteinases/peptidases from starter and nonstarter bacteria). The chemical composition (i.e., salt, pH, and moisture contents) of cheese also influences proteolysis. In addition, the structure of cheese and the accessibility of various cleavage sites on the caseins in the cheese matrix may determine the rate and extent of proteolysis (Wilkinson and Kilcawley, 2005).

In cheese, the concerted action of residual coagulant, indigenous milk proteinases, and starter proteinases on paracasein (primary proteolysis) provides suitable substrates for the starter peptidases, which generate small peptides and free amino acids (FAA; secondary proteolysis; Wilkinson, 1999). However, most of the starter enzymes are located intracellularly (Tan et al., 1992), suggesting that autolysis of starter bacteria, with release of intracellular enzymes, may have an important role in cheese ripening (Fox, 1989; Crow et al., 1995). The earlier release of intracellular enzymes into the cheese matrix could increase the proteolysis of cheeses, thus accelerating the ripening processes.

A number of reports have indicated that high-pressure (HP) treatment may be used to accelerate cheese ripening, in particular proteolysis (O'Reilly et al., 2001). The HP treatment causes membrane damage and an increase in the cellular permeability (Cheftel, 1992), thus favoring the release of intracellular material such as peptidases to the medium (Trujillo et al., 2000a). On the other hand, it has been indicated that HP treatment can induce conformational changes in casein structure, making the protein more susceptible to the action of proteases (Kunugi, 1993).

The application of HP can induce changes in the main proteolytic enzymes involved in cheese ripening (i.e., residual chymosin, plasmin, and starter peptidases) as Malone et al. (2003) in solution buffers, and Trujillo et al. (2000b) and Huppertz et al. (2004) in cheese have shown. The effect of HP on cheese enzymes could influence primary and secondary proteolysis of cheeses.

The possibility of accelerating the ripening of Cheddar cheese by exposure to a treatment at 50 MPa for 3 d at 25°C at different stages of ripening was studied by O'Reilly et al. (2000). These authors found that there was an immediate increase in pH 4.6-soluble nitrogen (WSN) and FAA in 2-d-old cheese, although this effect decreased during ripening time. The study of primary proteolysis of cheeses assessed by urea PAGE also indicated that HP treatment accelerated degradation of α_{s1} -casein and accumulation of α_{s1} -I-casein.

Studies performed on HP-treated Gouda cheese (50 to 500 MPa) for holding times of 20 to 100 min or 3 d

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showed that HP treatment did not influence indices of proteolysis (pH 4.6-soluble and phosphotungstic acid nitrogens, FAA, and casein breakdown by SDS-PAGE) during ripening (Kolakowski et al., 1998; Messens et al., 1999). These results indicate that in this cheese variety proteolysis by chymosin, plasmin, and the proteinase/peptidase system of the starter bacteria were apparently not influenced by these HP treatments, although enzyme activities were not evaluated.

Hispanico cheese HP-treated at 400 MPa for 5 min at 10°C exhibited faster caseinolysis and FAA formation than untreated cheeses (Ávila et al., 2006). Saldo et al. (2002) found twice the levels of FAA in HP-treated (400 MPa for 15 min at 14°C) goat milk cheeses; however, the breakdown of α_s -caseins was lower in HP-treated than in control cheeses.

The objective of this study was to investigate the effects of HP treatments, applied at 2 stages of ripening, on the main proteolytic enzymes implicated in cheese ripening, and their relationship to the primary and secondary proteolysis in ewe milk cheese.

MATERIALS AND METHODS

Cheese Making

Two independent batches of semi-hard cheeses (~0.5 kg) were manufactured from pasteurized (75.5°C, 1 min) ewe milk. Cheeses were produced by 1% starter culture [*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris* (Sacco SRL, CO, Milan, Italy)], 0.05% (wt/vol) of CaCl₂, 0.02% (vol/vol) of calf rennet (Renifor-10, 520 mg of chymosin/L, Laboratorios Arroyo, Santander, Spain), and 0.01% of lysozyme. Temperature was held at 38°C, and the coagulation process lasted about 30 min. The coagulum was cut into 8- to 10-mm cubes, and the curd was drained, molded, and pressed in a horizontal pneumatic press (Garvia S.A., Barcelona, Spain) at 1.2 kPa for 30 min, followed by 1.8 kPa for 30 min, and finally 2.45 kPa for 60 min. Cheeses were salted by immersion in brine (20% NaCl solution) for 2 h and ripened in a room at 12°C and 85% relative humidity for 60 d.

HP Treatment

Cheeses were packed into vacuum pouches, vacuum-sealed, and pressure-treated in a batch isostatic press (GEC Alsthom ACB, Nantes, France) at 200, 300, 400, or 500 MPa (**2P** to **5P**) for 10 min at 12°C. One group of cheeses was treated on the first day (**P1**) after manufacture and the others after 15 d (**P15**) of ripening. Untreated cheeses were used as a control.

Cheese Composition and Microbiological Analysis

Gross composition of cheeses was determined in grated samples. Triplicate samples were assayed for moisture content (IDF, 1982). The pH was measured with a pH meter (MicroPH 2001, Crison, Alella, Spain) on a cheese: distilled water (1:1) slurry. Analyses were performed at 1, 15, 30, and 60 d after cheese making. Microbiological analyses of total counts, *Enterobacteriaceae*, lactococci, and lactobacilli were performed as described by Juan et al. (2004).

Autolysis and Aminopeptidase Activity

Starter autolysis was assayed 24 h postpressurization by determination of lactate dehydrogenase (**LDH**) activity as O'Reilly et al. (2002) described, and results were expressed as international units (U/g of cheese), where 1 unit was defined as the amount of enzyme that catalyzes the reduction of 1 micromole of NAD per minute.

Cheese extract for aminopeptidase activity was obtained by homogenizing 20 g of cheese with 30 mL of 0.1 M sodium phosphate buffer (pH = 7) at 4°C for 3 min in a Stomacher, followed by centrifuging (12,000 × g, 15 min, 4°C) and filtering through Whatman No. 1 paper. Aminopeptidase activity was determined in duplicate on the cheese extract by using leucine-*p*-nitroanilide (**Leu-p-NA**) as substrate (Desmazeud and Juge, 1976), and results expressed as nanomoles of *p*-NA per hour per gram of cheese.

Water-Soluble Nitrogen and Free Amino Acids

Water-soluble extracts of the cheeses were prepared according to the method of Kuchroo and Fox (1982), and the WSN fractions were obtained from water-soluble extracts and determined by the Kjeldahl method (IDF, 1993).

Total FAA were determined on the water soluble extracts by the cadmium-ninhydrin method described by Folkertsma and Fox (1992).

Residual Chymosin and Plasmin Activities

Residual chymosin activity was determined on an extract obtained by mixing 50 mg of cheese with 1.5 mL of 0.1 M trisodium citrate and placed in a water bath at 37°C for 30 min, during which it was agitated briefly at 5-min intervals using a vortex mixer to disperse the cheese. The samples were then centrifuged at 1,000 × g for 2 min to separate the fat. The aqueous layer was used for analysis using a synthetic heptapeptide substrate (Pro-Thr-Glu-Phe-[NO₂-Phe]-Arg-Leu;

Bachem AG, Bubendorf, Switzerland) and measured by HPLC as described by Hurley et al. (1999).

Residual plasmin activity was determined on a cheese extract by mixing 1 g of cheese with 9 mL of 2% (wt/vol) sterile trisodium citrate solution and placed in a water bath at 37°C for 15 min, during which it was constantly agitated. The samples were then centrifuged at $1,000 \times g$ for 5 min at 4°C to remove the fat. The aqueous layer was centrifuged at $27,000 \times g$ for 10 min at 4°C. The clear extract was assayed for plasmin activity as described by Rampilli and Raja (1998). Plasmin activity was expressed as units, with 1 unit being the amount of enzyme that produced a change in absorbance at 405 nm of 0.1 in 60 min.

Residual Caseins

The water-insoluble fractions recovered during the water-soluble extraction were washed 3 times with 1 M sodium acetate buffer (pH 4.6), and the remaining fat was eliminated by washing with dichloromethane-sodium acetate buffer (1:1, vol/vol). The final protein precipitate was then lyophilized. Capillary electrophoresis analyses were performed following the method of Recio and Olieman (1996). Separations were carried out using a Agilent CE Instrument (Agilent Technologies, Germany) controlled by Chemstation Software (Agilent). The separations were performed using a fused-silica capillary column (BGB Analytik, Essen, Germany) of $0.6 \text{ m} \times 50 \text{ }\mu\text{m}$ i.d. (effective length 0.53 m) applying voltage of 20 kV at 45°C and a final current of approximately 33 μA . Sample injection was performed by pressure of 50 mb, 4 s. Protein components were detected at 214 nm. The area of each peak was integrated using Agilent ChemStation Operation Software and designation of capillary electrophoresis peaks of intact caseins (κ -casein, α_{s1} -casein, α_{s2} -casein, and β -casein) was carried out by comparing the electrophoregrams of 1-d-old cheeses with those of isolated pure proteins (Trujillo et al., 2000c). The extent of breakdown of caseins was expressed as a relative percentage of peak areas of 1-d-old cheeses.

Peptides Analysis

Peptides in the water-soluble fraction were separated by reverse-phase HPLC using an automated system (LCM1, Waters Corporation, Milford, MA). Separations were carried out on a 250×4.6 -mm column packed with C18-bonded silica gel with a particle diameter of 5 μm and pore width of 3,000 nm (Symmetry 300, Waters) at a constant temperature of 40°C, following the method of González del Llano et al. (1995). After running the samples, the integration area of peptides, ex-

cluding that of FAA, was determined. The area of peptides eluted between 10 and 35 min was considered the hydrophilic peptide area, whereas the area of peptides eluted from 35 to 80 min was considered the hydrophobic peptide area. The amounts of hydrophobic and hydrophilic peptides were expressed as units of chromatogram area to milligrams of cheese DM.

Statistical Analysis

An ANOVA was performed on all data from 2 batches obtained at each stage of ripening using SPSS Win version 13.0 (SPSS Inc., Chicago, IL). Mean comparisons were carried out using the Student-Newman-Keuls test. Level of significance was set for $P < 0.05$. Principal components analysis (PCA) was carried out using Statistica Software (6.0 version, Statsoft Inc., Tulsa, OK) on enzymatic activities and proteolysis variables.

RESULTS AND DISCUSSION

Microbiological Analysis

The viability of microorganisms in cheese was significantly affected by the HP treatment (Table 1). The initial *Enterobacteriaceae* counts in control cheeses were 5 log units, which decreased until 1 log unit at 60 d of ripening. A reduction of 2 log units was observed on the first day after treatments at 200 and 300 MPa. Pressures of 400 and 500 MPa reduced 3 and 4 log units, respectively. The total inactivation of *Enterobacteriaceae* became evident from 300 MPa, showing undetectable levels in 3P, 4P, and 5P cheeses at 15 d of ripening. At this moment, 2P cheeses showed 1 (for 2P1 cheeses) and 2 (for 2P15 cheeses) log units less than control cheeses, after which counts declined to undetectable levels.

Initial counts of lactic acid bacteria were 9 log units, which were reduced 1 log unit by pressures of 200 and 300 MPa, and 2 log units by 400 MPa. A drastic decrease (5 log units) was found in cheeses HP-treated at 500 MPa. In general, at 60 d of ripening, the counts of lactococci in HP-treated cheeses were similar to those found in control cheeses, except in 5P cheeses, which showed 4 log units lower than the control.

Counts of lactobacilli on d 1 were 2 log units lower in 4P1 and 5P1 cheeses than in untreated cheeses. Lactobacilli populations recovered with time, and at 60 d of ripening, counts of cheeses HP-treated at ≤ 400 MPa were identical (P15 cheeses) or higher (P1 cheeses) than those found in control cheeses. However, a significant decrease (3 and 4 log units) was observed in HP-treated cheeses at 500 MPa at 1 and 15 d of ripening, respectively. For total bacteria, a progressive decrease of

Table 1. Mean values of microbial counts (log cfu/g) of control and high-pressure (HP)-treated ewe milk cheeses during ripening

	HP treatment ¹								
	Control	2P1	3P1	4P1	5P1	2P15	3P15	4P15	5P15
<i>Enterobacteriaceae</i>									
1	5.26 ^a	3.00 ^b	3.22 ^b	2.26 ^{bc}	1.23 ^c				
15	3.92 ^a	2.35 ^{bc}	nd ^c	nd ^c	nd ^c	1.99 ^{bc}	nd ^c	nd ^c	nd ^c
30	2.66 ^a	1.68 ^b	nd ^c	nd ^c	nd ^c	nd ^c	nd ^c	nd ^c	nd ^c
60	1.77 ^a	nd ^c	nd ^c	nd ^c	nd ^c	nd ^c	nd ^c	nd ^c	nd ^c
<i>Lactococci</i>									
1	9.47 ^a	8.34 ^b	8.68 ^b	7.80 ^c	4.75 ^d				
15	8.47 ^a	8.13 ^{ab}	8.51 ^a	7.94 ^b	4.30 ^d	8.61 ^a	8.26 ^a	6.45 ^c	4.43 ^d
30	8.79 ^{ab}	8.69 ^{ab}	8.56 ^{ab}	8.50 ^{ab}	4.53 ^d	9.26 ^a	7.97 ^b	6.12 ^c	4.54 ^d
60	8.17 ^a	8.70 ^a	8.43 ^a	8.57 ^a	4.50 ^c	8.31 ^a	7.81 ^b	8.01 ^a	4.54 ^c
<i>Lactobacilli</i>									
1	3.93 ^a	3.63 ^a	3.15 ^a	1.85 ^b	1.24 ^b				
15	7.64 ^a	7.73 ^a	6.60 ^b	6.69 ^b	2.40 ^d	7.53 ^a	7.13 ^{ab}	5.03 ^c	2.53 ^d
30	7.77 ^{ab}	7.86 ^{ab}	7.47 ^{ab}	7.54 ^{ab}	4.33 ^c	8.32 ^a	7.62 ^{ab}	5.66 ^b	2.54 ^d
60	7.93 ^{ab}	8.14 ^a	8.29 ^a	8.76 ^a	4.25 ^b	7.91 ^{ab}	7.65 ^{ab}	7.77 ^{ab}	3.86 ^b
<i>Total bacteria</i>									
1	9.16 ^a	8.10 ^b	6.97 ^c	5.59 ^d	4.41 ^e				
15	8.35 ^a	7.96 ^{ab}	7.21 ^{ab}	6.76 ^b	4.57 ^c	8.33 ^a	7.30 ^{ab}	6.04 ^b	4.90 ^c
30	8.53 ^a	8.34 ^a	8.58 ^a	8.59 ^a	4.88 ^c	8.68 ^a	7.90 ^{ab}	5.96 ^b	4.88 ^c
60	8.20 ^a	8.29 ^a	8.51 ^a	8.76 ^a	4.60 ^c	8.34 ^a	8.12 ^a	7.96 ^b	4.76 ^c

^{a-e}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Treatments: 2P1, 3P1, 4P1, and 5P1 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, on the first day of manufacturing; 2P15, 3P15, 4P15, and 5P15 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, at 15 d of manufacturing. nd = below detection limit.

counts was observed on d 1 as the pressure increased (1, 3, 4, and 5 log units for 200, 300, 400, and 500 MPa, respectively). However, counts of total bacteria were recovered during ripening in cheeses HP-treated at ≤ 400 MPa, reaching similar values to control cheeses at 60 d of ripening (except 4P15 cheeses, which presented 1 log unit lower than the control). A decrease of 4 log units in the total bacteria counts was observed in cheeses HP-treated at 500 MPa. This significant decrease of microbial counts obtained at ≥ 400 MPa agreed with previous reports in other cheese varieties (Reps et al., 1998; Wick et al., 2004).

Cheese Composition

Moisture content decreased during ripening in all cheeses (Table 2). On the first day, HP-treated cheeses presented slightly lower values of moisture content than control cheeses, probably due to the water expulsion caused by the HP treatments. Afterwards, 4P1 and 5P1 cheeses presented the highest moisture content during the entire ripening period. This could be due to HP-induced changes in the cheese protein network, leading to the formation of a new structure that retains better the water in cheeses. Similar results have been obtained in goat milk cheese (Saldo et al., 2002) and cow milk smear-ripened cheeses (Messens et al., 2000).

On the first day of ripening, the pH of cheeses HP-treated at ≥ 400 MPa was significantly higher than con-

trol, 2P1, and 3P1 cheeses (Table 2). An increase of pH after HP treatment has also been described in Gouda (Messens et al., 1999) and goat milk (Saldo et al., 2002) cheeses. This has been attributed to the release of colloidal calcium phosphate into the soluble phase due to HP treatment, which causes a disintegration of casein micelles (Messens et al., 1998). The pH of cheeses presented a decrease during ripening due to the degradation of residual lactose in lactic acid by lactic acid bacteria. The pH values of P1 cheeses increased with pressure and could be related to the inactivation of glycolytic enzymes (Casal and Gómez, 1999; Krasowska et al., 2005) and to the reduction in lactic acid bacteria caused by the pressure treatment (Table 1). The 5P1 cheeses always had the highest pH values. On the other hand, P15 cheeses showed similar pH behavior to control cheeses, which indicates that at 15 d of ripening the acidification process was finished.

Autolysis and Aminopeptidase Activity

Cell lysis was assayed postpressurization and after 60 d of ripening (Table 3). On the first day of ripening an increase of LDH activity was observed with the increase of pressure up to 400 MPa. At 15 d of ripening, 3P1 cheeses showed the highest LDH activity, whereas cheeses HP-treated at 500 MPa had the lowest values. As has been previously reported, the activity of LDH appeared largely unaffected by pressure up to 400 MPa

Table 2. Mean values $\pm$ SD of pH and moisture content of control and high-pressure (HP)-treated ewe milk cheeses during ripening

Item	HP treatment ¹								
	Control	2P1	3P1	4P1	5P1	2P15	3P15	4P15	5P15
pH									
1	5.31 $\pm$ 0.06 ^b	5.36 $\pm$ 0.01 ^b	5.35 $\pm$ 0.01 ^b	5.46 ^a	5.46 $\pm$ 0.01 ^a				
15	5.02 $\pm$ 0.01 ^f	5.16 $\pm$ 0.02 ^d	5.31 $\pm$ 0.01 ^c	5.39 $\pm$ 0.01 ^b	5.48 $\pm$ 0.03 ^a	5.02 $\pm$ 0.01 ^f	5.07 $\pm$ 0.02 ^e	5.06 $\pm$ 0.03 ^e	5.09 ^e
30	4.99 $\pm$ 0.06 ^d	5.07 $\pm$ 0.01 ^{dc}	5.14 $\pm$ 0.02 ^b	5.18 $\pm$ 0.01 ^b	5.52 $\pm$ 0.06 ^a	4.99 $\pm$ 0.02 ^d	5.03 $\pm$ 0.02 ^{cd}	5.06 $\pm$ 0.04 ^{cd}	5.07 $\pm$ 0.04 ^c
60	4.99 $\pm$ 0.04 ^c	5.01 $\pm$ 0.04 ^c	4.93 $\pm$ 0.02 ^d	4.90 $\pm$ 0.01 ^d	5.51 $\pm$ 0.04 ^a	5.01 $\pm$ 0.05 ^c	5.01 $\pm$ 0.01 ^c	5.04 $\pm$ 0.02 ^c	5.10 $\pm$ 0.01 ^b
Moisture (%)									
1	44.75 $\pm$ 0.16 ^a	43.42 $\pm$ 0.21 ^b	44.30 $\pm$ 0.64 ^{ab}	43.91 $\pm$ 0.52 ^{ab}	44.10 $\pm$ 0.61 ^{ab}				
15	40.12 $\pm$ 0.24 ^{cd}	38.38 $\pm$ 0.32 ^e	39.85 $\pm$ 0.38 ^{cd}	41.53 $\pm$ 0.58 ^b	42.38 $\pm$ 0.18 ^a	40.07 $\pm$ 0.13 ^{cd}	40.01 $\pm$ 0.45 ^{cd}	40.80 $\pm$ 0.69 ^c	39.64 $\pm$ 0.66 ^d
30	36.14 $\pm$ 0.43 ^c	34.93 $\pm$ 0.70 ^d	34.88 $\pm$ 0.46 ^d	38.61 $\pm$ 0.66 ^a	39.44 $\pm$ 0.47 ^a	36.57 $\pm$ 0.68 ^c	36.57 $\pm$ 1.59 ^b	35.95 $\pm$ 0.26 ^{cd}	36.17 $\pm$ 0.87 ^c
60	31.29 $\pm$ 1.18 ^b	30.21 $\pm$ 0.87 ^c	30.87 $\pm$ 0.62 ^{bc}	32.63 $\pm$ 0.29 ^a	32.64 $\pm$ 0.8 ^a	31.31 $\pm$ 0.24 ^b	31.87 $\pm$ 0.16 ^{ab}	31.79 $\pm$ 0.89 ^{ab}	31.52 $\pm$ 0.29 ^{ab}

^{a-f}Means for the same parameter and day of ripening with different superscripts differ ($P \leq 0.05$).

¹Treatments: 2P1, 3P1, 4P1, and 5P1 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, on the first day of manufacturing; 2P15, 3P15, 4P15, and 5P15 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, at 15 d of manufacturing.

Table 3. Mean values $\pm$ SD of lactate dehydrogenase (LDH) activities (U/g) and aminopeptidase activity (nmol of *p*-NA/g per h) of control and high-pressure (HP)-treated ewe milk cheeses during ripening

Item	HP treatment ¹								
	Control	2P1	3P1	4P1	5P1	2P15	3P15	4P15	5P15
LDH									
1	28.62 $\pm$ 6.45 ^d	60.85 $\pm$ 16.2 ^c	77.97 $\pm$ 13.09 ^b	87.38 $\pm$ 9.34 ^a	50.64 $\pm$ 9.02 ^c				
15	140.83 $\pm$ 54.28 ^{bc}	196.78 $\pm$ 53.85 ^{ab}	263.34 $\pm$ 123 ^a	111.06 $\pm$ 22.29 ^b	78.77 $\pm$ 7.26 ^c	86.81 $\pm$ 27.06 ^c	89.71 $\pm$ 36.18 ^c	74.27 $\pm$ 15.09 ^c	59.48 $\pm$ 26.53 ^c
60	393.57 $\pm$ 5.08 ^{bc}	422.83 $\pm$ 22.96 ^{ab}	356.67 $\pm$ 20.12 ^{cd}	351.12 $\pm$ 14.32 ^{cd}	218.49 $\pm$ 8.87 ^e	445.42 $\pm$ 17.39 ^a	428.54 $\pm$ 6.48 ^{ab}	362.94 $\pm$ 13.98 ^{cd}	324.11 $\pm$ 12.96 ^d
Aminopeptidase									
1	0.49 $\pm$ 0.11 ^a	0.44 $\pm$ 0.02 ^{ab}	0.21 $\pm$ 0.01 ^c	0.30 $\pm$ 0.09 ^{bc}	0.35 $\pm$ 0.10 ^{abc}				
15	1.35 $\pm$ 0.21 ^d	2.18 $\pm$ 0.05 ^{abc}	2.43 $\pm$ 0.18 ^a	1.77 $\pm$ 0.21 ^c	2.09 $\pm$ 0.33 ^{abc}	2.31 $\pm$ 0.51 ^{ab}	2.51 $\pm$ 0.15 ^a	1.84 $\pm$ 0.11 ^{bc}	1.03 $\pm$ 0.23 ^d
30	2.39 $\pm$ 0.95 ^c	3.78 $\pm$ 0.72 ^{ab}	4.85 $\pm$ 0.64 ^a	4.82 $\pm$ 0.59 ^a	2.74 $\pm$ 0.81 ^{cd}	4.86 $\pm$ 1.04 ^a	4.93 $\pm$ 0.65 ^a	3.61 $\pm$ 1.13 ^{ab}	1.42 $\pm$ 0.44 ^d
60	3.57 $\pm$ 0.59 ^c	5.76 $\pm$ 0.50 ^b	8.03 $\pm$ 0.58 ^a	7.89 $\pm$ 0.17 ^a	6.38 $\pm$ 0.37 ^b	6.15 $\pm$ 0.21 ^b	5.95 $\pm$ 1.01 ^b	6.09 $\pm$ 0.21 ^b	2.19 $\pm$ 0.24 ^d

^{a-e}Means for the same parameter and day of ripening with different superscripts differ ($P \leq 0.05$).

¹Treatments: 2P1, 3P1, 4P1, and 5P1 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, on the first day of manufacturing; 2P15, 3P15, 4P15, and 5P15 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, at 15 d of manufacturing.

when treated in cheese and cheese extracts, indicating that LDH could be used as an index of HP-induced autolysis (O'Reilly et al., 2002). However, HP treatments at 500 MPa produced a great decrease of LDH activity when treated in cheese and cheese extracts, suggesting that severe HP treatments induce LDH inactivation (Juan et al., 2004), which could explain the lower LDH activities obtained in 5P cheeses. Malone et al. (2002) established that HP-induced cell lysis is pressure- and strain-dependent. Pressurizing cell suspensions of *Lactococcus lactis* ssp. *cremoris* MG1363, Malone et al. (2002) found that samples treated at 300 MPa lysed significantly more than those treated at 100, 200, and 600 to 800 MPa.

Aminopeptidase activity increased as cheese aged in all cheeses (Table 3). At the first day of ripening, amino-peptidase activity in HP-treated cheeses were lower than the activity recorded in control cheeses, but afterward, HP-treated cheeses (with the exception of 5P15 cheeses) presented higher amino-peptidase activity than untreated cheeses. The HP treatment increases cell membrane permeability (Cheftel, 1992), favoring the release of endocellular material, such as peptidases, to the medium (Trujillo et al., 2000a). At 15 d of ripening, 3P cheeses showed the highest amino-peptidase activity values, and at 60 d of ripening the highest activity was found in 3P1 cheeses. The lower amino-peptidase activities were obtained in 5P15 cheeses, suggesting inactivation of amino-peptidase under these HP conditions. According to Reps et al. (1998, 2003) amino-peptidase activity was entirely inactivated under pressure of 600 MPa in different cheese varieties HP-treated in 3 cycles of 5 min. Peptidase activities of cell-free extracts of some strains of *Lactococcus lactis* and *Lactobacillus casei* were not negatively affected by pressures up to 300 MPa; nevertheless, 400 MPa decreased peptidase activities of lactobacilli, whereas those of lactococci were unaffected (Casal and Gómez, 1999). On the other hand, the pressure treatment of 500 MPa for 15 min exerted diversified effects on the activity of peptidases of selected strains of lactic acid bacteria (Krasowska et al., 2005). These results indicate that pressure treatment has different effects on different peptidolytic enzymes as a consequence of pressure time, pressurization medium, and bacterial species; hence, it is necessary to study peptidase activity in each medium and pressure conditions applied.

Rennet and Plasmin Activities

Residual coagulant and plasmin activities are important for proteolysis in cheese because both contribute to the primary hydrolysis of caseins. Residual chymosin is believed to be responsible for the initial soften-

ing of cheese through hydrolysis of the Phe₂₃-Phe₂₄ bond of α_{s1} -casein yielding α_{s1} -I-casein (Creamer et al., 1982).

The HP treatment ≥ 400 MPa applied on the first day of ripening drastically reduced the chymosin activity in ewe milk cheeses (Table 4). A reduction of 62 and 84% of the chymosin activity, compared with control cheese, was observed at 15 d of ripening in 4P1 and 5P1 cheeses, respectively. These results agree with those of Saldo et al. (2002) who found a reduction in the residual coagulant activity to about half of the initial value in goat milk cheese treated at 400 MPa for 5 min on the first day of ripening. On the other hand, the chymosin activity was not influenced by pressure treatment (50 to 400 MPa, 20 to 100 min) in Gouda cheese (Messens et al., 1999), and it appeared to be unaffected by HP treatment on Cheddar cheese pressurized at 100 to 400 MPa (O'Reilly et al., 2002; Huppertz et al., 2004). However, pressures ≥ 600 MPa for 15 to 60 min reduced to about 90% the chymosin activity in Cheddar cheese (Huppertz et al., 2004). In diluted rennet, the chymosin activity was not affected from 100 to 400 MPa; however, a reduction in their activity was observed with pressures >400 MPa (Trujillo et al., 2000b; Malone et al., 2003).

Plasmin is the most important of the indigenous milk proteinases and readily hydrolyzes β -casein and α_{s2} -casein, and more slowly, α_{s1} -casein (Fox and McSweeney, 1996). Plasmin activity decreases in milk when treated at pressures ≥ 400 MPa; nevertheless, plasmin is relatively barostable in buffer and cheese (Scollard et al., 2000). Plasmin activity was not affected significantly by any pressure treatment assayed (Table 4); results that agree with Saldo et al. (2002), who did not find differences in plasmin activity in goat milk cheeses HP-treated at 50 MPa for 72 h and at 400 MPa for 5 min. However, a treatment of 15 to 60 min at 600 to 800 MPa reduced the plasmin activity by approximately 15% in Cheddar cheese (Huppertz et al., 2004).

Primary Proteolysis

Primary proteolysis in cheese may be defined as those changes in caseins and peptides, which can be detected by electrophoretic methods (Fox, 1989) and is mainly the result of the action of indigenous proteinases and the residual coagulant. However, proteinases from starter lactic acid bacteria and nonstarter microorganisms are also active in the degradation of cheese proteins (Fox et al., 1993).

In control cheeses, levels of the residual α_s -caseins declined considerably during ripening, whereas β -casein was scarcely degraded, with 36, 54, and 92% of α_{s1} -, α_{s2} -, and β -caseins intact at 60 d of ripening, respectively (Table 5). This low degree of breakdown of β -

Table 4. Mean values $\pm$ SD of residual coagulant and plasmin activity in high-pressure (HP)-treated ewe milk cheeses

Item	HP treatment ¹								
	Control	2P1	3P1	4P1	5P1	2P15	3P15	4P15	5P15
Chymosin ²									
1	142.4 $\pm$ 36.5	145.9 $\pm$ 42.9	151.2 $\pm$ 35.1	93.9 $\pm$ 21.9 ^b	29.3 $\pm$ 15.3 ^c				
15	146.8 $\pm$ 50.3 ^a	117.7 $\pm$ 27.2 ^{ab}	99.4 $\pm$ 49.8 ^b	65.5 $\pm$ 23 ^c	28.13 $\pm$ 26.1 ^d	128.1 $\pm$ 21.2 ^{ab}	134.6 $\pm$ 25.4 ^{ab}	128.6 $\pm$ 19.6 ^{ab}	106.8 $\pm$ 19.4 ^{ab}
Plasmin (U/g)									
1	15.85 $\pm$ 5.34	13.08 $\pm$ 3.37	16.55 $\pm$ 4.27	15.16 $\pm$ 2.66	12.57 $\pm$ 3.96				
15	30.32 $\pm$ 4.55	25.77 $\pm$ 1.59	27.2 $\pm$ 4.31	26.04 $\pm$ 3.26	26.62 $\pm$ 0.95	27.89 $\pm$ 3.22	27.31 $\pm$ 3.17	30.79 $\pm$ 6.64	32.64 $\pm$ 5.99

^{a-d}Means for the same parameter and day of ripening with different superscripts differ ($P \leq 0.05$).

¹Treatments: 2P1, 3P1, 4P1, and 5P1 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, on the first day of manufacturing; 2P15, 3P15, 4P15, and 5P15 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, at 15 d of manufacturing.

²Peak area (arbitrary units $\times 10^3$).

Table 5. Mean values $\pm$ SD of residual caseins (expressed as a percentage of the amount in the corresponding 1-d-old cheeses) in control and high-pressure (HP)-treated ewe milk cheeses during ripening

Item	HP treatment ¹								
	Control	2P1	3P1	4P1	5P1	2P15	3P15	4P15	5P15
α_{s1} -casein (%)									
15	66.03 $\pm$ 0.31 ^{bc}	61.15 $\pm$ 7.85 ^c	86.68 $\pm$ 14.94 ^{ab}	83.01 $\pm$ 8.78 ^{ab}	99.05 $\pm$ 3.80 ^a	69 $\pm$ 2.58 ^{bc}	57.92 $\pm$ 6.87 ^c	50.63 $\pm$ 2.25 ^c	48.07 $\pm$ 0.41 ^c
60	36.29 $\pm$ 6.13 ^b	40.74 $\pm$ 9.20 ^b	24.78 $\pm$ 2.80 ^c	20.75 $\pm$ 4.93 ^c	80.04 $\pm$ 12.15 ^a	23.28 $\pm$ 3.35 ^c	20.77 $\pm$ 0.52 ^c	21.06 $\pm$ 2.38 ^c	24.49 $\pm$ 1.70 ^c
α_{s2} -casein (%)									
15	74.04 $\pm$ 19.6 ^{ab}	83.58 $\pm$ 23.7 ^a	75.99 $\pm$ 6.25 ^{ab}	82.71 $\pm$ 2.38 ^a	80.72 $\pm$ 16.9 ^a	47.62 $\pm$ 1.96 ^b	49.43 $\pm$ 4.41 ^b	52.43 $\pm$ 7.46 ^b	46.27 $\pm$ 6.46 ^b
60	53.93 $\pm$ 9.89 ^a	47.30 $\pm$ 11.46 ^{ab}	35.42 $\pm$ 4.97 ^{ab}	39.23 $\pm$ 8.05 ^{ab}	42.19 $\pm$ 5.71 ^{ab}	43.05 $\pm$ 4.06 ^{ab}	41.42 $\pm$ 8.8 ^{ab}	41.55 $\pm$ 10.9 ^{ab}	33.07 $\pm$ 3.68 ^b
α_{s1} -I-casein (%)									
15	52 $\pm$ 2.8 ^a	48.7 $\pm$ 3.1 ^{ab}	34.1 $\pm$ 0.7 ^c	34.5 $\pm$ 0.7 ^c	32.5 $\pm$ 0.7 ^c	48.3 $\pm$ 5.1 ^{ab}	54.3 $\pm$ 6.1 ^a	41.2 $\pm$ 0.2 ^{bc}	38.5 $\pm$ 0.7 ^c
60	286.5 $\pm$ 17.9 ^a	240.4 $\pm$ 57.8 ^a	217.3 $\pm$ 5.1 ^{ab}	288.8 $\pm$ 8.3 ^a	123.6 $\pm$ 13 ^b	210.3 $\pm$ 49 ^{ab}	267.4 $\pm$ 38 ^a	258.7 $\pm$ 20 ^a	191.3 $\pm$ 25 ^{ab}
β -casein (%)									
15	99.8 $\pm$ 2.71 ^a	97.07 $\pm$ 3.06 ^a	82.49 $\pm$ 6.66 ^{bc}	82.09 $\pm$ 0.94 ^{bc}	78.75 $\pm$ 2.14 ^c	89.86 $\pm$ 1.23 ^b	86.52 $\pm$ 0.86 ^{bc}	83.19 $\pm$ 2.42 ^{bc}	77.16 $\pm$ 0.45 ^c
60	92.44 $\pm$ 0.30 ^a	92.99 $\pm$ 6.70 ^a	72.88 $\pm$ 1.82 ^{bc}	65.33 $\pm$ 0.21 ^{cd}	77.47 $\pm$ 1.82 ^b	63.70 $\pm$ 4.06 ^d	74.56 $\pm$ 0.79 ^b	75.27 $\pm$ 2.39 ^b	68.97 $\pm$ 0.68 ^{bcd}
Para- κ -casein (%)									
15	79.4 $\pm$ 2.1 ^b	79.3 $\pm$ 3.2 ^b	65.6 $\pm$ 4.5 ^b	74.3 $\pm$ 3.2 ^b	119.5 $\pm$ 5.3 ^a	74.2 $\pm$ 2.4 ^b	70.7 $\pm$ 3.1 ^b	73.3 $\pm$ 1.6 ^b	68.2 $\pm$ 4.4 ^b
60	15.7 $\pm$ 1 ^b	16.7 $\pm$ 1.1 ^b	12.5 $\pm$ 1 ^b	11.5 $\pm$ 0.8 ^b	27.4 $\pm$ 7.2 ^a	13.9 $\pm$ 3.8 ^b	13.2 $\pm$ 4.1 ^b	16.1 $\pm$ 3.9 ^b	11.9 $\pm$ 5.6 ^b

^{a-d}Means for the same parameter and day of ripening with different superscripts differ ($P \leq 0.05$).

¹Treatments: 2P1, 3P1, 4P1, and 5P1 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, on the first day of manufacturing; 2P15, 3P15, 4P15, and 5P15 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, at 15 d of manufacturing.

casein is typical for ewe milk cheeses, in which β -casein is very resistant to proteolysis (Marcos et al., 1978).

Hydrolysis of caseins was affected significantly by the pressure treatment. At 15 d of ripening, P1 cheeses treated at ≥ 300 MPa presented higher levels of residual α_{s1} -caseins than untreated cheeses, whereas P15 cheeses had similar or lower intact α_{s1} -casein than the control. At 60 d of ripening, 3P1, 4P1, and P15 cheeses showed higher hydrolysis of α_{s1} -caseins than control cheeses. In contrast, 5P1 cheeses showed the highest intact α_{s1} -casein related to the lower level of α_{s1} -I-casein (primary degradation product of α_{s1} -casein) detected in 5P1 cheeses (Table 5). Hydrolysis of α_{s1} -casein is due mainly to the residual chymosin retained in the curd. As we have previously described, HP induced inactivation of chymosin at a pressure ≥ 400 MPa, and it was largely reduced in 5P1 cheeses (Table 4), which could explain the lower degradation of α_{s1} -caseins found in these cheeses. O'Reilly et al. (2003) showed that the application of relatively low pressures (50 to 100 MPa) in Cheddar cheese increased degradation of α_{s1} -casein and accumulation of α_{s1} -I-casein. However, at higher pressures, increased breakdown of α_{s1} -caseins was not apparent, and at 350 to 400 MPa the accumulation of α_{s1} -I-casein was reduced. These authors suggested that the increase in primary proteolysis in cheese may be due to conformational changes in casein structure post-pressurization, making the protein more susceptible to the action of proteases.

The α_{s2} -casein appears to be relatively resistant to proteolysis by the chymosin action, but it is susceptible to plasmin attack (Fox et al., 1993). The α_{s2} -casein gradually decreased during ripening, and HP-treated cheeses showed higher degradation of α_{s2} -casein than control cheeses at 60 d of ripening.

The β -casein was hydrolyzed to a lesser extent than α_s -caseins in all cheeses, except in 5P1 cheeses, which exhibited a poor α_s -casein hydrolysis due to the effect of HP on the chymosin activity (Table 5). Higher β -casein hydrolysis was observed in HP-treated cheeses than in control cheeses, except 2P1 cheeses, which showed similar levels to the untreated cheeses. It is known that β -casein is very susceptible to plasmin attacks and to a lesser extent to chymosin action (Fox et al., 1993). The HP did not change the activity of plasmin at any treatment conditions assayed (Table 4), but it could improve the proteolytic action of this enzyme against the β -casein (i.e., changing protein conformations), explaining the higher β -casein hydrolysis in HP-treated cheeses. Messens et al. (1998) observed the acceleration of the hydrolysis of β -casein by plasmin in HP-treated (300 MPa) Gouda cheese, a fact that they attributed to conformational changes in the paracasein gel structure produced by pressure, which led to the

exposure of susceptible peptide bonds from β -casein, which are readily cleavable by plasmin. An increase in breakdown products from plasmin activity was also found by Saldo et al. (2002) in HP-treated (400 MPa, 5 min) goat milk cheese with nonsignificant differences in plasmin activity and explained by the higher pH found in HP-treated cheeses, which could favor the plasmin action.

Para- κ -casein decreased during ripening in all cheeses, and significant differences were only found in 5P1-cheeses, where the para- κ -casein decreased at the lowest rate.

Secondary Proteolysis

Secondary proteolysis is attributed to the proteinases and peptidases of the cheese microorganisms, which degrade large-medium casein peptides to low molecular weight peptides and free amino acids.

The WSN is produced mainly by rennet and, to a lesser extent, by plasmin (McSweeney and Fox, 1997) or cell envelope proteinases from the starter (Sousa et al., 2001). Levels of WSN, expressed as percentage of total N, increased during ripening in all cheeses (Table 6), and at 15 d of ripening, the highest amount of WSN was found in 3P15 cheeses. The WSN was influenced by the moment that the HP treatment was applied; P15 cheeses had higher values of WSN than P1 cheeses at 30 and 60 d of ripening. However, no significant differences were found between P15 cheeses and control cheeses at 60 d of ripening. In Gouda cheese, a slight increase in WSN was observed at 400 MPa after pressure treatment; however, no significant differences in the WSN level were observed between HP and unpressurized cheese samples at longer ripening times (Messens et al., 1999). O'Reilly et al. (2003) found a greater increase in the levels of WSN by HP treatments in Cheddar cheese, which increased with pressurization time did.

Levels of hydrophobic and hydrophilic peptides decreased with the HP treatment, and the lowest values of both variables were found in 5P1 cheeses as a result of the lowest degree of proteolysis developed in these cheeses (Table 7). On the other hand, 5P1 cheeses exhibited the highest ratio of hydrophobic to hydrophilic peptides, which is associated with cheese bitterness. In contrast, the lowest levels of the ratio of hydrophobic to hydrophilic peptides were found in 3P1 cheeses, which also showed lower levels of hydrophobic peptides than control and the other HP-treated cheeses, and this could be beneficial for flavor quality. In this case, the lower levels of hydrophobic peptides could be associated to a higher peptidolytic activity, which agreed with the highest LDH and aminopeptidase activities (Table 3)

Table 6. Mean values $\pm$ SD of water-soluble nitrogen (WSN) and free amino acids (FAA; mg of Leu/g) in control and high-pressure (HP)-treated ewe milk cheeses during ripening

Item	HP treatment ¹								
	Control	2P1	3P1	4P1	5P1	2P15	3P15	4P15	5P15
WSN (% of total N)									
1	4.62 $\pm$ 0.41	4.22 $\pm$ 0.55	4.78 $\pm$ 0.25	4.56 $\pm$ 0.61	4.58 $\pm$ 0.44				
15	10.60 $\pm$ 0.29 ^b	10.57 $\pm$ 1.11 ^b	10.22 $\pm$ 0.11 ^b	10.05 $\pm$ 0.46 ^b	8.23 $\pm$ 0.19 ^c	10.53 $\pm$ 0.54 ^b	11.67 $\pm$ 0.59 ^a	10.89 $\pm$ 0.25 ^b	10.64 $\pm$ 0.30 ^b
30	12.35 $\pm$ 0.38 ^b	11.58 $\pm$ 0.61 ^{bc}	12.42 $\pm$ 1.06 ^b	11.68 $\pm$ 0.46 ^{bc}	10.29 $\pm$ 0.59 ^{bc}	11.81 $\pm$ 1.57 ^d	15.37 $\pm$ 1.87 ^a	14.61 $\pm$ 0.90 ^a	14.41 $\pm$ 1.43 ^a
60	16.78 $\pm$ 0.79 ^a	15.09 $\pm$ 0.51 ^b	15.22 $\pm$ 0.79 ^b	14.91 $\pm$ 0.93 ^b	12.58 $\pm$ 0.83 ^c	17.32 $\pm$ 0.60 ^a	16.94 $\pm$ 0.64 ^a	17.71 $\pm$ 0.83 ^a	17.35 $\pm$ 0.56 ^a
FAA									
1	0.18 $\pm$ 0.02 ^a	0.16 $\pm$ 0.02 ^b	0.16 $\pm$ 0.004 ^b	0.01 $\pm$ 0.01 ^c	0.01 $\pm$ 0.005 ^d				
15	0.76 $\pm$ 0.20 ^{bc}	0.91 $\pm$ 0.22 ^{ab}	1.01 $\pm$ 0.19 ^a	0.66 $\pm$ 0.12 ^c	0.30 $\pm$ 0.06 ^d	0.91 $\pm$ 0.03 ^{ab}	0.99 $\pm$ 0.12 ^{ab}	0.89 $\pm$ 0.08 ^{ab}	0.80 $\pm$ 0.11 ^{abc}
30	1.35 $\pm$ 0.22 ^{bcd}	1.70 $\pm$ 0.34 ^{ab}	1.90 $\pm$ 0.12 ^a	1.17 $\pm$ 0.50 ^{cd}	0.44 $\pm$ 0.31 ^e	1.41 $\pm$ 0.18 ^{bcd}	1.76 $\pm$ 0.19 ^{ab}	1.58 $\pm$ 0.24 ^{abc}	1.08 $\pm$ 0.28 ^d
60	3.39 $\pm$ 0.38 ^b	3.77 $\pm$ 0.15 ^b	4.51 $\pm$ 0.32 ^a	2.63 $\pm$ 0.81 ^{cd}	1.32 $\pm$ 0.16 ^e	3.20 $\pm$ 0.35 ^{bc}	3.63 $\pm$ 0.88 ^b	2.47 $\pm$ 0.35 ^d	1.51 $\pm$ 0.23 ^e

^{a-c}Means for the same parameter and day of ripening with different superscripts differ ($P \leq 0.05$).

¹Treatments: 2P1, 3P1, 4P1, and 5P1 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, on the first day of manufacturing; 2P15, 3P15, 4P15, and 5P15 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, at 15 d of manufacturing.

Table 7. Mean values $\pm$ SD of hydrophobic and hydrophilic peptides and the ratio of hydrophobic to hydrophilic peptides (chromatograph units/mg of DM) in the water-soluble fraction of control and high-pressure (HP)-treated ewe milk cheeses at 30 d of ripening

Item	HP treatment ¹								
	Control	2P1	3P1	4P1	5P1	2P15	3P15	4P15	5P15
Hydrophobic	2,956 $\pm$ 84 ^a	2,169 $\pm$ 340 ^b	1,997 $\pm$ 197 ^{bc}	2,082 $\pm$ 104 ^b	1,720 $\pm$ 299 ^c	2,129 $\pm$ 19 ^b	2,169 $\pm$ 41 ^b	2,159 $\pm$ 28 ^b	2,103 $\pm$ 101 ^b
Hydrophilic	254 $\pm$ 34 ^a	201 $\pm$ 64 ^{ab}	225 $\pm$ 13 ^a	207 $\pm$ 39 ^{ab}	134 $\pm$ 24 ^b	195 $\pm$ 4 ^{ab}	227 $\pm$ 7 ^a	224 $\pm$ 26 ^a	220 $\pm$ 40 ^a
Ratio	11.76 $\pm$ 1.45 ^{ab}	11.72 $\pm$ 2.14 ^{ab}	8.85 $\pm$ 0.36 ^b	10.18 $\pm$ 1.76 ^{ab}	12.99 $\pm$ 1.93 ^a	10.90 $\pm$ 0.32 ^{ab}	9.57 $\pm$ 0.44 ^{ab}	9.75 $\pm$ 1.24 ^{ab}	10.29 $\pm$ 0.79 ^{ab}

^{a-c}Means for the same parameter with different superscripts differ ($P \leq 0.05$).

¹Treatments: 2P1, 3P1, 4P1, and 5P1 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, on the first day of manufacturing; 2P15, 3P15, 4P15, and 5P15 = cheeses treated at 200, 300, 400, and 500 MPa, respectively, at 15 d of manufacturing.

that were found in these cheeses and also evidenced by the highest amount of FAA present in 3P1 cheeses (Table 6). These results suggest that treatment at 300 MPa applied on the first day of ripening could induce changes to the cheese matrix, which enhanced the susceptibility of cheese to proteolysis. In addition, the peptidases may have been released due to cell lysis by means of the pressure treatment, accelerating the contact between substrate and enzyme.

The release of amino acids in cheeses clearly indicates aminopeptidase activity. On the first day of ripening, the HP-treated cheeses showed lower levels of FAA than control cheeses (Table 6) in accordance with the lower aminopeptidase activity observed (Table 3). An initial reduction in peptidolytic activity in cheese immediately after HP treatment has been described elsewhere (Sendra et al., 2000). The amount of FAA increased during ripening, and at 15 d of ripening the highest value of FAA was found in 3P1 cheeses agreeing with the highest aminopeptidase activity found in these cheeses. This increase in FAA levels could be explained as result of rapid peptides degradation, which agreed with the lowest level of hydrophobic peptides (Table 7) and associated with the earlier starter lysis done by the HP treatment. Crow et al. (1995) established that when starter lysis occurs, the levels of FAA in cheese increase and bitterness is reduced, leading to a better flavor. On the other hand, at 60 d of ripening, FAA levels in 4P1, 4P15, 5P1, and 5P15 were 1.28-, 1.37-, 2.56-, and 2.24-fold lower than untreated cheeses, respectively. These results agree with previous works (Juan et al., 2004; Wick et al., 2004) and show that pressures ≥ 400 MPa seem to delay FAA development.

A PCA was carried out to correlate the activity of proteolytic enzymes with the primary and secondary proteolysis of cheeses (Figures 1 and 2). Principal component (PC) 1 explained 32.19% of the variance and correlated positively with chymosin, FAA, residual α_{s1} -I-casein, and hydrophobic and hydrophilic peptides, and negatively with the residual para- κ - and α_{s1} -caseins, and the ratio of hydrophobic to hydrophilic peptides (Figure 1a). Accordingly, PC 1 was defined as a degree of proteolysis factor. The α_{s2} - and β -caseins showed high negative loading with PC 2, which explained 19.18% of the variance (Figure 1a) and could be defined as plasmin factor. The LDH and Leu-p-NA activities correlated positively with PC 3 (Figure 2a), and accordingly, this factor was defined as a lytic factor. Plotting of cheeses at 60 d of ripening in the 2-dimensional coordinate system (Figures 1b and 2b) shows that pressures of 500 MPa applied on the first day of ripening decelerated the proteolysis of cheeses. The 5P1 cheeses showed the lowest degradation of para- κ - and α_{s1} -casein and the lowest α_{s1} -I-casein, peptides, and

FAA levels. On the other hand, HP treatments at >200 MPa applied on the first day of ripening, and all HP treatments applied at 15 d of ripening accelerated the degradation of α_{s2} - and β -caseins mainly due to the plasmin action (Figure 1b). The treatment of 300 MPa applied on the first day of ripening produced a prompt starter lysis, increasing the aminopeptidase activity and the levels of FAA (Figure 2b).

CONCLUSIONS

Primary proteolysis, analyzed by the casein degradation, was enhanced by pressures of 300 and 400 MPa applied on the first day of ripening and with 200 to 500 MPa applied on d 15 of manufacturing, probably as a consequence of the barostability of plasmin activity combined with conformational changes on the cheese matrix, which enhanced the susceptibility of casein to chymosin and plasmin attacks. However, pressures of 500 MPa applied on the first day of ripening resulted in large inactivation of residual chymosin and reduced the primary proteolysis of ewe milk cheeses.

Secondary proteolysis, which gives the proportion of peptides and FAA, was also enhanced with the treatment at 300 MPa applied on the first day of ripening. The 3P1 cheeses had the lowest hydrophobic peptides and the highest FAA content due to the highest peptidolytic activity. The HP treatment favored the lysis of starter bacteria enhancing the release of intracellular aminopeptidases into the cheese matrix. This treatment could also have also a beneficial effect on flavor quality because 3P1 cheeses contained lower amounts of hydrophobic peptides and the lowest hydrophobicity index, both parameters associated with cheese bitterness. On the other hand, the treatment of 500 MPa decreased the secondary proteolysis of ewe milk cheeses. These cheeses showed the lowest amounts of peptides and FAA, probably due to the reduction of starter bacteria and enzyme inactivation by the pressure.

These results suggest that HP treatment at 300 MPa applied on the first day of ripening could be used to accelerate the proteolysis of ewe milk cheeses. In contrast, the treatment at 500 MPa applied on the first day of ripening would decelerate it.

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Figure 1. a) Principal components (PC) analysis plot defined by PC 1 and PC 2 showing the distribution of enzymatic activity [lactate dehydrogenase (LDH), aminopeptidase, chymosin, and plasmin] and proteolysis variables [residual caseins, hydrophobic and hydrophilic peptides and their ratio, and total free amino acids (FAA)]. b) Distribution of 60-d-old cheeses in the plane defined by PC 1 and PC 2. Control (+), 2P (□, ■), 3P (△, ▲), 4P (◇, ◆), and 5P (○, ●) cheeses. Open symbols represent P1 cheeses; closed symbols represent P15 cheeses. Leu-*p*-NA = leucine-*p*-nitroanilide.

Figure 2. a) Principal components (PC) analysis plot defined by PC 1 and PC 3 showing the distribution of enzymatic activity [lactate dehydrogenase (LDH), aminopeptidase, chymosin, and plasmin] and proteolysis variables [residual caseins, hydrophobic and hydrophilic peptides and their ratio, and total free amino acids (FAA)]. b) Distribution of 60-d-old cheeses in the plane defined by PC 1 and PC 3. Control (+), 2P (□, ■), 3P (△, ▲), 4P (◇, ◆), and 5P (○, ●) cheeses. Open symbols represent P1 cheeses; closed symbols represent P15 cheeses. Leu-*p*-NA = leucine-*p*-nitroanilide.

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