Food Chemistry 111 (2008) 696–702

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Proteolysis of ultra-high pressure homogenised treated milk during refrigerated storage

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ARTICLE INFO

Article history:
Received 14 January 2008
Received in revised form 13 February 2008
Accepted 15 April 2008

Keywords:
Ultra-high pressure homogenisation
Proteolysis
Milk proteins

ABSTRACT

Plasmin residual activity and its relation to proteolysis of milk subjected to ultra-high pressure homogenisation (UHPH; 200–300 MPa, inlet temperature = 30 °C and 40 °C) and to a high-pasteurisation treatment (90 °C, 15 s) were studied during refrigerated storage. Proteolysis was examined by capillary electrophoresis, HPLC peptide profiles, pH 4.6-soluble nitrogen and free amino acids. Inactivation of plasmin increased as homogenisation pressure did. Extensive proteolysis, was observed in 200 MPa 40 °C milk, due to its higher native and microbial enzyme contents, compared with the other samples. In general, hydrolysis of β-casein, hydrophobic peptide and pH 4.6-soluble nitrogen levels increased with higher residual plasmin activity, while hydrophilic peptides were not affected by the different treatments applied. β-Lactoglobulin was denatured to a greater extent by thermal treatment than by UHPH treatments. This study provides further insight into how UHPH treatments influence milk properties.

1. Introduction

Proteolysis in milk products can have positive or negative effects, depending on the reason for processing. Proteolysis can produce desirable flavours and texture during cheese ripening (Farkye & Fox, 1992), while uncontrolled proteolysis can alter the rennet coagulation properties of milk, leading to poor curd formation (Srinivasan & Lucey, 2002), development of bitter flavours, increase in viscosity, and gelation of heated milks (Datta & Deeth, 2003; Kohlmann, Nielsen, & Ladisch, 1991). The rate and extent of proteolysis will be determined by the types and activities of the proteolytic enzymes present. Breakdown of proteins in milk can be mainly caused by indigenous proteases and by those produced by psychotrophic bacteria during cold storage of raw milk. Plasmin (EC 3.4.21.7), the principal indigenous milk proteinase, has optimum activity at 37 °C and pH 7.4 and hydrolyses mainly β- and α_{s2}-caseins, and more slowly α_{s1}-casein (Bastian & Brown, 1996). Plasmin (PL) is part of a complex system called the PL system which includes its active form PL as well as its enzymatically inactive precursor, plasminogen (PG), plasminogen activators, plasminogen activator inhibitors and plasmin inhibitors.

The kinetic behaviour of PL has been studied by several authors and most of them have described a first-order inactivation profile (Kennedy & Kelly, 1997; Saint Denis, Humbert, & Gaillard, 2001). Metwalli, de Jongh, and van Boekel (1998) observed that irreversible inactivation of the enzyme starts at temperatures above 65 °C. However, there appears to be a strong interference with sulfhydryl groups, since in the absence of SH-components, PL and PG are reported to be very heat-stable (Bastian et al., 1996).

Normally, psychotrophic bacteria would not be a serious problem in heat treated milk, because they can be eliminated with the thermal treatment applied. Nevertheless, most proteinases from psychotrophic microorganisms survive UHT treatment and are readily able to degrade κ-, α_{s1}- and β-caseins, causing proteolysis by themselves (Sorhaug & Stepaniak, 1997), and furthermore these heat-stable proteases can affect the PL system.

Ultra-high pressure homogenisation (UHPH), which is based on the same principle as conventional homogenisation, but works at higher pressures (up to 400 MPa), is being studied as an alternative technology to thermal treatments, with possible applications in milk (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007), cheese (Zamora, Ferragut, Jaramillo, Guamis, & Trujillo, 2007) and yogurt (Serra, Trujillo, Quevedo, Guamis, & Ferragut, 2007) production. UHPH technology at 200 and 300 MPa is able to produce milk with a microbial and physico-chemical shelf-life similar to that of high-pasteurised milk (Pereda et al., 2007). However, the interest in this technology is not only due to its effect on microorganism inactivation, but also due to its alteration of food constituents. Different studies on milk have shown that UHPH reduces the fat globule size (Hayes & Kelly, 2003b; Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003), denaturates whey proteins, with β-lactoglobulin (β-Lg) being more affected than α-lactoalbumin (α-La) (Hayes, Fox, & Kelly, 2005; Zamora et al., 2007), and inactivates indigenous milk

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doi:10.1016/j.foodchem.2008.04.040

enzymes, depending on the pressure applied. Inactivation of PL and PG-derived activities has been observed in conventionally homogenised samples; however, by using high pressure homogenisation more inactivation is produced (Hayes & Kelly, 2003a; Hayes et al., 2005).

The objective of the present study was to investigate and compare the effects of UHPH at 200 and 300 MPa on PL activity and its influence on milk proteolysis with the effects produced by a conventional high-pasteurisation treatment, in order to evaluate the potential application of this emerging technology in the milk industry.

2. Materials and methods

2.1. Milk supply

Fresh raw bovine milk (11.6 ± 1.0% total solids and 3.29 ± 0.03% protein) was collected from a local farm (Can Badó, Barcelona, Spain), and after fat standardisation at 3.5 ± 0.2%, it was kept overnight at 4 °C.

2.2. Ultra-high pressure homogenisation and heat-pasteurisation of milk

Milk was subjected to UHPH treatments at 200 and 300 MPa, with inlet temperatures of 30 and 40 °C by using a Stansted high-pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Stansted, Essex, UK) which is able to operate at up to 400 MPa and works with a flow rate of 120 l/h. The inlet temperatures of milk were reached by passing milk through a heat exchanger located behind the feeding tank. During UHPH treatments an increase in milk temperature (~19 °C per 100 MPa) is produced as a consequence of the adiabatic heating generated in the machine, in addition to the high turbulence, shear and cavitation forces that the fluid suffers in the homogenisation valve. Therefore, to minimise temperature retention after treatment, two spiral type heat-exchangers (Garvía, Barcelona, Spain) located behind the second valve were used, which were able to cool milk in less than 0.7 s. During treatments, the inlet temperature (T_i), the temperature before the first homogenisation valve (T_1), the temperature before the second homogenisation valve (T_2), as well as the final temperature of the milk after passing through the heat exchanger (T_f), were monitored.

Ultra-high pressure-treated samples were compared with a high-pasteurised milk (PA). Two-stage homogenisation (18 MPa plus 2 MPa) and pasteurisation (90 °C for 15 s) of raw milk were carried out with a Niro Soavi homogeniser (model X68P, Parma, Italy) and a Finamat heat exchanger (model 6500/010, Gea Finnah GmbH, Ahaus, Germany), respectively.

Milk samples were collected and then stored at 4 °C. Analyses of treated samples were conducted after treatment and at different days during storage (7, 14, 18 and 21 days).

2.3. Residual plasmin and plasminogen activities

Sample preparation to determine residual PL and PG activities was performed according to the method described by Richardson and Pearce (1981), with minor changes. Milk samples were diluted with sodium citrate solution (0.4 M) in a 3:1 ratio (milk:citrate), and centrifuged at 27,000*g* for 15 min at 25 °C. The supernatant solution containing the PL content was centrifuged twice at 15,000*g* for 15 min in a microcentrifuge.

Plasmin and plasminogen activities were measured using a version of the colorimetric method described by Baldi et al. (1996), using a microplate assay. Plasmin activity was assayed by mixing 250 μl of 0.1 M Tris–HCl buffer (pH 7.4), containing 0.6 mM Val-Leu-Lys-*p*-nitroanilide (Sigma, St. Louis, MO) with 30 μl of the milk supernatant. In the case of PG-derived activity (that is the PL activity generated after addition of urokinase), 100 μl of milk supernatant and 5 μl of urokinase (Sigma) solution (4.35 mg urokinase/ml) were previously incubated for 1 h at 37 °C, prior to assaying total PL activity.

The reaction mixtures were incubated at 37 °C and absorbance was measured at 405 nm at 15 min intervals for 4 h. The absorbance was followed in a microprocessor controlled reader (SLT-Labinstruments GmbH, Salzburg, Austria) equipped with a thermostatted cell holder and 96-well microtitre plates. A sample without supernatant served as a control for the detection of spontaneous breakdown of the substrate. Plasmin and plasminogen activities were expressed as units: 1 unit of PL being the amount of enzyme that produced a change in absorbance at 405 nm of 0.1 in 60 min. Measurements were carried out in triplicate.

2.4. Separation of milk fractions

The pH 4.6-insoluble fraction containing the isoelectric caseins was prepared by precipitation of milk with 1 M sodium acetate buffer (pH 4.6) followed by centrifugation at 4500*g* and 5 °C for 15 min. The casein pellets recovered were first washed three times with 1 M sodium acetate buffer (pH 4.6) and then, to remove the remaining fat, they were washed twice with a mixture of sodium acetate buffer/dichloromethane (1:1, v/v). The final protein precipitate was then lyophilised. The pH 4.6-soluble fraction was filtered through Whatman No. 1 paper and kept frozen until used. From this fraction the pH 4.6-soluble nitrogen (pH 4.6-SN) was determined by the Dumas method (IDF, 2002), and total free amino acids (FAA) by the cadmium–ninhydrin method of Folkertsma and Fox (1992). These determinations were done in triplicate.

2.5. Capillary electrophoresis

Analysis of individual proteins in the pH 4.6-insoluble fraction was performed by capillary electrophoresis (CE) by using an Agilent CE instrument (Agilent Technologies, Waldbronn, Germany) controlled by Chemstation software (Agilent). The electrophoresis conditions and sample buffers were similar to those used in the method described by Recio and Olieman (1996), using a fused silica capillary column (BGB Analytik, Essen, Germany) of 0.6 m × 50 μm i.d. with an effective length of 50 cm. Electrophoresis runs were done at 45 °C with a linear voltage gradient from 0 to 20 kV in 3 min, followed by a constant voltage of 20 kV. Detection of proteins was at 214 nm and identification of peaks was carried out by comparison with capillary electrophoregrams obtained elsewhere (Recio, Pérez-Rodríguez, Ramos, & Amigo, 1997). The extent of breakdown of caseins was expressed as a relative percentage of peak areas of raw milk analysed on the first day of storage, and as a percentage of the respective area at day 1, for samples analysed during storage. All samples were analysed in duplicate.

2.6. HPLC peptide analysis

Peptides in the pH 4.6-soluble fraction were analysed in triplicate by reversed-phase (RP)-HPLC with a Summit ×2 dual gradient HPLC system (Dionex, Idstein, Germany). Samples (50 μl) previously filtered through a 0.45 μm filter were injected into a C_{18}-bonded silica gel (250 × 4.6 mm) with a particle diameter of 5 μm and pore width of 3000 nm (Simmetry 300, Waters), at a constant temperature of 40 °C. Peptides were eluted using a binary gradient program at a flow rate of 1 ml/min. Eluent A was 0.1% trifluoroacetic acid (Sigma) in Milli-Q water and eluent B was 0.1% trifluoroacetic acid in acetonitrile (Panreac, Barcelona, Spain). The

linear gradient, expressed as solvent A, was 100% at 0 min, 97% at 3 min, 50% at 60 min and 0% at 65 min. Detection was performed at 220 nm. After each run, the integration area of peptides, excluding that of FAA and whey proteins, was determined. The area of peptides between 3.5 and 20 min (between Tyr and Trp) was considered the hydrophilic peptide portion while the area of peptides eluting after 20 min was considered the hydrophobic peptide portion. The amount of hydrophobic and hydrophilic peptides were expressed as units of chromatogram peak area.

2.7. Accelerated test of proteolysis

Proteolysis in milk was estimated after incubation of milk samples for 48 h at 37 °C in the presence of 0.07% NaN_3 (Sigma) as inhibitor of bacterial growth. The proteolytic activity was calculated as the percent increase in pH 4.6-SN (obtained as above) after incubation, and it was also estimated by analysing the peptide pattern as explained above.

2.8. Statistical analysis

Results were analysed by an analysis of variance, using the general linear models procedure of the (Statistical Analysis Systems Institute, 2004). The Student–Newman–Keuls test was used for comparison of sample data. Evaluations were based on a significant level of $p < 0.05$. Correlations between caseins, peptides, FAA and pH 4.6-SN were evaluated with Pearsońs test.

3. Results and discussion

3.1. Residual plasmin and plasminogen activities

Plasmin and plasminogen activities decreased in treated samples, compared to raw milk, and PL was significantly affected by UHPH and PA treatments (Table 1). Data of PG showed that most of the potential PL activity in raw milk is in the form of the inactive precursor PG, which is then converted to active PL. PG-derived activity demonstrated a greater sensitivity to the treatments applied than PL. However, decreases in PG-derived activity do not necessarily mean PG inactivation. An increase in activity of PL and a subsequent decrease in concentration of PG were observed in pasteurised milk, compared to raw milk, after incubation at 37 °C for 48 h (Richardson, 1983). Prado, Sombers, Ismail, and Hayes (2006) observed that PG activator inhibitors are less heat-stable than PL inhibitor, which indicated that activation of PG could overcome any inhibition of PL. This implies that these large amounts of PG in raw milk should be taken into account because the simple activation of a fraction of this PG would lead to a significant increase of the proteolytic activity.

Approximately 70% of the PL activity in raw milk was inactivated by UHPH at 300 MPa and PA treatments, and 45% at 200 MPa. Hayes et al. (2005) also obtained a 70% inactivation in conventional pasteurised milk, and 85 and 95% inactivation at 150 and 250 MPa (T_i = 45 °C), respectively. In another study, Hayes and Kelly (2003a) working with the same machine, but at a lower inlet temperature, observed lower PL inactivation (65%). They suggested that inactivation of PL must be due to the temperature achieved combined with the forces that the fluid suffers in the high pressure valve. Inactivation of PL by UHPH has also been studied by Datta, Hayes, Deeth, and Kelly (2005), who working at 200 MPa and T_i = 25 °C and 50 °C, obtained 74% and 90% inactivation, respectively. In the present study, a higher temperature was achieved during processing (see Table 1), compared to those achieved in the previously mentioned works; however, lower PL inactivation was observed. Differences in PL inactivation between this study and the other works could be explained by a combination of differences in the valve construction and machine design, as well as in the inlet temperature and the time at which milk is maintained at the highest temperature reached during the UHPH treatment. In our study milk was quickly cooled; therefore, the holding time at the highest temperature was very short and probably inactivation of PL was mainly produced by the mechanical forces associated with this treatment. Datta et al. (2005) observed enzyme inactivation at temperatures lower than those needed for thermal inactivation and therefore they confirmed that in UHPH, mechanical forces are more important in PL inactivation than the thermal effect. Additionally, PL is relatively instable in the presence of whey proteins, in particular β-Lg, which plays a major role in the thermal inactivation of PL in milk. During thermal denaturation, β-Lg exposes its free sulphydryl groups which can form a complex with the unfolded enzyme, via disulphide/sulphydryl interactions, leading to irreversible denaturation of PL (Metwalli et al., 1998). In the present study β-Lg was most denatured by the high-pasteurisation treatment, followed by UHPH treatments at 300 MPa and both T_i, and by 200 MPa and T_i = 40 °C (Table 2).

Both PL and PG were susceptible to inactivation by UHPH treatments. However, since UHPH is a new technology and PL system in milk is quite complex, it is difficult to know the exact nature of their inactivation.

3.2. pH 4.6-Insoluble fraction

A typical electropherogram showing the separation of milk proteins from pH 4.6-insoluble fraction is shown in Fig. 1. The relative migration of the proteins was in accordance with previous reports (De Noni, Pellegrino, Cattaneo, & Resmini, 2007; García-Risco, Ramos, & López-Fandiño, 1999; Recio et al., 1997). The peak belonging to β-Lg was found in all treated samples studied, whereas the presence of α-La was almost negligible, indicating that β-Lg is more susceptible than α-La to heat and pressure homogenisation denaturation. This is in accordance with the results presented by Zamora et al. (2007), where 35% of denaturation was observed for β-Lg and 12% for α-La in cheese whey from milk treated at 300 MPa. As can be seen in Table 2, immediately after processing, there were higher levels of β-Lg in the casein fraction of PA milk than in those of UHPH-treated samples, demonstrating that β-Lg was more drastically denatured by the conditions used in the thermal treatment than by pressure homogenisation. During refrigerated storage, an increase in the amount of peaks in the area of β-Lg was observed; these whey protein peaks were not analyzed during storage, because probably some protein peaks could co-migrate at the same time as β-Lg, complicating the determination of whey protein changes during storage. In this sense, García-Risco

Table 1
Plasmin and plasminogen-derived activities (U/ml) for raw and treated milk samples[A] together with the temperature changes of milk during ultra-high pressure homogenisation treatment

Treatment	T_1[B] (°C)	T_2[C] (°C)	Plasmin	Plasminogen
Raw milk			$5.70^{a} \pm 0.00$	$20.91^{a} \pm 5.38$
200 MPa 30 °C	34.5	78.5	$3.17^{b} \pm 1.10$	$0.63^{b} \pm 1.10$
200 MPa 40 °C	42.5	85	$3.17^{b} \pm 1.10$	$1.90^{b} \pm 1.90$
300 MPa 30 °C	38	95.5	$1.43^{c} \pm 0.59$	$0.29^{b} \pm 0.80$
300 MPa 40 °C	44	101.5	$1.90^{c} \pm 0.00$	$2.85^{b} \pm 1.10$
Pasteurised[D]			$1.43^{c} \pm 0.36$	$0.06^{b} \pm 0.29$

[a,b,c] Different superscripts indicate significant differences ($p < 0.05$).
[A] Data are means ± standard deviations of triplicate analysis from three independent experiments.
[B] T_1: temperature before the first homogenisation valve.
[C] T_2: temperature before the second homogenisation valve.
[D] Pasteurised: high-pasteurised milk (90 °C for 15 s).

Table 2
Mean values ± standard deviation of residual β-lactoglobulin (peak area units) and caseins, expressed as a percentage of peak areas of raw milk, for samples analysed the first day of storage, and as a percentage of the respective area at day 1, for samples analysed at day 21 of storage at 4 °C

	Treatment					
	Day	200 MPa, 30 °C	300 MPa, 30 °C	200 MPa, 40 °C	300 MPa, 40 °C	Pasteurised[A]
α_{S2}-Casein (%)	1	73.7 ± 8.3	80.8 ± 12.7	76.7 ± 18.2	64.4 ± 3.4	84.1 ± 17.1
	21	53.8^{c} ± 6.7	48.2^{c} ± 16.1	45.8^{c} ± 7.8	72.1^{b} ± 4.3	94.4^{a} ± 11.2
α_{S1}-Casein (%)	1	61.4 ± 6.6	61.1 ± 15.3	61.9 ± 2.8	57.1 ± 9.9	69.9 ± 6.2
	21	93.9^{ab} ± 7.4	78.5^{b} ± 6	83.8^{ab} ± 16.8	82.7^{ab} ± 2	98.4^{a} ± 15
κ-Casein (%)	1	63.1 ± 5.7	64.1 ± 16.2	65.5 ± 6.6	56.1 ± 14.5	70.1 ± 4.8
	21	106^{a} ± 12.4	96.7^{a} ± 15.9	63.4^{b} ± 2.8	101^{a} ± 4.5	114^{a} ± 26.7
β-Casein (%)	1	55.8 ± 9.9	65.8 ± 9	63.8 ± 6	58.8 ± 10.1	67 ± 6.9
	21	63.5^{b} ± 5.5	66.8^{b} ± 3.2	47.4^{c} ± 18.1	81.7^{b} ± 2	97.2^{a} ± 17
β-Lg (Peak area units)	1	128^{c} ± 63.4	154^{b} ± 38.0	194^{b} ± 11.2	171^{b} ± 42.7	329^{a} ± 45.3

[a,b,c]Different superscripts for the same parameter and day indicate significant differences ($p < 0.05$).
[A] Pasteurised: high-pasteurised milk (90 °C for 15 s).

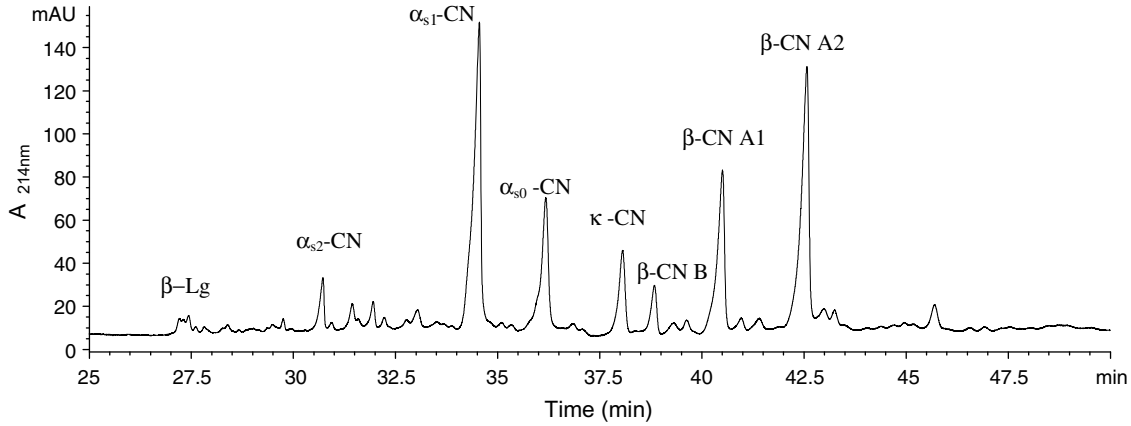

Fig. 1. Capillary electropherogram of the pH 4.6-insoluble fraction from an ultra-high pressure homogenised treated sample (200 MPa, 30 °C), after 1 day of storage (β-Lg = β-lactoglobulin, CN = casein) at 4 °C.

et al. (1999) working with a protease of *Pseudomonas fluorescens* in casein solutions observed that *para*-κ-casein forms migrate in the region of β-Lg.

Residual casein data presented in Table 2 revealed that proteolysis immediately after treatment occurred in all treated samples, compared to raw milk. Significant differences between samples were detected only during storage at 4 °C. Levels of the residual α_{s2}- and β-caseins, which are preferential substrates for PL, declined considerably during milk storage in all UHPH-treated samples, whereas α_{s1}- and κ-caseins were hydrolysed to a lesser extent.

Milk treated at 300 MPa and 40 °C possessed a similar PL content to milk treated at the same pressure, but with an inlet temperature of 30 °C; nevertheless, lower hydrolysis of α_{s2}-casein was produced compared to the other UHPH-treated milk samples. This difference could be related to the formation of fat aggregates, which were observed by Pereda et al. (2007) in samples treated at 300 MPa, being more pronounced at $T_i = 40$ °C than at 30 °C. When clusters are formed through shared protein adsorbed onto the fat surface they could, in some way, protect casein fractions. The protein could be entrapped between a group of fat globules instead of being positioned on the surface of the fat globule and therefore, the accessibility of the enzyme to caseins could be partially restricted.

The α_{s1}- and κ-caseins are more resistant to proteolysis by PL, but they are susceptible to attack by cathepsin D and microorganism proteases. Larsen et al. (2000) have demonstrated that cathepsin D has low heat stability. Approximately half of the procathepsin D-derived activity detected in milk serum remained after heat treatment at 72 °C for 15 or 60 s; however, heat treatment at higher temperatures further reduced the enzyme activity. Therefore, cathepsin D is not considered as an important enzyme in high-pasteurised milk or UHPH-treated milk samples, where temperatures achieved during treatments are higher than 72 °C.

A slight decrease in the residual level of α_{s1}-casein was observed for all samples during storage; however, this decrease was only significant for samples treated at 300 MPa at both inlet temperatures.

In general, the κ-casein did not undergo hydrolysis during storage, except in milk treated at 200 MPa and 40 °C, in which a significant difference was observed between days. Proteases from different species and strains of *Pseudomonas* spp. differ in their substrate specificities toward milk proteins, most proteinases being from psychotrophs able to degrade κ-casein (Sorhaug & Stepaniak, 1997). Pereda et al. (2007) observed bacterial growth, using Pseudomonas agar base medium, in samples treated at 200 and 300 MPa with $T_i = 40$ °C during the last days of storage. However, hydrolysis of κ-casein was detected in samples treated at 200 MPa, but not in milk treated at 300 MPa, probably due to the clusters previously mentioned.

According to De Noni et al. (2007) and Enright, Bland, Needs, and Kelly (1999), hydrolysis of β-casein, which can be produced by PL or microorganism proteases, is one of the main phenomena observed in milk samples. All UHPH-treated samples exhibited a significant decrease in β-casein residual level during storage, which was more pronounced in samples treated at 200 MPa 40 °C. Both the higher PL content, compared to milk samples treated at 300 MPa, and the presence of *Pseudomonas* spp. that can

secretate proteases, could explain the higher β-casein hydrolysis in 200 MPa, 40 °C milk sample.

Although PA milk had the same PL content as UHPH-treated samples at 300 MPa, during storage PA milk did not suffer additional proteolysis to that experienced after treatment. Differences in proteolysis between UHPH and PA samples are difficult to explain because high-pressure homogenisation could have exerted various effects. By using UHPH a decreased average micelle size by increasing pressure is produced (Hayes et al., 2003b; Sandra & Dalgleish, 2005), which can lead to a higher available protein surface area on which the enzyme can act, thus generating more proteolysis. It has been established that when heated, denatured β-Lg and casein form a complex by sulphydryl–disulphide interchange which could impede or delay the accessibility of proteases to caseins *via* repulsion or steric hindrance. Also, homogenisation pressure could modify protein susceptibility to proteolytic action, i.e., changing protein conformation, which could expose new substrate sites. On the other hand, the PL system is quite complicated and, besides the effects of homogenisation pressure on PL and plasminogen themselves, it could have also affected the inhibitors of PL and PG activators.

3.3. pH 4.6-Soluble fraction

3.3.1. Peptide profile

Peptide profiles of the pH 4.6-soluble fraction from raw and treated milk samples were almost similar in relation to qualitative aspects. In general, pasteurisation and pressure homogenisation treatments of milk had no significant effects (immediately after treatment and during storage) on the levels of hydrophilic peptides, which are those produced by bacterial proteinases that elute early in the RP-HPLC profile (Datta & Deeth, 2003). In contrast, hydrophobic peptides were highly affected by the treatments applied (Fig. 2). The level of hydrophobic peptides increased during storage in all samples; however, this increase was significant ($p < 0.05$) only in samples treated at 200 MPa. The hydrophobic peptide content, which is associated with bitter flavour, is highly related to the hydrolysis of β- and α_{s1}-casein (Lemieux & Simard, 1991). This is in accordance with the results obtained in the present study, where the degree of β- and α_{s1}-casein hydrolysis was negatively correlated with the hydrophobic peptide level; the milk that showed the highest β-casein hydrolysis and the highest hydrophobic peptide level was from the sample treated at 200 MPa and 40 °C, while PA and 300 MPa milk samples were the ones with a lower hydrophobic to hydrophilic peptide ratio, which suggests that these samples could have better flavour characteristics.

Peptide profile at day 21 of storage of 200 and 300 MPa treated samples with T_i = 40 °C were characterised by a marked increase of two hydrophobic peaks at retention times of 21.5 and 30.5 min, which could be associated to the presence of *Pseudomonas* spp. in these samples, given that these peaks were not present in the others milk samples or in the milk samples with added NaN_3.

In order to evaluate proteolysis by means of an accelerated test, a microbial preservative (NaN_3) was added to a fraction of treated milk samples (control samples) and they were incubated for 48 h

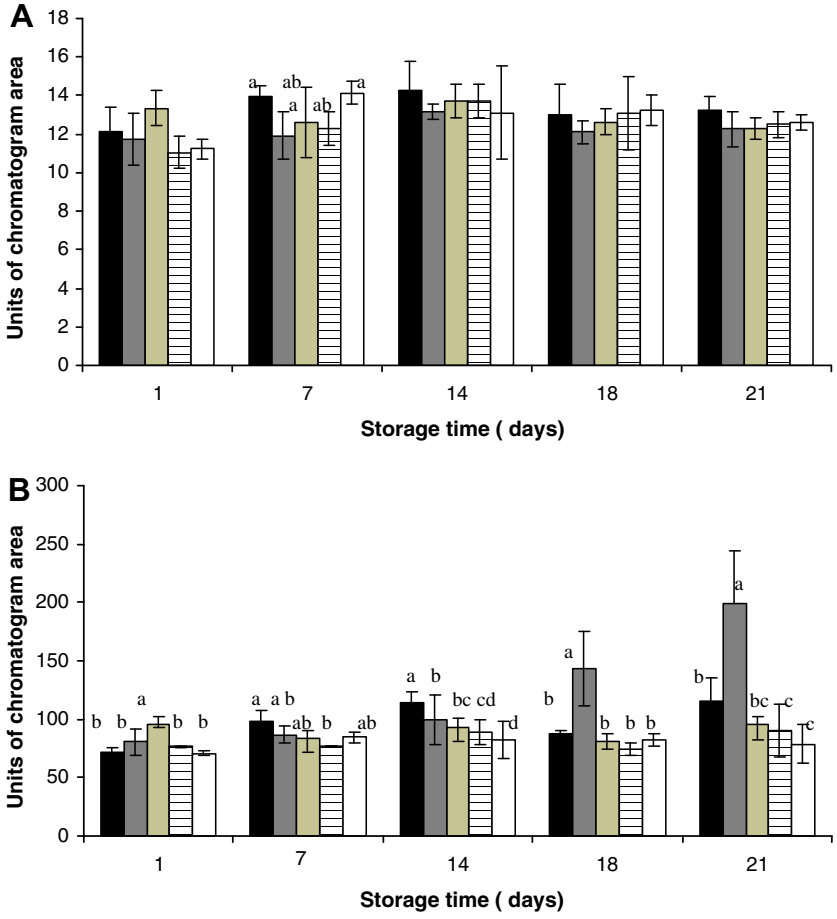

Fig. 2. Area count of hydrophilic (A) and hydrophobic (B) peptides present in the pH 4.6-soluble fraction of UHPH (200 MPa 30 °C (■), 200 MPa 40 °C (▩), 300 MPa 30 °C (▨), 300 MPa 40 °C (▤) and high-pasteurised milk (□) during storage at 4 °C. [a,b,c,d]Different superscripts for the same day indicate significant differences ($p < 0.05$).

at 37 °C (incubated samples) and then peptides were studied (Table 3). Although no significant differences were detected in hydrophilic peptide level for each milk sample before and after incubation, there seems to be a slight increase, which was more noticeable in samples treated at 200 MPa. As was mentioned before, hydrophilic peptides are mostly produced by bacterial proteinases. Since by adding NaN_3 no additional microbial proteinases can be produced during storage, only proteases that could survive treatments can act on caseins to produce these peptides. The lower pressure (200 MPa) probably produced less microbial protease inactivation, which could explain the higher increase in hydrophilic peptides, compared to milk samples treated at 300 MPa. In relation to hydrophobic peptides, incubation at 37 °C favours PL activity. All milk samples exhibited a significant difference between the control and incubated sample. The higher the PL content was, the higher the hydrophobic peptide level after incubation.

3.3.2. pH 4.6-Soluble nitrogen and total free amino acids

Data obtained for FAA by the cadmium–ninhydrin method were inconsistent, without a clear trend during milk storage (data not shown; values of FAA were around 0.08–0.11 mg leucine/ml milk). The same oscillatory tendency was reported by Cladman, Scheffer, Goodrich, and Griffiths (1998) in whole and 2% fat pasteurised milk during storage at 4 °C, while Papachristou et al. (2006) observed a constant degree of proteolysis during storage of whole milk. In general, no significant differences were detected between samples at each sampling day and during refrigerated storage. By comparing day 1 with day 21 a slight, although not significant, increase in FAA content was observed for UHPH-treated samples with an inlet temperature of 40 °C. The production of FAA is related with the presence of microbial proteases, which can degrade large-medium casein peptides to low molecular weight peptides and FAA, the presence of *Pseudomonas* spp. in these samples could probably be related to the increase in FAA observed.

The level of pH 4.6-SN in raw milk (~0.2% of total N) was significantly higher ($p < 0.05$) than in UHPH and high-pasteurised samples (~0.15–0.17% of total N), due to the presence of native whey proteins, which in treated samples are partially denatured particularly in PA milk. During storage a slight increase of pH 4.6-SN was observed in all samples, and milk treated at 200 MPa and 40 °C was the one that exhibited the highest increase ($p < 0.05$). The content of pH 4.6-SN will be partially determined by the level of hydrophilic and hydrophobic peptides presents in the pH 4.6-soluble fraction. Hydrophobic peptides were present at a higher percentage than the hydrophilic peptides; therefore, the amount of hydrophobic peptides could have an influence on the pH 4.6-SN. The same relationship of hydrophobic peptides with pH 4.6-SN was observed for samples with added NaN_3, used in the test of accelerated proteolysis.

Table 3
Mean values ± standard deviation of hydrophobic and hydrophilic peptides (chromatographic area units) of raw and treated milk samples with added NaN_3 before and after incubation at 37 °C

	Hydrophilic peptides		Hydrophobic peptides	
	Control[A]	Incubated[B]	Control	Incubated
Raw	14.1 ± 0.71	14.6 ± 1.3	98.4^{b} ± 6.65	161.2^{a} ± 22.9
200 MPa, 30 °C	12.7 ± 0.6	13.3 ± 0.38	83.1^{b} ± 9.29	144^{a} ± 35.5
300 MPa, 30 °C	13.6 ± 0.33	13.7 ± 0.8	80.2^{b} ± 8.82	108^{a} ± 29.0
200 MPa, 40 °C	12.8 ± 0.36	13.0 ± 0.46	67.4^{b} ± 7.48	132^{a} ± 21.5
300 MPa, 40 °C	12.9 ± 0.74	13.4 ± 0.99	70.2^{b} ± 6.21	116^{a} ± 14.8
Pasteurized[C]	13.0 ± 1.17	13.6 ± 1.12	67.2^{b} ± 5.88	94.5^{a} ± 15.0

[a,b]Different superscripts for the same parameter and sample indicate significant differences ($p < 0.05$).
[A] Control: milk sample with 0.07% of NaN_3.
[B] Incubated: milk sample with 0.07% of NaN_3 that is incubated at 37 °C for 48 h.
[C] Pasteurised: high-pasteurised milk (90 °C for 15 s).

4. Conclusions

Proteolysis of milk during storage is a major deteriorative reaction (together with lipolysis and oxidation) limiting its shelf-life; therefore the knowledge of the cause that produces this biochemical change is important, in order to help manufacturers find appropriate conditions of treatment and storage. The present study of proteolysis in milk during storage at 4 °C indicated the greater importance of PL compared to microbial proteases in this biochemical phenomenon. The indigenous milk proteolytic enzyme, PL, is as susceptible to inactivation in milk treated at 300 MPa as in PA milk. The results obtained showed that PA milk was the sample that had the lowest casein hydrolysis during storage, while UHPH-treated sample at 200 MPa and 40 °C was the one that showed the most important casein breakdown ($p < 0.05$).

In general, no great differences were observed between samples in the levels of hydrophilic peptides; however, the proportion of hydrophobic peptides was correlated to casein hydrolysis, mainly β- and α_{s1}-casein. As the pH 4.6-SN is affected by the peptides present, and since hydrophilic peptide level did not show differences between samples, pH 4.6-SN content also had a relation with hydrophobic peptide amount and therefore with casein hydrolysis. Evolution of FAA for all treated samples during storage did not exhibit a clear behaviour.

Although, UHPH-treated samples exhibited higher casein hydrolysis compared to PA milk, there were no significant differences in peptide and pH 4.6-SN content between UHPH-treated samples at 300 MPa and PA milk. These results suggest that these samples could probably have similar flavour characteristics, while samples treated at 200 MPa and 40 °C would not be used as an alternative to thermal treatment, since the high content of hydrophobic peptides could be related to bitterness, gelation and sedimentation, and therefore to a reduced shelf-life. To determine if these chemical differences between samples are appreciated by consumers or not, sensory analyses of milk are in progress in our laboratories.

Acknowledgements

The authors acknowledge the European Union for the financial support given to this investigation (CRAFT Project No. 512626), and Julieta Pereda acknowledges a predoctoral fellowship from Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR).

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