

## Plasmin activity and proteolysis in high pressure-treated bovine milk

Thom HUPPERTZ, Patrick F. FOX, Alan L. KELLY\*

Department of Food and Nutritional Sciences, University College, Cork, Ireland

Received 30 June 2003 – Accepted 9 October 2003

Published online 26 March 2004

**Abstract** – In this study, proteolysis resulting from the action of the indigenous milk proteinase, plasmin, in high pressure (HP)-treated raw skim bovine milk during storage was examined. Plasmin activity was reduced by treatment at pressures  $\geq 400$  MPa and decreased further throughout 28 d of storage at 5 °C. In untreated milk or milk treated at 100 MPa plasmin activity increased during the first 3 d of storage at 37 °C, indicating activation of plasminogen; considerably less activation occurred in milk treated at 200–600 MPa. However, considerable decreases in plasmin activity, probably due to autolysis, were apparent in all samples on storage at 37 °C for  $>3$  d. Proteolysis, as measured by increases in the level of pH 4.6-soluble N and decreases in the level of  $\beta$ -casein, was very limited on storage of milk at 5 °C, with little difference between untreated and HP-treated samples. Proteolysis on storage of milk at 37 °C was influenced only slightly by treatment of milk at a pressure  $\leq 250$  MPa and was reduced considerably in milk treated at 600 MPa for 30 min, but in milk treated at 300–400 MPa, proteolysis was more extensive than in untreated milk, possibly as a result of HP-induced disruption of casein micelles. Overall, HP can either induce or reduce proteolysis in milk, and may therefore have implications for products made from such milk.

### High pressure / milk / proteolysis / plasmin / casein micelle

**Résumé** – **Activité de la plasmine et protéolyse dans un lait de bovin traité par hautes pressions.** Dans cette étude, nous nous sommes intéressés à la protéolyse résultant de l'activité d'une protéinase indigène du lait, la plasmine, au cours de l'incubation d'un lait de bovin frais écrémé et traité par hautes pressions. L'activité de la plasmine était réduite par un traitement à des pressions  $\geq 400$  MPa et diminuait progressivement au cours des 28 j de stockage à 5 °C. Pour un lait non traité ou traité à 100 MPa, l'activité de la plasmine augmentait pendant les trois premiers jours d'incubation à 37 °C, indiquant l'activation du plasminogène ; l'activation était bien moins importante pour un lait traité à des pressions comprises entre 200 et 600 MPa. Cependant, des diminutions considérables de l'activité de la plasmine, probablement due à une autolyse, étaient observées pour tous les échantillons incubés à 37 °C pour des durées de stockage  $>3$  j. La protéolyse, mesurée par les augmentations du taux d'azote soluble à pH 4,6 et par les diminutions du taux de caséine  $\beta$  résiduelle, était très limitée pour un stockage du lait à 5 °C, avec une petite différence entre les échantillons non traités et ceux traités aux hautes pressions. La protéolyse durant l'incubation du lait à 37 °C était seulement légèrement influencée par le traitement du lait à des pressions  $\leq 250$  MPa et était considérablement réduite pour un lait traité à 600 MPa pour 30 min, mais pour un lait traité à 300–400 MPa, la protéolyse était plus importante que dans un lait non traité, cela étant probablement dû à la désintégration des micelles de caséine induite par les hautes pressions. Le traitement par hautes pressions peut à la fois induire ou réduire le phénomène de protéolyse dans le lait, et peut avoir des implications au niveau des produits fabriqués à partir de tels laits.

### Haute pression / lait / protéolyse / plasmine / micelle de caséine

\* Corresponding author: a.kelly@ucc.ie

## 1. INTRODUCTION

High pressure (HP) processing of food is currently of great interest as a potential process for both preservation and alteration of the properties of foods. On HP treatment, many constituents and properties of milk are affected (for reviews see [13, 28]). HP affects indigenous enzymes in milk; the principal proteinase in bovine milk, plasmin, is an alkaline proteinase which readily hydrolyses  $\beta$ -casein and, more slowly,  $\alpha_{s1}$ - and  $\alpha_{s2}$ -caseins (see [3, 18]). The majority of plasmin in milk is in the form of the inactive precursor, plasminogen, which is converted to plasmin through a complex system of plasminogen activators [3, 18]. Plasmin is not affected by treatment at pressures <400 MPa, but treatment at a higher pressure causes considerable inactivation [8, 26, 27].

The main source of substrate for plasmin, the casein micelle, is also influenced by HP. Treatment at 100–200 MPa has little effect on casein micelle size [6, 14, 23], but treatment at 250 MPa for  $\geq 15$  min increases casein micelle size, possibly through the formation of micellar aggregates [14, 15]. Treatment at a pressure  $\geq 300$  MPa reduces casein micelle size by  $\sim 50\%$  [6, 8, 10, 14, 15, 23]. Dissociation of caseins, particularly  $\beta$ - and  $\kappa$ -caseins, occurs at a pressure  $\geq 100$  MPa [2, 21].

HP-induced effects on plasmin, combined with effects on the casein micelle, may influence proteolysis in milk. Scollard et al. [27] reported that proteolysis in milk was not affected by HP treatment at 50 MPa, was increased in milk treated at 300–500 MPa, but was reduced in milk treated at higher pressures. According to Garcia-Risco et al. [9], increased proteolysis in milk treated at such pressures is probably related to HP-induced disruption of casein micelles.

The aim of this study was to examine the relationship between HP-induced changes in raw skim bovine milk and proteolysis in such milk on subsequent storage at 5 or

37 °C. Plasmin activity in untreated and HP-treated milk was monitored during such storage, which has not been done previously; also, the results of plasmin activity in milk, i.e., increases in level of pH 4.6-soluble nitrogen and the degradation of  $\beta$ -casein, were measured during storage.

## 2. MATERIALS AND METHODS

### 2.1. Milk supply

Raw whole bovine milk, obtained from a local dairy (CMP Dairies, Cork, Ireland), was skimmed by centrifugation at 2 000 *g* for 20 min at 20 °C, followed by filtration through glass wool to remove fat particles. Sodium azide (0.05%, w/v) was added to prevent microbial growth.

### 2.2. High pressure treatment

Skimmed milk samples were packaged and HP-treated for 10 or 30 min at 100–600 MPa at 20 °C, as described by Huppertz et al. [15] using a Stansted Fluid Power Iso-Lab 900 High Pressure Food Processor (Stansted Fluid Power, Stansted, Essex, UK). After HP treatment, milk samples were incubated in sterile containers for up to 14 d at 37 °C or 28 d at 5 °C.

### 2.3. Determination of plasmin activity in milk

Plasmin activity in the centrifugal (30 min at 15 800 *g* at 20 °C) supernatant of a 3:1 mixture of milk and 0.4 mol·L<sup>-1</sup> trisodium citrate was determined as described by Richardson and Pearce [25], using *N*-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (Sigma Chemical Co., St. Louis, MO, USA) as substrate. Plasmin activity was monitored during storage of milk and was determined in duplicate for each sample; values are expressed as a percentage of the mean value for untreated milk at day zero (d 0).

**Table I.** Effect of high pressure treatment at 100–600 MPa for 10 or 30 min at 20 °C and subsequent storage for up to 28 or 14 d at 5 or 37 °C, respectively, on plasmin activity in raw skim bovine milk. Values are expressed as a percentage of the mean value for untreated milk at day 0,  $\pm$  standard deviation. Results are the means of data from triplicate experiments on individual milk samples.

Pressure Treatment (MPa)	Time (min)	Plasmin activity (% of control)							
		Day 0	Days at 5 °C		Days at 37 °C				
			14	28	1	3	7	14	
0 (Control)	0	100.0 $\pm$ 0.0	92.2 $\pm$ 5.9	86.7 $\pm$ 5.6	120.9 $\pm$ 5.8	116.3 $\pm$ 6.4	70.0 $\pm$ 5.4	51.3 $\pm$ 5.3	
100	10	102.3 $\pm$ 2.4	89.2 $\pm$ 1.6	84.3 $\pm$ 3.1	121.3 $\pm$ 6.1	111.8 $\pm$ 3.3	68.3 $\pm$ 5.8	55.7 $\pm$ 3.6	
	30	101.8 $\pm$ 2.4	84.6 $\pm$ 5.5	82.9 $\pm$ 4.7	113.0 $\pm$ 4.7	111.5 $\pm$ 6.4	63.2 $\pm$ 5.0	48.2 $\pm$ 5.3	
200	10	96.4 $\pm$ 2.7	78.7 $\pm$ 3.2	79.2 $\pm$ 3.0	100.5 $\pm$ 1.6	99.9 $\pm$ 3.8	60.5 $\pm$ 3.6	49.0 $\pm$ 4.4	
	30	94.4 $\pm$ 2.5	80.8 $\pm$ 1.7	79.2 $\pm$ 3.8	97.8 $\pm$ 2.9	94.1 $\pm$ 5.4	59.6 $\pm$ 6.8	47.8 $\pm$ 3.0	
250	10	92.1 $\pm$ 1.0	79.0 $\pm$ 2.1	75.6 $\pm$ 7.1	90.6 $\pm$ 6.6	91.0 $\pm$ 7.2	57.1 $\pm$ 3.8	48.7 $\pm$ 4.1	
	30	94.0 $\pm$ 3.1	77.3 $\pm$ 0.9	72.9 $\pm$ 3.3	91.1 $\pm$ 5.0	87.5 $\pm$ 5.0	54.9 $\pm$ 3.7	46.9 $\pm$ 1.0	
300	10	91.9 $\pm$ 3.0	71.7 $\pm$ 4.4	69.1 $\pm$ 4.3	83.2 $\pm$ 4.1	86.5 $\pm$ 7.0	61.4 $\pm$ 7.7	45.2 $\pm$ 4.0	
	30	89.6 $\pm$ 6.6	74.3 $\pm$ 6.6	67.4 $\pm$ 3.8	84.1 $\pm$ 3.1	82.1 $\pm$ 5.9	69.7 $\pm$ 5.6	41.2 $\pm$ 1.9	
400	10	75.3 $\pm$ 5.0	59.9 $\pm$ 6.2	54.9 $\pm$ 1.7	68.7 $\pm$ 4.4	65.8 $\pm$ 2.2	55.1 $\pm$ 2.1	35.5 $\pm$ 5.5	
	30	69.4 $\pm$ 3.0	59.2 $\pm$ 4.8	53.3 $\pm$ 0.9	65.2 $\pm$ 2.5	66.1 $\pm$ 4.6	58.0 $\pm$ 6.7	39.1 $\pm$ 4.9	
600	10	36.1 $\pm$ 7.4	31.7 $\pm$ 4.9	29.2 $\pm$ 4.8	36.3 $\pm$ 4.9	34.3 $\pm$ 6.9	29.3 $\pm$ 4.4	32.4 $\pm$ 1.9	
	30	24.9 $\pm$ 3.2	26.2 $\pm$ 3.5	24.7 $\pm$ 4.3	28.7 $\pm$ 5.0	26.8 $\pm$ 5.9	19.7 $\pm$ 4.9	21.4 $\pm$ 5.8	

#### 2.4. Evaluation of proteolysis in milk

Proteolysis in milk was determined by quantitatively assaying the level of pH 4.6-soluble nitrogen (pH 4.6-SN) during storage using the Lowry assay for protein [22], as described by Kelly et al. [19]. The pH 4.6-SN fraction of milk includes native whey proteins plus proteolysis products, and changes in the level thereof on storage post-HP treatment can be assumed to be due to proteolysis. The level of pH 4.6-SN was determined in triplicate for each sample; values are expressed as a percentage of the mean value for untreated milk at d 0.

Degradation of  $\beta$ -casein during storage of milk was monitored using Urea Polyacrylamide Gel Electrophoresis (Urea-PAGE; [1]). Gels were stained directly by the

method of Blakesley and Boezi [4] and the level of residual  $\beta$ -casein determined by densitometric analysis using Total Lab V1.10 software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) and expressed as a percentage of the mean value for untreated milk at day 0.

#### 2.5. Denaturation of $\beta$ -lactoglobulin

Denaturation of  $\beta$ -lactoglobulin ( $\beta$ -Lg) was determined as described by Huppertz et al. [14].

### 3. RESULTS

#### 3.1. Effects of high pressure treatment on plasmin activity in milk

When plasmin activity was determined immediately after HP treatment (Tab. I; d 0),

**Table II.** Effect of high pressure treatment at 100–600 MPa for 10 or 30 min at 20 °C and subsequent storage for up to 28 or 14 d at 5 or 37 °C, respectively, on the level of pH 4.6-soluble N in raw skim bovine milk. Values are expressed as a percentage of the mean value for untreated milk at day 0,  $\pm$  standard deviation. Results are the means of data from triplicate experiments on individual milk samples.

		pH 4.6-soluble N (% of control)							
Pressure (MPa)	Treatment time (min)	Day 0	Days at 5 °C		Days at 37 °C				
			14	28	1	3	7	14	
0	(Control) 0	100.0 $\pm$ 0.0	112.1 $\pm$ 5.4	112.4 $\pm$ 7.6	110.2 $\pm$ 3.0	123.6 $\pm$ 2.2	137.6 $\pm$ 9.6	148.8 $\pm$ 1.4	
100	10	97.2 $\pm$ 1.2	108.6 $\pm$ 7.7	107.6 $\pm$ 7.3	103.6 $\pm$ 2.4	125.6 $\pm$ 2.5	141.3 $\pm$ 2.6	143.3 $\pm$ 6.3	
	30	97.3 $\pm$ 3.1	110.1 $\pm$ 7.7	103.9 $\pm$ 5.2	106.6 $\pm$ 2.1	128.4 $\pm$ 5.7	131.7 $\pm$ 6.7	144.7 $\pm$ 5.7	
200	10	97.7 $\pm$ 4.1	108.0 $\pm$ 2.7	102.5 $\pm$ 7.2	103.1 $\pm$ 2.4	123.7 $\pm$ 6.0	132.0 $\pm$ 1.7	141.7 $\pm$ 5.4	
	30	90.8 $\pm$ 4.3	100.5 $\pm$ 6.3	98.2 $\pm$ 7.5	105.3 $\pm$ 6.3	115.9 $\pm$ 6.6	132.8 $\pm$ 2.5	142.4 $\pm$ 8.5	
250	10	88.0 $\pm$ 1.9	99.8 $\pm$ 7.4	100.7 $\pm$ 5.5	102.9 $\pm$ 1.9	116.0 $\pm$ 5.4	131.6 $\pm$ 5.1	139.9 $\pm$ 7.8	
	30	77.9 $\pm$ 5.3	90.4 $\pm$ 5.5	93.4 $\pm$ 7.9	87.8 $\pm$ 2.2	106.2 $\pm$ 4.7	127.8 $\pm$ 4.1	134.5 $\pm$ 7.2	
300	10	84.1 $\pm$ 5.5	87.1 $\pm$ 4.6	93.5 $\pm$ 6.9	96.2 $\pm$ 3.5	109.2 $\pm$ 7.2	127.7 $\pm$ 7.5	137.5 $\pm$ 2.3	
	30	72.4 $\pm$ 4.9	79.8 $\pm$ 6.3	80.6 $\pm$ 3.4	91.8 $\pm$ 5.7	104.5 $\pm$ 2.9	123.8 $\pm$ 5.6	132.0 $\pm$ 7.3	
400	10	71.5 $\pm$ 5.8	81.7 $\pm$ 7.2	83.5 $\pm$ 1.9	86.3 $\pm$ 6.2	104.6 $\pm$ 2.9	122.4 $\pm$ 6.0	133.7 $\pm$ 9.2	
	30	61.8 $\pm$ 2.0	73.6 $\pm$ 2.9	79.7 $\pm$ 3.0	76.1 $\pm$ 5.8	97.7 $\pm$ 2.2	108.7 $\pm$ 4.8	129.0 $\pm$ 8.4	
600	10	52.9 $\pm$ 0.8	60.8 $\pm$ 3.6	64.7 $\pm$ 0.9	62.4 $\pm$ 7.6	70.9 $\pm$ 1.3	87.9 $\pm$ 8.3	98.1 $\pm$ 6.2	
	30	47.8 $\pm$ 3.0	53.6 $\pm$ 6.0	55.2 $\pm$ 2.1	53.4 $\pm$ 4.2	60.7 $\pm$ 4.6	77.0 $\pm$ 4.1	79.7 $\pm$ 5.5	

considerable effects of HP were apparent. Treatment at 100 MPa for 10 or 30 min had little effect on plasmin activity, but treatment at 200–400 MPa progressively reduced plasmin activity, e.g., to ~75 or ~70% of that in untreated milk after 10 or 30 min at 400 MPa, respectively. Treatment of milk at 600 MPa for 10 or 30 min reduced plasmin activity to 36 or 25% of that in untreated milk, respectively.

In untreated and HP-treated milk, plasmin activity decreased throughout 28 d of storage at 5 °C (Tab. I); the magnitude of the decrease in plasmin activity on storage had a maximum at 300 MPa, and no post-HP treatment reduction in plasmin activity was observed on cold storage of milk treated at 600 MPa for 30 min. In untreated milk or milk treated at 100 MPa, storage at 37 °C for 1 d increased plasmin activity,

whereas after 3 d at 37 °C, plasmin activity in such milk was lower than that after 1 d, but higher than that at day 0 (Tab. I); for samples treated at 200–600 MPa, little influence of storage for up to 3 d at 37 °C on plasmin activity was apparent. Storage of both untreated and HP-treated milk at 37 °C for 7 or 14 d progressively reduced plasmin activity, to values of 21–55% of that in untreated milk.

### 3.2. Proteolysis in high pressure-treated milk

HP treatment of milk reduced the level of pH 4.6-SN therein (Tab. II; d 0). During subsequent storage at 5 °C, the level of pH 4.6-SN increased only slightly (by ~10%) in untreated or HP-treated milk, with little difference in the extent of the increase between samples. However, on incubation

**Table III.** Effect of high pressure treatment at 100–600 MPa for 10 or 30 min at 20 °C and subsequent storage for up to 14 days at 37 °C on the residual level of  $\beta$ -casein in raw skim bovine milk. Values are expressed as a percentage of the mean value for untreated milk at day 0,  $\pm$  standard deviation. Results are the means of data from triplicate experiments on individual milk samples.

Pressure (MPa)	Treatment time (min)	Residual $\beta$ -casein (% of control)				
		Day 0	Days at 37 °C			
			1	3	7	14
0	(Control) 0	100.0 $\pm$ 0.0	91.4 $\pm$ 6.3	68.4 $\pm$ 2.1	55.6 $\pm$ 3.0	48.1 $\pm$ 3.8
100	10	94.6 $\pm$ 5.5	90.0 $\pm$ 6.1	63.4 $\pm$ 3.8	54.3 $\pm$ 3.4	44.3 $\pm$ 2.2
	30	95.7 $\pm$ 6.9	95.1 $\pm$ 1.8	65.1 $\pm$ 4.3	55.8 $\pm$ 1.1	44.9 $\pm$ 4.5
200	10	100.9 $\pm$ 7.8	94.0 $\pm$ 4.0	63.1 $\pm$ 2.5	55.0 $\pm$ 5.0	43.2 $\pm$ 1.0
	30	99.7 $\pm$ 4.1	91.7 $\pm$ 4.4	66.9 $\pm$ 7.2	55.7 $\pm$ 7.7	42.6 $\pm$ 3.3
250	10	100.0 $\pm$ 5.0	95.1 $\pm$ 5.5	66.5 $\pm$ 6.6	54.4 $\pm$ 5.8	41.4 $\pm$ 2.6
	30	100.4 $\pm$ 3.3	96.4 $\pm$ 0.9	69.6 $\pm$ 9.2	52.3 $\pm$ 3.5	40.2 $\pm$ 6.7
300	10	100.1 $\pm$ 5.0	92.9 $\pm$ 3.0	62.7 $\pm$ 8.8	49.5 $\pm$ 2.5	35.7 $\pm$ 3.2
	30	98.3 $\pm$ 4.6	81.1 $\pm$ 5.2	63.5 $\pm$ 7.6	41.4 $\pm$ 5.6	34.7 $\pm$ 1.9
400	10	96.5 $\pm$ 7.0	82.6 $\pm$ 6.7	62.3 $\pm$ 6.7	40.2 $\pm$ 4.5	32.7 $\pm$ 2.2
	30	95.3 $\pm$ 3.8	86.8 $\pm$ 6.4	66.8 $\pm$ 8.1	46.0 $\pm$ 8.6	37.7 $\pm$ 2.2
600	10	96.8 $\pm$ 5.8	96.1 $\pm$ 1.3	77.1 $\pm$ 4.6	70.3 $\pm$ 2.3	65.7 $\pm$ 6.2
	30	96.2 $\pm$ 4.7	93.5 $\pm$ 3.8	86.5 $\pm$ 0.5	81.5 $\pm$ 6.0	79.7 $\pm$ 0.7

at 37 °C, levels of pH 4.6-SN increased progressively throughout 14 d of storage. In milk treated for 10 min at 100, 200, 250, 300 or 600 MPa or 30 min at 100 or 200 MPa, increases in pH 4.6-SN throughout 14 d of storage at 37 °C were similar to those observed in untreated milk (Tab. II). For samples treated for 10 min at 400 MPa or 30 min at 250–400 MPa, increases in the level of pH 4.6-SN were larger than in untreated milk, whereas in milk treated at 600 MPa for 30 min, increases in the level of pH 4.6-SN (Tab. II) were considerably less than that in untreated milk.

HP treatment of milk had, as expected, no immediate effect on the level of  $\beta$ -casein in milk (Tab. III; d 0) and storage at 5 °C for 28 d after HP treatment reduced the level of  $\beta$ -casein only slightly (data not shown); however, on storage at 37 °C, the level of  $\beta$ -casein decreased progressively for up to 14 d (Tab. III). The extent of the reduction in the level of  $\beta$ -casein generally

increased with pressure up to 400 MPa and was most extensive in milk treated at 300 or 400 MPa for 10 or 30 min; during incubation at 37 °C, the level of residual  $\beta$ -casein was consistently higher in samples treated at 600 MPa than in untreated samples or in samples treated at 100–400 MPa.

### 3.3. Effects of high pressure on whey protein denaturation in milk

No denaturation of  $\beta$ -Lg was observed after treatment at 100 MPa for 10 or 30 min, but at pressures  $\geq$ 200 MPa, denaturation of  $\beta$ -Lg increased with increasing pressure, reaching > 95% after treatment at 600 MPa (data not shown).

## 4. DISCUSSION

Proteolysis in milk is due primarily to the action of plasmin on caseins, especially  $\beta$ -casein [18]; thus, HP-induced changes in

the level of proteolysis in milk may be due to effects of HP on plasmin. Thermal inactivation of plasmin in milk is thought to be due to thiol-disulphide interchange reactions with  $\beta$ -Lg [11]. Scollard et al. [26] reported that HP treatment at 600 MPa did not inactivate plasmin in phosphate buffer, but inactivated >95% of plasmin in a phosphate buffer containing 5 mg·mL<sup>-1</sup>  $\beta$ -Lg, which suggests that HP-induced inactivation of plasmin is also linked to  $\beta$ -Lg. In this study, treatment of milk at  $\geq 400$  MPa reduced plasmin activity therein considerably (Tab. I), as previously reported by Scollard et al. [26, 27], and denatured  $\geq 95\%$  of  $\beta$ -Lg (data not shown), again consistent with previous studies [10, 14, 20, 27], supporting the possibility that HP-induced inactivation of plasmin in milk may be linked to denaturation of  $\beta$ -Lg. However, it remains unclear whether there is a direct causal relationship between the two phenomena.

After HP treatment, milk was stored at 5 and 37 °C and changes in plasmin activity and proteolysis were monitored. The lower temperature (5 °C) was chosen as it mimics general refrigerated storage of consumption milk, whereas 37 °C is the optimum temperature for plasmin activity [18]; storage at this temperature was therefore performed to evaluate the maximum extent of changes in untreated and HP-treated milk due to plasmin activity. Reductions in plasmin activity on refrigerated storage of untreated milk (Tab. I) were also observed by Guinot-Thomas et al. [12], who suggested that this may be a result of autolysis of plasmin; Ueshima et al. [29] also showed that, in 0.9% NaCl, bovine plasmin is susceptible to autolysis.

On storage of milk at 37 °C, both increases and decreases in plasmin activity were observed, depending on the applied pressure. Increases in plasmin activity in untreated milk or in milk treated at 100 MPa during the first 3 d of storage at 37 °C (Tab. I), are probably due to the conversion of the inactive zymogen, plas-

minogen, to active plasmin [5, 24], and suggest that plasminogen and plasminogen activators are stable to treatment at such pressures. In milk treated at  $\geq 200$  MPa, however, plasmin activity did not increase on storage at 37 °C (Tab. I), possibly through inactivation either of plasminogen or of its activators; Scollard et al. [27] showed that treatment at <500 MPa inactivated <20% of plasminogen, with more extensive inactivation at higher pressures.

Decreases in plasmin activity in milk during more prolonged storage of milk at 37 °C (Tab. I; 7 or 14 d), as at 5 °C, may be due to autolysis of the enzyme. The greater apparent autolysis of plasmin at 37 °C than at 5 °C (Tab. I) is in agreement with results of Ueshima et al. [29]. The fact that decreases in plasmin activity on storage at 5 or 37 °C were considerably less in milk treated at 600 MPa than in untreated milk or milk treated at 100–400 MPa (Tab. I) is probably due to the low initial level of plasmin in the system; autolysis may be limited in milk treated at 600 MPa, due to considerable HP-induced inactivation of plasmin.

HP-induced decreases in the level of pH 4.6-SN immediately after treatment (Tab. II; d 0), as also observed by Johnston et al. [17], are a result of whey protein denaturation, which results in a loss of their solubility at pH 4.6 [16]. A low level of proteolysis was observed on storage of untreated or HP-treated milk at 5 °C, as indicated by slight increases in pH 4.6-SN (Tab. II) and slight decreases in  $\beta$ -casein levels (results not shown), as also reported by Guinot-Thomas et al. [12] for untreated milk. The low level of proteolysis is probably due to the low activity of plasmin at refrigeration temperatures. Garcia-Risco et al. [7] observed little difference between the level of proteolysis in untreated milk and milk treated at 400 MPa for 30 min, during storage at 7 °C for 15 d; however, in the present study (Tab. II) milk treated under such conditions had slightly faster proteolysis than untreated milk on storage at 5 °C.

On storage at 37 °C, proteolysis was far more rapid, and was most extensive in samples treated at 300 or 400 MPa for 30 min. Proteolysis was reduced considerably in milk treated at 600 MPa for 30 min, in agreement with the results of Scollard et al. [27]. Increased proteolysis in milk treated at 400 MPa (Tabs. II and III), despite reduced plasmin activity in such milk (Tab. I), is possibly due to the disruptive effect of HP on the micellar structure, thereby facilitating increased availability of substrate to plasmin [27]. Garcia-Risco et al. [9] showed that when a high level of exogenous plasmin was added to untreated or HP-treated milk, proteolysis was more extensive in the latter, which is consistent with the above suggestion. Reduced proteolysis in milk treated at 600 MPa for 30 min (Tabs. II and III), despite considerable disruption of casein micelles [10, 14, 23], is probably related to the significantly reduced plasmin activity in such samples. On treatment of milk at a pressure  $\leq 250$  MPa, plasmin activity was affected only slightly (Tab. I) and little disruption of casein micelles occurs [10, 14, 15, 23]; it is therefore not surprising that treatment of milk at such pressures influenced proteolysis during subsequent storage only slightly (Tabs. II and III).

In conclusion, proteolysis in milk was influenced by effects of HP on the casein micelles, whey proteins and plasmin; treatment at 100–250 MPa affected proteolysis relatively little, but proteolysis was accelerated by treatment of milk at 300–400 MPa, but inhibited by treatment at 600 MPa for 30 min. As considerable changes in plasmin activity occurred on storage of untreated and HP-treated milk, monitoring of plasmin activity throughout storage of milk is necessary for full understanding of changes in proteolysis in milk. Both the activation of plasminogen and autolysis of plasmin appeared to be influenced by HP treatment of milk; more detailed studies on how HP affects these processes may be of interest for future study.

**Acknowledgements:** This research was funded by the Food Institutional Research Measure (FIRM), which is administered by the Irish Government under the National Development Plan 2000–2006. The linguistic assistance of D. Afoufa-Bastien and A. Chevin was greatly appreciated.

## REFERENCES

- [1] Andrews A.T., Proteinases in normal bovine milk and their action on the caseins, *J. Dairy Res.* 50 (1983) 45–55.
- [2] Arias M., Lopez-Fandino R., Olano A., Influence of pH on the effects of high pressure on milk proteins, *Milchwissenschaft* 55 (2000) 191–194.
- [3] Bastian E.D., Brown R.J., Plasmin in milk and dairy products: an update, *Int. Dairy J.* 6 (1996) 435–457.
- [4] Blakesley R.W., Boezi J.A., A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250, *Anal. Biochem.* 82 (1977) 580–581.
- [5] De Rham O., Andrews A.T., The roles of native milk proteinase and its zymogen during proteolysis in normal bovine milk, *J. Dairy Res.* 49 (1983) 577–585.
- [6] Desobry-Banon S., Richard F., Hardy J., Study of acid and rennet coagulation of high pressurized milk, *J. Dairy Sci.* 77 (1994) 3267–3274.
- [7] Garcia-Risco M.R., Cortes E., Carrascosa A.V., Lopez-Fandino R., Microbiological and chemical changes in high-pressure-treated milk during refrigerated storage, *J. Food Prot.* 61 (1998) 735–737.
- [8] Garcia-Risco M.R., Olano A., Ramos M., Lopez-Fandino R., Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk, *J. Dairy Sci.* 83 (2000) 2184–2189.
- [9] Garcia-Risco M.R., Recio I., Molina E., Lopez-Fandino R., Plasmin activity in pressurized milk, *J. Dairy Sci.* 86 (2003) 728–734.
- [10] Gaucheron F., Famelart M.H., Mariette F., Raulot K., Michel F., Le Graet Y., Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk, *Food Chem.* 59 (1997) 439–447.
- [11] Grufferty M.B., Fox P.F., Potassium iodate-induced proteolysis in ultra high heat treated milk during storage: The role of  $\beta$ -lactoglobulin and plasmin, *J. Dairy Res.* 53 (1986) 601–613.

- [12] Guinot-Thomas P., Al Ammoury M., Le Roux Y., Laurent F., Study of proteolysis during storage of raw milk at 4 °C: effect of plasmin and microbial proteinases, *Int. Dairy J.* 5 (1995) 685–697.
- [13] Huppertz T., Kelly A.L., Fox P.F., Effects of high pressure on constituents and properties of milk, *Int. Dairy J.* 12 (2002) 561–572.
- [14] Huppertz T., Fox P.F., Kelly A.L., High pressure treatment of bovine milk: effects on casein micelles and whey proteins, *J. Dairy Res.* 71 (2004) 98–106.
- [15] Huppertz T., Grosman S., Fox P.F., Kelly A.L., Heat and ethanol stabilities of high pressure-treated bovine milk, *Int. Dairy J.* 14 (2004) 125–133.
- [16] Jelen P., Rattray W., Thermal denaturation of whey proteins, in: Fox P.F. (Ed.), *Heat-Induced Changes in Milk*, Special Issue 9501, *Int. Dairy Fed.*, Brussels, Belgium, 1995, pp. 66–85.
- [17] Johnston D.E., Austin B.A., Murphy R.J., Effects of high hydrostatic pressure on milk, *Milchwissenschaft* 47 (1992) 760–763.
- [18] Kelly A.L., McSweeney P.L.H., Indigenous proteinases in milk, in: Fox P.F., McSweeney P.L.H. (Eds.), *Advanced Dairy Chemistry*, Volume 1: Proteins, 3rd edn., Kluwer Academic/Plenum Publishers, New York, USA, 2003, pp. 495–522.
- [19] Kelly A.L., Reid S., Joyce P., Meaney W.J., Foley J., Effect of decreasing milking frequency of cows in late lactation on milk somatic cell count, polymorphonuclear leucocyte numbers, composition and proteolytic activity, *J. Dairy Res.* 65 (1998) 365–373.
- [20] Lopez-Fandino R., Carrascosa A.V., Olano A., The effects of high pressure on whey protein denaturation and cheese-making properties of raw milk, *J. Dairy Sci.* 79 (1996) 929–936.
- [21] Lopez-Fandino R., De la Fuente M.A., Ramos M., Olano A., Distribution of minerals and proteins between the soluble and colloidal phases of pressurized milks from different species, *J. Dairy Res.* 65 (1998) 69–78.
- [22] Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., Protein measurement with the folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [23] Needs E.C., Stenning R.A., Gill A.L., Ferragut V., Rich G.T., High-pressure treatment of milk: effects on casein micelle structure and on enzymic coagulation, *J. Dairy Res.* 67 (2000) 31–42.
- [24] Richardson B.C., The proteinases of bovine milk and the effect of pasteurization on their activity, *N.Z. J. Dairy Sci. Technol.* 18 (1983) 233–245.
- [25] Richardson B.C., Pearce K.N., The determination of plasmin in dairy products, *N.Z. J. Dairy Sci. Technol.* 16 (1981) 209–220.
- [26] Scollard P.G., Beresford T.P., Murphy P.M., Kelly A.L., Barostability of plasmin activity, *Lait* 80 (2000) 609–619.
- [27] Scollard P.G., Beresford T.P., Needs E.C., Murphy P.M., Kelly A.L., Plasmin activity,  $\beta$ -lactoglobulin denaturation and proteolysis in high pressure treated milk, *Int. Dairy J.* 10 (2000) 835–841.
- [28] Trujillo A.J., Capellas M., Saldo J., Gervilla R., Guamis B., Applications of high-hydrostatic pressure on milk and dairy products: a review, *Innov. Food Sci. Emerg. Technol.* 3 (2002) 295–307.
- [29] Ueshima S., Okada K., Matsuo O., Stabilization of plasmin by lysine derivatives, *Clin. Chim. Acta* 245 (1996) 7–18.