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Effect of high-pressure treatment at various temperatures on indigenous proteolytic enzymes and whey protein denaturation in bovine milk

Golfo Moatsou[1,*], Constantinos Bakopanos[1], Dimitis Katharios[2], George Katsaros[3], Ioannis Kandarakis[1], Petros Taoukis[3] and Ioannis Politis[4]

[1] Department of Food Science and Technology, Agricultural University of Athens, Iera Odos 75, 11855, Athens, Greece
[2] Vivartia S.A., 16, Irinis Avenue, 17778, Athens, Greece
[3] School of Chemical Engineering, National Technical University of Athens, Chemical Engineering Building, Zographou Campus, Athens, Greece
[4] Department of Animal Science, Agricultural University of Athens, Iera Odos 75, 11855, Athens, Greece

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The objective of the present study was to determine the effect of high pressure (HP) processing (200, 450 and 650 MPa) at various temperatures (20, 40 and 55 °C) on the total plasmin plus plasminogen-derived activity (PL), plasminogen activator(s) (PA) and cathepsin D activities and on denaturation of major whey proteins in bovine milk. Data indicated that transfer of both PL and PA from the casein micelles to milk serum occurred at all pressures utilized at room temperature (20 °C). In addition to the transfer of PL and PA from micelles, there were reductions in activities of PL (16–18%) and PA (38–62%) for the pressures 450 and 650 MPa, at room temperature. There were synergistic negative effects between pressure and temperature on residual PL activity at 450 and 650 MPa and on residual PA activity only at 450 MPa. Cathepsin D activity in the acid whey from HP-treated milk was in general baroresistant at room temperature. The residual activity of cathepsin D decreased significantly at 650 MPa and 40 °C and at the pressures 450 and 650 MPa at 55 °C. Synergistic negative effects on the amount of native β-lactoglobulin were observed at 450 and 650 MPa and on the amount of native α-lactalbumin at 650 MPa. There were significant correlations between enzymatic activities (PL, PA and cathepsin D) and the residual native β-lactoglobulin and α-lactalbumin in bovine milk. In conclusion, HP significantly affected the activity of indigenous proteolytic enzymes and whey protein denaturation in bovine milk. Reduction in activity of indigenous enzymes (PL, PA and cathepsin D) and transfer of PL and PA from the casein to milk serum induced by HP is expected to have a profound effect on cheese yield, proteolysis during cheese ripening and quality of UHT milk during storage.

Keywords: High pressure, plasmin, plasminogen activators, cathepsin D, whey proteins.

Milk contains several types of indigenous proteolytic enzymes. Plasmin (PL) is the predominant proteolytic enzyme in bovine milk; it is the active enzyme formed by the activation of the inactive proenzyme plasminogen. Furthermore, bovine milk contains plasminogen activator (PA), enzymes capable of converting plasminogen to plasmin. The main form of PA localized within the casein fraction is tissue-PA (White et al., 1995). Increases in the amount of plasmin negatively affects the quality of UHT milk during storage, cheese yield, and yogurt quality. Furthermore, plasmin may affect proteolysis during cheese ripening (Bastian & Brown, 1996). Another native milk protease is cathepsin D. It is a lysosomal aspartic protease mainly present in the whey. Its specificity is similar to that of chymosin regarding casein hydrolysis and milk coagulation. Thus, it may contribute to degradation of κ-casein during long term storage of various milk products and

*For correspondence; e-mail: mg@aua.gr

affect the quality of high-cooked cheese varieties, where chymosin is inactivated (Larsen et al. 1996; Hurley et al. 2000b).

High pressure (HP) processing is a non-thermal method for food preservation that aims to achieve microbial inactivation equivalent to thermal processing without any practical loss of its nutritional and sensory characteristics (O'Reilly et al. 2001). In addition to the well known antimicrobial effect, research has focused on the potential of HP to cause reversible and irreversible changes in milk. Comprehensive reviews of the effects of HP on the physicochemical characteristics of milk are available (Huppertz et al. 2006; López-Fandiño, 2006; Considine et al. 2007). The application of HP affects the individual components of milk, mainly by causing structural changes, which are related to the conditions of pressurization (pressure, duration, temperature).

The effect of high pressure on plasmin activity has been studied in the past (Scollard et al. 2000a, b; García-Risco et al. 2003; Borda et al. 2004; Huppertz et al. 2004c). The general conclusion is that HP $\geqslant$400 MPa decreases the activity of PL and the reduction is more pronounced in the presence of β-lactoglobulin (β-lg) or by pressurizing at elevated temperatures. Furthermore, there is a synergistic effect of HP and temperature on the PL activity in the bovine milk. In contrast, the effect of HP on PA and cathepsin D activities has not been investigated in the past. It is not known whether changes of PA and cathepsin D activities caused by HP are related to whey protein denaturation. The determination of the effect of HP on PA in addition to PL, will provide a much more clear picture of alterations of the PL-PA as a system.

Therefore, the objective of the present study was to determine the effect of high-pressure treatment at various temperatures on indigenous proteolytic enzymes (PL, PA, cathepsin D) and whey proteins denaturation in bovine milk. An additional objective was to determine correlation coefficients between enzymatic activities and the amount of residual native whey proteins in bovine milk.

Materials and Methods

High pressure treatment of milk

Raw bovine whole milk samples were obtained from the premises of Vivartia, which is the largest dairy company located in Athens, Greece. Milk samples maintained at 4 °C throughout, were transported to our laboratory within 30 min and processed as described below. Milk samples were subjected to various combinations of pressure (200, 450 and 650 MPa) and thermal treatment (20, 40 and 55 °C). The pressurization time was 10 min. Five independent experiments were performed using different milk samples. High pressure treatments were performed using a laboratory scale HP equipment with a maximum operating pressure of 1000 MPa (Food Pressure Unit FPU 1.01, Resato International BV, Roden, Holland), consisting of an operation high pressure unit with a pressure intensifier, a high pressure vessel of 1·5 l and a multivessel system consisting of six vessels of 42 ml capacity each. All high pressure vessels were surrounded by a water circulating jacket connected to a temperature controlling system. The pressure transmitting fluid used was polyglycol ISO viscosity class VC 15 (Resato International BV, Roden, Holland). Heat sealed polypropylene pouches were filled with 150 ml raw, untreated milk and placed in the 1·5 l high-pressure vessel for processing. The desired value of pressure was set and after pressure build-up, the pressure vessel was isolated. This point defined the zero time of the process. Pressure of the vessel was released after a preset time interval by opening the pressure valve. The initial temperature increase during pressure build-up (about 3 deg C/100 MPa) was taken into consideration in order to achieve the desired operating temperature. Pressure and temperature were constantly monitored and recorded during the process.

Determination of the activities of plasmin and plasminogen activators

The total plasmin plus plasminogen-derived (PL) activity was determined using the conventional method of Politis et al. (1993) with a slight modification. The modification included the omission of the addition of 50 mM-ε-aminocaproic acid (EACA) and of the 2 h EACA incubation step. The EACA is added in this assay system to induce transfer of PL from casein to the milk serum. Omission of EACA allows determination of PL activity in both fractions (casein and serum) and thus, estimation of the effect of HP on dissociation of the enzyme from the casein micelles. Plasminogen was converted to plasmin by the addition of urokinase (150 Plough units). All other details of this method are described by Politis et al. (1993). One unit of plasmin plus plasminogen-derived (PL) activity was defined as the amount of enzyme required to change absorbance by 0·001 in 1 min. Activity was expressed as units per ml milk.

Activity of plasminogen activators (PA) was determined using the method of White et al. (1995) that utilizes the PA of casein or milk serum to convert exogenously supplied inactive plasminogen to active plasmin. One unit of PA activities was defined as the amount of enzymes required to change absorbance by 0·1 in 60 min. Activities were expressed as units per ml milk.

Detection and assay of cathepsin D in the acid whey of pressurized milk samples were carried out in duplicate using the method of O'Driscoll et al. (1999) with the modification of the incubation time proposed by Hurley et al. (2000a). For the determination of enzymatic activity at pH 3·2, the synthetic heptapeptide Pro-Thr-Glu-Phe-[p-nitro-Phe]-Arg-Leu (Bachem Feinchemicalien AG, Bubendorf, Switzerland) was used as the substrate. Aliquots of the supernatants of the reaction mixture (250 µl) were analysed by reversed phase HPLC. A Nucleosil

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Table 1. Effect of high pressure treatment (200, 450 and 650 MPa) at various temperatures (20, 40 and 55 °C) on plasmin plus plasminogen-derived (PL) and plasminogen activators (PA) activities in the casein and the milk serum fraction (means ± standard deviations), expressed in units/ml. Means with different letters within each column differed significantly (Duncan's multiple comparison procedure at 95% confidence level)

		Activity of			
		plasmin plus plasminogen (PL) (units/ml)		plasminogen activators (PA) (units/ml)	
Pressure (MPa)	Temperature (°C)	Milk serum	Casein	Milk serum	Casein
0	Untreated, raw milk	$7{\cdot}14^{a} \pm 1{\cdot}25$	$49{\cdot}68^{e} \pm 7{\cdot}91$	$0{\cdot}02^{a} \pm 0{\cdot}01$	$0{\cdot}19^{e} \pm 0{\cdot}06$
200	20	$18{\cdot}78^{c,d} \pm 4{\cdot}60$	$32{\cdot}18^{d} \pm 4{\cdot}25$	$0{\cdot}08^{c,d} \pm 0{\cdot}03$	$0{\cdot}11^{d} \pm 0{\cdot}02$
	40	$18{\cdot}98^{c,d} \pm 4{\cdot}55$	$31{\cdot}44^{d} \pm 4{\cdot}09$	$0{\cdot}10^{d} \pm 0{\cdot}04$	$0{\cdot}09^{c,d} \pm 0{\cdot}01$
	55	$18{\cdot}64^{c,d} \pm 3{\cdot}96$	$32{\cdot}34^{d} \pm 4{\cdot}65$	$0{\cdot}10^{d} \pm 0{\cdot}04$	$0{\cdot}09^{c,d} \pm 0{\cdot}02$
450	20	$22{\cdot}94^{d} \pm 4{\cdot}04$	$24{\cdot}58^{c} \pm 3{\cdot}29$	$0{\cdot}06^{b,c} \pm 0{\cdot}01$	$0{\cdot}07^{b,c} \pm 0{\cdot}02$
	40	$16{\cdot}02^{c} \pm 2{\cdot}77$	$15{\cdot}48^{b} \pm 2{\cdot}32$	$0{\cdot}04^{a,b} \pm 0{\cdot}02$	$0{\cdot}05^{a,b} \pm 0{\cdot}02$
	55	$9{\cdot}96^{a} \pm 0{\cdot}96$	$8{\cdot}60^{a} \pm 0{\cdot}53$	$0{\cdot}05^{a,b} \pm 0{\cdot}02$	$0{\cdot}05^{a,b} \pm 0{\cdot}02$
650	20	$23{\cdot}06^{d} \pm 3{\cdot}50$	$23{\cdot}30^{c} \pm 2{\cdot}53$	$0{\cdot}03^{a,b} \pm 0{\cdot}01$	$0{\cdot}05^{a,b} \pm 0{\cdot}01$
	40	$15{\cdot}04^{b,c} \pm 3{\cdot}28$	$14{\cdot}32^{b} \pm 2{\cdot}16$	$0{\cdot}04^{a,b} \pm 0{\cdot}02$	$0{\cdot}04^{a,b} \pm 0{\cdot}01$
	55	$11{\cdot}54^{a,b} \pm 3{\cdot}65$	$7{\cdot}76^{a} \pm 0{\cdot}76$	$0{\cdot}03^{a,b} \pm 0{\cdot}01$	$0{\cdot}03^{a} \pm 0{\cdot}01$

C8 analytical column 4·6 mm × 250 mm (5 μm particle size, 300 Å pore size) and a Nucleosil C8 guard column 4·6 mm × 10 mm (Macherey-Nagel GmbH & Co, D-5252313, Düren, Germany) were used for analysis. For comparison purposes, the acid whey of milk samples pasteurized at 68 °C for 10 min were analysed.

For the interpretation of the resulting RP-HPLC profiles, control samples containing the reaction mixture without substrate or without acid whey were analysed. Assays with commercial cathepsin D and cathepsin B from bovine spleen (C3138 and C6286, Sigma-Aldrich Co., St Louis, MO 63178, USA) instead of acid whey were carried out. Assays were also carried out in the presence of various protease inhibitors. Pepstatin A (195368, MP Biomedicals, Inc., 64702 Illkirch, France) at a concentration of 1 μm was used for the inhibition of aspartic protease activity; chymostatin (152845, MP Biomedicals, Inc.) at a concentration of 100 μm, for cysteine protease and chymotrypsin-like serine proteases and E-64 (trans-epoxysuccinyl-Lleucylamido0(4-guanidino) butane, 152846, MP Biomedicals, Inc.) at a concentration of 10 μm for inhibition of cysteine proteases. Finally, a protease inhibitor cocktail for use with mammalian cell and tissue extracts (P-8340, Sigma-Aldrich Co.) at 3% (v/v) was used for the inhibition of serine, cysteine, aspartic proteases and aminopeptidases. The methodology regarding enzyme inhibitors described above and their use in this assay is essentially identical to that of O'Driscoll et al. (1999).

Denaturation of whey proteins

The acid whey fractions of the pressurized milk samples were analysed in duplicate by RP-HPLC on a Vydac C4 214 TP 5415 column (Separation group, Hesperia,CA 92345, USA) following the methodology described by Moatsou et al. (2003). The elution times of native α-la and β-lg were verified by analysis of purified standard proteins and their quantification was based on the area of the relevant peaks. The extent of denaturation was expressed as percentage of the respective peaks of the control raw untreated milk samples. For comparison purposes, the acid whey of milk samples pasteurized at 68 °C for 10 min were analysed.

Statistical analysis

The effect of pressure and temperature and their interactions on all parameters was studied by multifactor analysis of variance. The differences between the means resulting from each pressure/temperature combination were tested by the Duncan's test ($P<0{\cdot}05$). The relationship between the various variables was investigated by regression analysis. The estimated correlations were considered statistically significant at $P<0{\cdot}01$. The software Statgraphics Plus for Windows v. 2.1 (1995, Manugistics, Inc., Rockville, Maryland 20852, USA) was used.

Results

High pressure and plasmin system

The effect of various HP conditions on total plasmin activity (plasmin plus plasminogen-derived) is presented in Table 1. Results indicated that transfer of PL and PA from the casein micelles to milk serum occurred in pressurized milk, at 20 °C. More specifically, the percentage of PL detected in the casein fraction was 87% in the untreated

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control. The PL associated with casein in milk pressurized at 20 °C was significantly lower than that of the untreated control and ranged from 63·1% at 200 MPa to 50·3% at 650 MPa. There were small decreases up to 18% in total PL enzymatic activity, which suggests that transfer of PL from the casein to milk serum rather than reduction in enzymatic activity, is the key phenomenon for all pressures at room temperature.

In samples treated at 200 MPa and 40 or 55 °C, 36–37% of total PL activity was detected in milk serum, while the value in untreated milk was only 12·6%. (Table 1). Loss of total PL enzymatic activity (from casein plus serum fractions) never exceeded 10% in these conditions. Thus, the key phenomenon at 200 MPa and elevated temperatures (40 and 55 °C) remains transfer of PL activity from casein to serum rather than substantial reduction of enzymatic activity.

There was a synergistic effect between temperature and HP on PL activity for pressures of 450 and 650 MPa. The reduction of total PL activity, compared with the untreated controls was 66–67% at 450 and 650 MPa, when the temperature was 55 °C.

The effect of HP on PA activity is presented in Table 1. At 200 MPa, the major phenomenon was the transfer of PA activity from casein to milk serum; the extent of transfer was the same for all temperatures used. In fact, at 200 MPa the PA activity in the milk serum was 42–52% of the total activity, which was 4–5 times higher than that of the untreated control. At 450 and 650 MPa, the PA activity in the serum was 37·5–50% of the total activity, indicating that transfer of PA also occurs at higher pressures. A decrease in the total PA activity in casein and serum was observed for the processing at 450 MPa. More specifically, the reduction at 450 and 650 MPa was 38% and 61% respectively compared with the untreated control. Moreover, the total PA activity decreased with the increase of HP at 20 °C. Significant synergistic effect of temperature was apparent only at 450 MPa but not at the 650 MPa.

Changes in cathepsin D and other protease activities in the acid whey

The assessment of cathepsin D behaviour was based on the area of its product in the RP-HPLC profiles following hydrolysis of the synthetic heptapeptide (Fig. 1, Table 2). The profile was rather complex, since the heptapeptide serves as the substrate for protease activities other than cathepsin D under the conditions of the assay (O'Driscoll et al. 1999; Larsen et al. 2000). The peak area of cathepsin D product was affected significantly only by pressure ($P<0·05$). The same was true for the area of peak 2, whereas peaks 1 and 3 were significantly affected ($P<0·05$) by pressure and temperature.

For the interpretation of the peaks, the profiles of the control samples and of the assays with commercial enzymes and protease inhibitors were taken into consideration. The elution time of cathepsin D product (P) was verified by analysing an hydrolysate of the substrate with commercial cathepsin D. Peak P was the only peak of the profiles that was inhibited by pepstatin A, indicating that there was no other aspartyl protease derived activity under the assay conditions. Cathepsin D activity (Table 2) was affected by HP but to lesser extent than PL. There was a significant decrease of activity caused by 650 MPa at 40 and 55 °C: at 650 MPa/55 °C the residual activity was half of that of the untreated control. Pasteurization did not affect the area of cathepsin D product significantly.

Peak 1 was not totally inhibited by the inhibitors used in this study. However, it was partially inhibited by chymostatin, which apart from cysteine protease, is also a serine protease inhibitor. The addition of E-64, which inhibits cysteine protease activity and does not inhibit serine protease, did not affect the area of this peak. Therefore, it is reasonable to suggest that this peak does not correspond to cysteine protease activity; this peak is probably related to various other protease activities present in the whey. Pasteurization inhibited this type of activity (Table 2) and pressures >450 MPa decreased it significantly ($P<0·05$).

Peak 2 (Fig. 1) resulted from the action of commercial cathepsin B on the substrate and it was inhibited by both chymostatin and E-64. It is likely that it is a product of cathepsin B activity, a major cysteine protease originating from the lysosomes of milk somatic cells (Magboul et al. 2001; Kelly et al. 2006). Interestingly, at 650 Mpa/55 °C, when the area of cathepsin D product was half the initial, the area of the cathepsin B product was double that of the control. Pasteurization did not affect significantly the activity. Magboul et al. (2001) suggested that more than 20% of cathepsin B activity survives following HTST pasteurization.

The inhibition of peak 3 (Fig. 1) by chymostatin and E-64 indicated that this peak could be related to cysteine protease activity other than cathepsin B. This activity was absent from pasteurized whey and was negligible after treatment at 650 MPa/55 °C combinations. Therefore, both peaks 1 and 3 were eliminated after pasteurization; 650 MPa were necessary to decrease this activity significantly at 20 °C.

Denaturation of α-la and β-lg

Processing at 200 and 450 MPa did not significantly affect the quantity of native α-la. There was a strong synergistic effect between temperature and pressure at 650 MPa on the amount of native α-la, residual native α-la ranged from 73% at 20 °C to 27% at 55 °C (data not shown).

Processing at 200 MPa resulted in a decrease in the amount of native β-lg; the decrease was not affected by temperature. The decrease of native β-lg was very intense at 450 MPa and 650 MPa. At room temperature the residual native β-lg was 25 and 10% of that of the untreated control at 450 and 650 MPa, respectively. The effect of temperature was more pronounced at pressures ⩾450 MPa (data not shown).

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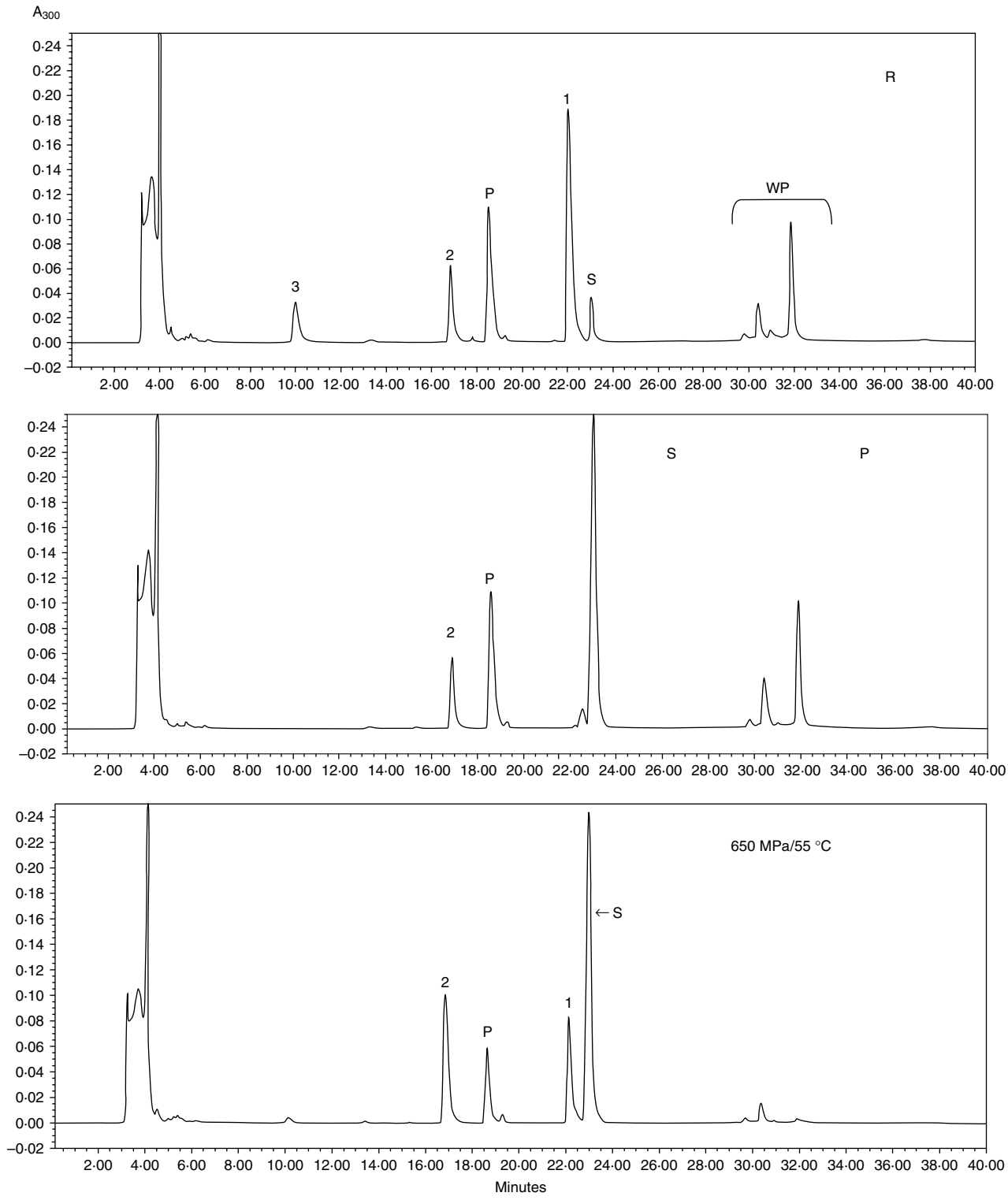

Fig. 1. Cathepsin D and other protease activities detected in the acid wheys (see text) of untreated raw, pasteurized and pressurized bovine milks (650 MPa at 55 °C) after incubation with the substrate Pro-Thr-Glu-Phe-[p-nitro-Phe]-Arg-Leu, at pH 3·2, for 12 h, at 37 °C. S: residual substrate, P: cathepsin D product. R, untreated raw bovine milk; P, pasteurized bovine milk; WP, whey proteins.

Correlation coefficients between activities of plasmin (PL), plasminogen activators (PA) cathepsin D, and various protease activities in the acid whey (Peaks 1, 2, 3 in Fig. 1) and the residual native whey proteins in bovine milk were calculated (data not shown). PL and PA activities in casein plus in serum fraction were correlated positively (n=50,

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Table 2. Effect of high pressure treatment (200, 450 and 650 MPa) at various temperatures (20, 40 and 55 °C) on various protease activities in the acid wheys, expressed in arbitrary units, i.e. chromatographic areas (means ± standard deviations). Means with different letters within each column differed significantly (Duncan's multiple comparison procedure at 95% confidence level)

Pressure (MPa)	Temperature (°C)	Cathepsin D product (P)†	Substrate (S)†	Peak 1†	Peak 2†	Peak 3†
0	Untreated, raw milk	$1{\cdot}635^{c,d} \pm 0{\cdot}144$	$0{\cdot}376^{a} \pm 0{\cdot}004$	$2{\cdot}874^{c,d} \pm 0{\cdot}293$	$0{\cdot}877^{a} \pm 0{\cdot}203$	$0{\cdot}526^{c} \pm 0{\cdot}253$
	Pasteurized milk	$1{\cdot}618^{c,d} \pm 0{\cdot}090$	$4{\cdot}129^{g} \pm 2{\cdot}78$		$0{\cdot}646^{a} \pm 0{\cdot}318$	
200	20	$1{\cdot}918^{d} \pm 0{\cdot}149$	$0{\cdot}485^{a} \pm 0{\cdot}121$	$3{\cdot}102^{d} \pm 0{\cdot}159$	$0{\cdot}786^{a} \pm 0{\cdot}177$	$0{\cdot}402^{b,c} \pm 0{\cdot}094$
	40	$1{\cdot}868^{d} \pm 0{\cdot}294$	$0{\cdot}569^{a,b} \pm 0{\cdot}188$	$3{\cdot}141^{d} \pm 0{\cdot}457$	$1{\cdot}106^{a,b} \pm 0{\cdot}256$	$0{\cdot}514^{c} \pm 0{\cdot}087$
	55	$1{\cdot}876^{d} \pm 0{\cdot}383$	$0{\cdot}552^{a,b} \pm 0{\cdot}177$	$3{\cdot}115^{d} \pm 0{\cdot}563$	$0{\cdot}979^{a,b} \pm 0{\cdot}185$	$0{\cdot}433^{c} \pm 0{\cdot}040$
450	20	$1{\cdot}614^{c,d} \pm 0{\cdot}327$	$1{\cdot}199^{b,c} \pm 0{\cdot}509$	$2{\cdot}770^{c,d} \pm 0{\cdot}620$	$1{\cdot}601^{b,c} \pm 0{\cdot}403$	$0{\cdot}416^{b,c} \pm 0{\cdot}079$
	40	$1{\cdot}528^{c,d} \pm 0{\cdot}523$	$1{\cdot}238^{c,d} \pm 0{\cdot}398$	$2{\cdot}675^{c,d} \pm 0{\cdot}664$	$2{\cdot}079^{c} \pm 0{\cdot}453$	$0{\cdot}504^{c} \pm 0{\cdot}170$
	55	$1{\cdot}318^{b,c} \pm 0{\cdot}358$	$1{\cdot}835^{d,e} \pm 0{\cdot}587$	$2{\cdot}444^{c} \pm 0{\cdot}564$	$2{\cdot}099^{c} \pm 0{\cdot}480$	$0{\cdot}337^{b,c} \pm 0{\cdot}063$
650	20	$1{\cdot}319^{b,c} \pm 0{\cdot}283$	$1{\cdot}972^{e} \pm 0{\cdot}350$	$2{\cdot}320^{b,c} \pm 0{\cdot}577$	$1{\cdot}636^{b,c} \pm 0{\cdot}500$	$0{\cdot}231^{a,b} \pm 0{\cdot}090$
	40	$1{\cdot}080^{a,b} \pm 0{\cdot}268$	$2{\cdot}979^{f} \pm 0{\cdot}843$	$1{\cdot}761^{b} \pm 0{\cdot}426$	$1{\cdot}802^{c} \pm 0{\cdot}754$	$0{\cdot}149^{a} \pm 0{\cdot}020$
	55	$0{\cdot}808^{a} \pm 0{\cdot}276$	$4{\cdot}240^{g} \pm 0{\cdot}967$	$1{\cdot}047^{a} \pm 0{\cdot}562$	$1{\cdot}931^{c} \pm 0{\cdot}871$	$0{\cdot}063^{a} \pm 0{\cdot}010$

† for definition of the various peaks see Fig. 1 and text

$P<0{\cdot}01$) with residual native whey proteins, especially β-lg (r = 0·742 and r = 0·836 for native α-la and β-lg respectively). There was a positive correlation ($n=40$, $P<0{\cdot}01$), between cathepsin D product and residual native whey proteins (r = 0·742 and r = 0·777 for native α-la and β-lg respectively). This was also true for peaks 1 and 3. On the contrary, peak 2 related to cathepsin B activity, was negatively correlated ($n=40$, $P<0{\cdot}01$) with major native whey proteins (r = −0·451 and r = −0·715 for native α-la and β-lg respectively).

Discussion

The first finding emerging from the present study is that HP affected PL and PA in bovine milk by at least two different mechanisms. The first mechanism involved transfer of both enzymes from the casein to the milk serum fraction, reported also by Garía-Risco et al. (2003). Several studies have documented that PL and PA are bound to the casein micelles in bovine milk (White et al. 1995; Ismail et al. 2006). This transfer was very apparent essentially in all combinations of temperature and pressure. The most logical explanation for this observation is the well known disruption of casein micelles caused by HP described by Needs et al. (2000) and Huppertz et al. (2004b). The second mechanism in which HP affects PL and PA involved decreases in activity of both enzymes. The extent of decrease was low at 200 MPa but much higher at the higher pressures. There were strong synergistic effects between temperature and time on residual PL activity for 450 and 650 MPa pressures and on PA for the 450 MPa. This is consistent with the findings of previous studies by García-Risco et al. (2000) and Borda et al. (2004). Transfer of PA and PL from casein to milk serum may have a profound effect on milk quality during storage and on cheese manufacturing and ripening. HP is known to induce disruption of caseins micelles resulting in an increased susceptibility of casein to plasmin. On the other hand, the decrease of both PA and PL activities caused by HP and their transfer to milk serum may remove both enzymes away from their natural substrates in milk. Furthermore, serum liberated PL and PA are susceptible to the action of inhibitors present in the serum fraction.

Due to the strong correlation between PL and PA and the residual whey proteins, it is reasonable to suggest that the decrease in enzymatic activity may be related to the increase of whey protein denaturation. Our findings are consistent with those of Borda et al. (2004) and Scollard et al. (2000a), who studied the inactivation kinetics of plasmin in phosphate buffer (pH 6·6) and with the reports of Scollard et al. (2000b), García-Risco et al. (2003) and Huppertz et al. (2004c), with regard to the application of HP to milk. According to their results, whey proteins inhibit plasmin activity in milk serum, because the presence of β-lg greatly destabilizes the enzyme under HP treatments, due to thiol-disulphide interactions of disulphide bonds in the plasmin molecule, similarly to heat inactivation. The findings of the present study regarding the denaturation of major WP were for the most part consistent with other reports (López-Fandiño et al. 1996; Gaucheron et al. 1997; García-Risco et al. 2000; Scollard et al. 2000b; Huppertz et al. 2004a, b; Hinrichs & Rademacher, 2005), although higher denaturation levels have been reported for α-la at 400 MPa. Overall, reductions in plasmin activity and transfer of PL and PA to milk serum are expected to have a positive effect on quality of milk during storage and on cheese yield. Reductions in PA plus PL activity may delay ripening in some cheese varieties.

The second novel finding of the present study is that cathepsin D activity is relatively baroresistant. Cathepsin D activity is also resistant to pasteurization. This enzyme is

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capable of at least partially surviving 3 cycles of pasteurization at 72 °C for 15 s and it is completely inactivated in milk at 70 °C for 10 min (Hurley et al. 2000b; Larsen et al. 2000; Hayes et al. 2001). Moreover, other protease activities in the acid whey of pressurized milks revealed under the conditions of this assay were in general relatively baroresistant. According to the results, peak 1 does not result from cysteine protease activity and it is attributed to various protease activities in the acid whey (O'Driscoll et al. 1999), characterized as ''unidentified milk protease'' (UMP) by Larsen et al. (2006). Using the same substrate with the present study, they reported activity about twice that of cathepsin D activity that is consistent with the present results. The changes of the area of peak 1 under different pressure/temperature conditions parallel those of cathepsin D product. The opposite is true for the area of peak 2 (Fig. 1) related to cathepsin B activity, which exhibits a weak activity under the pH conditions of the assay (O'Driscoll et al. 1999; Magboul et al. 2001). A possible explanation could be that the progressive inactivation of cathepsin D made the substrate accessible to cathepsin B action. However, this trend could be also a result of the complex inter-relationships and control mechanisms which control lysosomal proteases, as concluded by Kelly et al. (2006). Peak 3 was related to cysteine protease activity other than cathepsin B. There are at least 6 known lysosomal cysteine proteases (cathepsins), apart from cathepsin B, most of them with optimum activity in the pH range 3·0–6·0 (Magboul et al. 2001). The significant positive correlation between cathepsin D product and residual native whey proteins indicated that cathepsin D and the enzymes corresponding to peaks 1 and 3 could take part in thiol-disulphide interchange with pressure denatured β-lg as happens with plasmin.

In conclusion, HP caused significant changes in the distribution and activity of the indigenous enzymes, such as PL, PA, cathepsin D, and induced whey protein denaturation in bovine milk. Reductions in activity of indigenous enzymes present in milk is expected to have a profound effect on quality of fluid milk with extended self life and of cheese and yoghurt yield and quality.

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