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High pressure homogenisation of milk (b) effects on indigenous enzymatic activity

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The objective of this study was to determine the effect of high pressure homogenisation (HPH) on alkaline phosphatase activity and plasmin and plasminogen-derived activities in raw whole bovine milk. Milk (approximately 4% fat) was treated by two-stage conventional homogenisation (18 MPa) or single or two-stage HPH at 50, 100, 150 or 200 MPa. Inactivation of plasmin and plasminogen-derived activities was evident in conventionally homogenised samples, and increased as HPH pressure increased. Two-stage HPH reduced both activities to a greater extent than single-stage HPH. Milk inlet temperature had a significant effect on residual plasmin and plasminogen activities of HPH-treated milk samples, especially those treated at 50 MPa. Inactivation of plasmin and plasminogen on HPH-treatment (150 MPa) of milk samples of varying fat contents (0–10%) was also investigated; there was a curvilinear relationship between residual plasmin and plasminogen-derived activities and fat content in the ranges 0–2% and 0–4%, respectively, with little additional inactivation at higher fat contents. Thus, indigenous proteolytic activity of milk is clearly affected by HPH. However, all homogenised milk samples retained active alkaline phosphatase, indicating that thermal conditions during HPH did not equate to that of conventional high temperature short time pasteurisation, and that the wide range of forces experienced by milk during HPH treatment does not inactivate the latter enzyme.

Keywords: High pressure homogenisation, milk, plasmin, alkaline phosphatase.

In the last number of years, many new food-processing technologies have been studied for possible advantages over conventional techniques, due to continuing demand for products with improved quality and safety. High pressure homogenization (HPH) is one of the most recent of these new processing techniques. Fluids treated by HPH are subjected to a wide range of forces, such as turbulence, shear and cavitation (Walstra, 1983; Floury et al. 2000) and large temperature increases (Floury et al. 2000), some of which may be due to adiabatic heating. Still in its infancy, very little published data is available regarding the effects of this novel processing technology on liquid foods, such as milk, and its constituents, properties or enzymes.

Plasmin, the major proteolytic enzyme in raw milk, is present at levels in the region of 0·3 mg/l (Bastian & Brown, 1996). Its activity in dairy systems cannot be ignored because many of its effects can be detrimental to processed dairy products, as degradation of casein prior to, or during, storage of dairy products can result in poor product quality. Furthermore, due to its high heat stability, plasmin can alter the quality of heat-processed dairy products *via* protein degradation. For example, Enright et al. (1999) linked age gelation of UHT milk to residual active plasmin. Thus, regulation and control of its activity in fluid milk prior to processing is essential in order to avoid a product with substandard quality. However, the activity of plasmin in some cheeses subsequent to milk coagulation is essential for development of desired characteristics (Farkye & Landkammer, 1992).

Alkaline phosphatase is associated with the milk fat globule membrane (Andrews, 1991) and is inactivated by conventional high temperature short time pasteurisation (72 °C for 15 sec). Milk processed in this manner will give a negative phosphatase reaction, which is widely used as an indicator that vegetative pathogenic microorganisms in milk have been killed.

The objective of this work was to determine the effect of single or two-stage HPH on the indigenous milk enzymes, plasmin and alkaline phosphatase.

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Materials and Methods

High pressure homogenisation (HPH) of milk

Raw whole bovine milk samples were homogenised at conventional or high pressures, as described by Hayes & Kelly (2003).

Determination of plasmin and plasminogen-derived activities

Plasmin and plasminogen-derived activities in milk were assayed using the method of Richardson & Pearce (1981), which measures the rate of cleavage of a non-flourescent substrate, *N*-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (Sigma Chemical Co, St. Louis, MO 63103, USA), to a fluorescent product. The rate of increase in fluorescence intensity during the assay is proportional to the quantity of plasmin or plasminogen-derived activity present.

Determination of alkaline phosphatase activity

Alkaline phosphatase activity in all milk samples was determined using a Fluorophos (model FLM 200, Advanced Instruments Inc. Needham Heights, Massachusetts). In this assay, residual alkaline phosphatase in milk hydrolyses a non-fluorescent substrate into a highly fluorescent product, the rate of release of which is used to determine if alkaline phosphatase activity is present in milk (International Dairy Federation, 1992). This assay yields a positive or negative result for enzyme activity, where a negative result indicates an extent of inactivation consistent with heat treatment at commercial high-temperature/short-time (HTST) pasteurisation conditions.

Urea-polyacrylamide gel electrophoresis

Control and homogenised milk samples with added sodium azide (0·5 g/l) were incubated at 37 °C for 7 days. Aliquots were taken throughout this period (at 0 and 7 d) for urea-PAGE analysis. Urea-PAGE samples were prepared by dilution of incubated milk 1 : 4 with single strength electrophoresis sample buffer (McSweeney et al. 1993) before analysis by electrophoresis (Andrews, 1983). Gels were stained directly by the method of Blakesley & Boezi (1977).

Compositional analysis

Compositional analysis of milk samples was determined as described by Hayes & Kelly (2003).

Statistical analysis

Statistical analyses were performed as described by Hayes & Kelly (2003).

Results and Discussions

Effects of HPH treatment on alkaline phosphatase activity in milk

Positive alkaline phosphatase results were obtained for milk samples subjected to single or two-stage HPH (50–200 MPa) with inlet temperatures of 6–9 °C; thus, the activity of this enzyme in all samples tested fell outside the upper detection range of the instrument used (Fluorophos FLM 200). These results suggest that HPH treatment of raw whole milk (⩽200 MPa) does not equate to the thermal treatment applied to milk during HTST pasteurisation. Furthermore, alkaline phosphatase is not totally inactivated by the high shear forces encountered in the process. However, since the assay for this enzyme was only of a qualitative nature, partial inactivation of this enzyme by homogenisation may have been a possibility. Alkaline phosphatase is also quite resistant to inactivation by high pressure (HP), with no inactivation being reported by Lopez-Fandino et al. (1996) in raw milk after treatment at 400 MPa for 60 min at 20 °C, although HP treatment of milk at higher pressures and temperatures generally increases inactivation of alkaline phosphatase (Rademacher et al. 1998; Ludikhuyze et al. 2000).

Effects of homogenisation on plasmin and plasminogen-derived activities in milk

Conventionally-homogenised milk (18 MPa) consistently showed significant ($P<0·05$) decreases, by approximately 40%, in plasmin activity, compared to unhomogenised milk (Table 1). Single-stage HPH-treated samples (50 MPa) had significantly ($P<0·05$) lower plasmin activity than control samples, but significantly higher activity than samples homogenised at 18 MPa (Table 1a). Plasmin activity was significantly decreased in samples treated at ⩾150 MPa, compared with conventionally-homogenised samples (Table 1a). However, activity in samples homogenised at 200 MPa was not significantly lower than samples HPH-treated at 150 MPa.

Plasmin activities of samples treated by two-stage HPH were generally similar to those measured following single-stage HPH-treatment of milk samples (Table 1b). However, samples HPH-treated at 100 MPa had significantly lower activity than conventionally-homogenised samples. Two-stage HPH-treatment at ⩾100 MPa did not significantly further decrease plasmin activity. This indicated that, under these operating conditions, HPH-treated milk may always retain a significant proportion (∼35%) of its original plasmin activity. However, milk subjected to HPH-treatment at higher pressures and/or higher number of passes through the homogeniser may show greater inactivation of plasmin. In general, single and two-stage HPH-treatments resulted in similar extents of inactivation of plasmin.

In further experiments, milk samples were preheated prior to HPH-treatment to obtain a relatively constant outlet temperature (50±5 °C; Table 1c). In this trial, no

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Table 1. Plasmin and plasminogen-derived activities and plasminogen : plasmin ratios of control and homogenised raw whole milk samples. All results shown are means of data from 3 independent experiments

Total Homogenisation Pressure (MPa)	Primary Valve Pressure (MPa)	Secondary Valve Pressure (MPa)	T_{inlet} (°C)	T_{outlet} (°C)	ΔT (deg C)	Plasmin§	Plasminogen§	Plasminogen : plasmin ratio
(a) Effectiveness of single-stage high pressure homogenisation, compared to conventional homogenisation								
0†	0	0	—	—	—	0·089[a]	0·741[a]	8·270[a]
18‡	15	3	50·0	50·0	0·0	0·053[b]	0·366[b]	6·885[a,b]
50	50	0	6·10±0·51	26·4±0·60	20·3±0·85	0·077[c]	0·619[a]	7·974[a]
100	100	0	7·10±0·61	34·5±0·95	27·4±1·33	0·050[b]	0·277[b,c]	5·472[b,c]
150	150	0	7·60±0·41	44·4±0·46	36·7±0·70	0·039[d]	0·215[c]	5·537[b,c]
200	200	0	6·40±0·77	51·4±1·67	44·9±2·06	0·036[d]	0·170[c]	4·698[c]
P-value¶						<0·001	<0·001	<0·001
(b) Effectiveness of two-stage high pressure homogenisation								
0†	0	0	—	—	—	0·125[a]	1·128[a]	8·928[a]
18‡	15	3	50·0	50·0	0·0	0·072[b]	0·523[b,c]	7·543[a]
50	45	5	8·7±0·92	28·0±0·97	19·3±0·38	0·095[c]	0·883[a,b]	9·068[a]
100	90	10	8·9±1·12	36·4±1·02	27·5±0·21	0·043[d]	0·321[c]	7·484[a]
150	135	15	8·8±0·96	44·8±1·14	36·0±0·31	0·040[d]	0·213[c]	5·223[b]
200	180	20	9·5±1·10	53·6±1·64	44·2±1·25	0·042[d]	0·162[c]	3·847[b]
P-value¶						<0·001	<0·001	<0·001
(c) Effectiveness of prewarming prior to two-stage high pressure homogenisation								
0†	0	0	—	—	—	0·128[a]	1·172[a]	9·086[a]
18‡	15	3	50·0	50·0	0·0	0·071[b]	0·637[b]	8·814[a]
50	45	5	48·0±0·76	45·8±0·49	−2·2±0·78	0·055[b]	0·202[c]	3·579[b]
100	90	10	29·6±2·54	48·3±1·15	18·6±1·31	0·046[b]	0·141[c]	3·022[b]
150	135	15	9·80±0·36	50·6±1·11	40·8±0·76	0·048[b]	0·206[b,c]	4·170[b]
200	180	20	6·70±1·11	54·4±1·28	47·7±2·39	0·046[b]	0·156[c]	3·294[b]
P-value¶						<0·001	<0·001	<0·001

Values without common superscripts were significantly different; $P<0·05$
† Unhomogenised control
‡ Conventional homogenisation (18 MPa)
§ Plasmin and plasminogen-derived activities are presented in AMC units/ml milk
¶ *P*-value denotes the significance of treatment, as described in Material and Methods

significant differences in plasmin activities were observed with increasing HPH pressure or between HPH-treated and conventionally-homogenised samples. Plasmin activities of milk samples treated by two-stage HPH at 50 MPa (at an inlet temperature of 48 °C) or two-stage HPH at 100 MPa (at an inlet temperature of 29·6 °C) were lower than samples subjected to cold homogenisation at similar pressures but similar to samples subjected to cold HPH-treatment at 150 or 200 MPa (Table 1c). Thus, prewarming milk to >10 °C prior to HPH-treatment at <100 MPa will increase inactivation of plasmin. This may be related to the smaller fat globule sizes, and hence greater efficiency of homogenisation of milk fat, achieved in samples HPH-treated at elevated temperatures (Hayes & Kelly, 2003). However, prewarming samples before HPH-treatment at 100 MPa did not decrease residual plasmin activity below 35% of that in raw milk, again suggesting a maximum level of inactivation of this enzyme.

Generally similar trends to those observed for residual plasmin activity were evident for residual plasminogen-derived activity in all milk samples post-homogenisation. Again, conventional homogenisation decreased plasminogen-derived activity by ~50% and greater inactivation of plasminogen-derived activity was observed for samples prewarmed and HPH-treated at 50 MPa (two-stage) compared with milk homogenised cold at the same pressure (Tables 1b, c). However, residual plasminogen-derived activity decreased, although not significantly, as homogenisation pressure increased from 100–200 MPa, which was not seen for plasmin activity. Milk samples prewarmed to ~30 °C and HPH-treated at 100 MPa retained less plasminogen-derived activity than all other samples. There was no evidence of a maximum level of inactivation of plasminogen-derived activity by HPH, unlike that seen for inactivation of plasmin. Thus, raising inlet temperatures and processing pressures, or increasing the number of passes through the homogeniser, may totally inactivate plasminogen in milk.

The plasminogen : plasmin ratio of milks treated by either single- or two-stage HPH decreased as HPH pressure increased (Tables 1a, b). Ratios in samples subjected to single-stage HPH at ⩾100 MPa or two-stage HPH at

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⩾150 MPa were significantly different from that of unhomogenised control sample. Milk samples prewarmed prior to HPH treatment at 50 or 100 MPa had lower plasminogen : plasmin ratios than samples homogenised at the same pressures with an inlet temperature of ~10 °C (Table 1c). From these observed decreases in the plasminogen : plasmin ratio, it would seem reasonable to conclude that plasminogen-derived activity is inactivated to a greater extent than plasmin activity with increasing homogenisation pressure or inlet temperature. Alternatively, loss of plasminogen-derived activity may be due to its activation to plasmin, which is then inactivated by HPH, although this activation step would have to be extremely rapid.

The majority of HPH-treated samples in trials 1–3 (Tables 1a, b, c) had lower plasmin and plasminogen-derived activities than conventionally homogenised samples. Since outlet temperatures of HPH-treated samples only exceeded those of conventionally-treated samples by a few deg C at the highest processing pressure, and samples homogenised at lower pressures (50–150 MPa) had lower outlet temperatures than conventionally-treated samples, it may be concluded that the indigenous proteolytic activity of milk was reduced by the considerable level of shear and other forces, mentioned earlier, encountered during HPH-treatment.

Following treatment of skim milk under identical experimental parameters (except prewarming), no inactivation of plasmin and plasminogen-derived activities were observed (results not shown). This suggests that HPH-induced inactivation of both plasmin and plasminogen-derived activities is linked to the presence of fat in milk. Indigenous milk fat globule membrane proteins, which act as an emulsifying agent that prevents flocculation and coalescence of milk fat globules and also protect milk fat against enzymatic activity, are not present in sufficient amounts, post-homogenisation, to coat the increased fat-liquid interface present in milk. This additional interfacial area is, as a result, stabilised by adsorption of milk proteins, primarily caseins (Oortwijin et al. 1977). As a result of such adsorption, the structures of the casein micelles may be disrupted as they distribute around the surfaces of the fat globules. Consequently, fat globules in homogenised milk are coated with a heterogeneous mixture of casein micellar fragments (Dalgleish et al. 1996).

Plasmin and plasminogen in milk are mainly associated with the casein micelle (Bastian & Brown, 1996). The redistribution of casein micelles may have a destabilising influence on plasmin and plasminogen-derived activities in milk, with greater efficiency of homogenisation leading to more extensive destabilisation of enzyme activity. Casein micelles may also be damaged by the forces with which fat globules and micelles encounter one another following the primary homogenisation stage in full fat milk (since the greatest pressure drop is experienced by the liquid at this point).

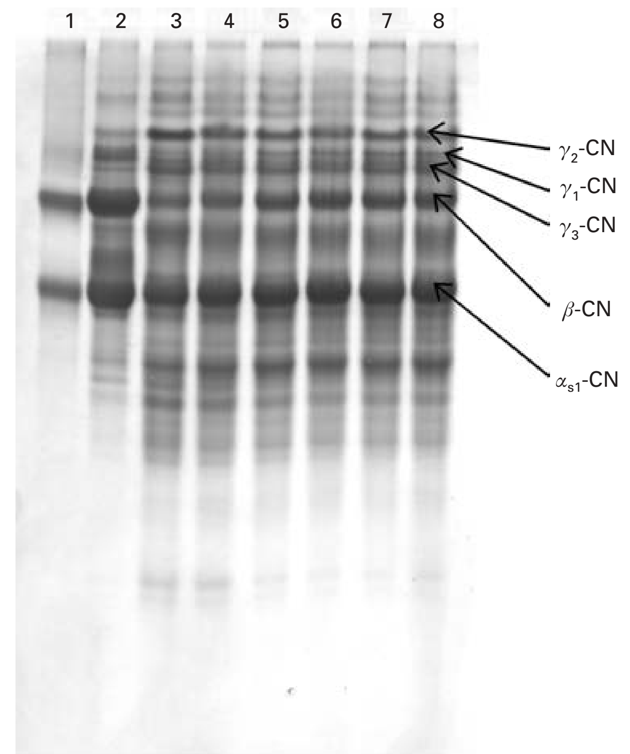

Fig. 1. Urea-polyacrylamide gel electrophoretograms (12·5 % T, 4 % C, pH 8·9) of Na caseinate (lane 1) or raw whole bovine milk after 0 or 7 d of incubation at 37 °C (lanes 2, or 3 respectively), or raw whole bovine milk homogenized at 18, 50, 100, 150 and 200 MPa after incubation at 37 °C for 7 d (lanes 4–8, respectively).

Effects of HPH treatment on proteolysis in milk

Proteolytic activity in milk was further investigated by incubating control and homogenised milk samples at 37 °C for 7 days. Urea polyacrylamide gel electrophoretograms (PAGE) of incubated milk samples are shown in Fig. 1. Untreated milk incubated for 7 d (lane 3) showed a considerable decrease in residual β-casein, due to plasmin action, during incubation. Conventionally-homogenised milk sample (lane 4) had slightly more residual intact β-casein after 7 d incubation than the control sample. However, HPH-treated milk samples, incubated for 7 d (Lanes 4–8), clearly showed decreased production of γ_2-casein and a number of fast moving proteolysis products and increased residual β-casein, relative to raw milk, as homogenisation pressure increased to 200 MPa. This confirms the decreased assayed plasmin activity following homogenisation (Tables 1a, b, c). However, the sharp initial drop in plasmin activity observed using the coumarin peptide method in conventionally homogenised milk (18 MPa) compared with both raw milk and milk homogenised at 50 MPa was not as evident on urea-PAGE gels.

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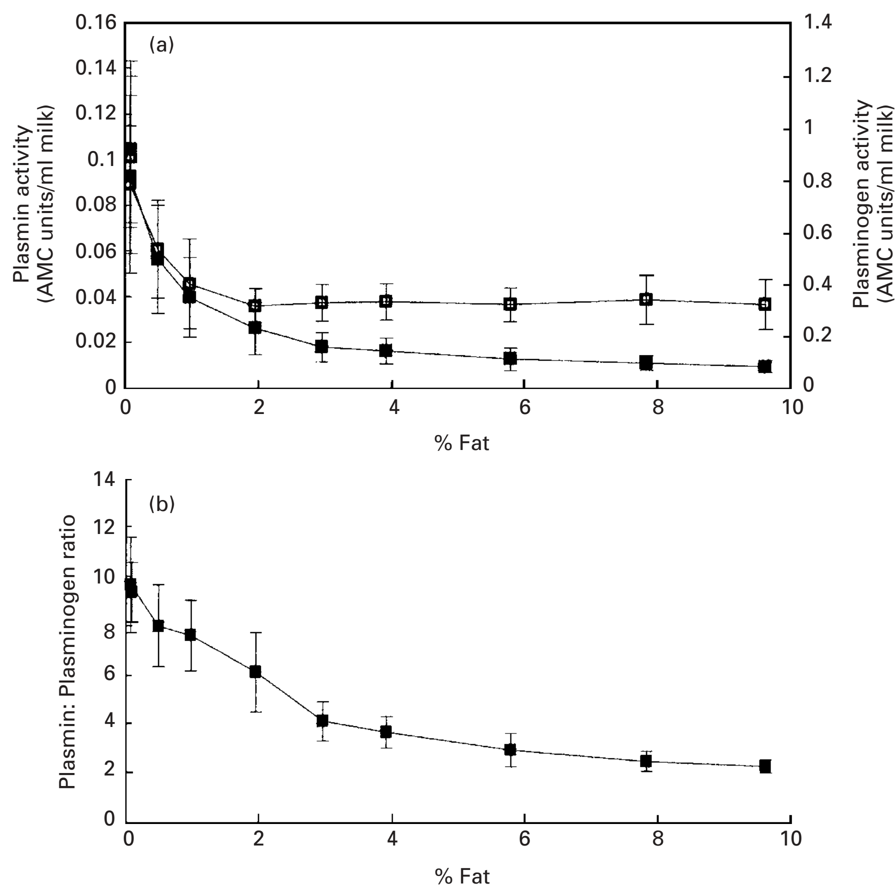

Fig. 2. (a) Plasmin (–□–) and plasminogen-derived (–■–) activities and (b) plasminogen : plasmin ratios of raw whole bovine milk samples of varying fat content treated by high pressure homogenisation at 150 MPa with inlet temperatures of 13–15 °C. Results shown are mean ± standard deviation of triplicate trials on individual milk samples.

Effects of milk fat content on plasmin and plasminogen-derived activities following HPH treatment

When milk samples with a range of fat contents (0–10%) were HPH-treated at 150 MPa, plasmin activity decreased in a curvilinear fashion, to approximately 40% residual activity, as milk fat content increased from 0–2% (Fig. 2a). No further inactivation of plasmin activity was evident at higher % milk fat, providing further evidence of an apparent limit to the maximum extent of inactivation of plasmin activity in milk by HPH.

Plasminogen-derived activities also decreased as milk fat content increased from 0–10% (Fig. 2a) with the majority of the decrease in activity being evident in the range 0–4% fat. Further decreases at higher fat contents were minimal. However, as for milk samples (~4% fat) homogenised at 0–200 MPa (Tables 1a, b, c) there was no evidence of a limit to the extent of inactivation of plasminogen-derived activity in milk following HPH treatment. The plasminogen : plasmin ratio of milk samples with varying fat content (0–10%) HPH treated at 150 MPa decreased with increasing fat content (Fig. 2b), again suggesting differences in the rates of inactivation of enzyme and proenzyme by HPH treatment.

Possible mechanisms and significance of effects of HPH treatment on indigenous proteolytic activity in milk

High pressure (HP) treatment of milk at pressures >300 MPa was observed by Scollard et al. (2000) to reduce rates of proteolysis, compared to untreated milk. The same study also reported that HP-treatment of milk at 600 MPa for 30 min was necessary to reduce plasmin activity in milk to ~40% of original activity, while treatment for 10 min at the same pressure had an equivalent inactivation effect on plasminogen-derived activity. Thus, plasminogen-derived activity has a greater sensitivity to HP-treatment than plasmin, similar to results observed in this study for HPH-treatment.

In this study, two-stage HPH of milk at 100 MPa resulted in significant inactivation of both plasmin and plasminogen-derived activities. Thus, the process of HPH

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considerably enhances inactivation of both forms of this enzyme compared to batch hydrostatic pressurization. This enhancement in HPH-treated milk is possibly due to pressure-induced physical forces not present in HP-treatment, such as shear, turbulence and cavitation.

Plasmin has a relatively high heat stability, with heating to 80 °C for 10 min at pH 7·0 being required for complete thermal inactivation of the purified enzyme (Kaminogawa et al. 1972). Metwalli et al. (1998) reported first-order kinetics for irreversible inactivation of plasmin, with inactivation starting at temperatures above 65 °C. Renaturation/refolding of plasmin, which completely restored activity, was observed after heating at 54 and 65 °C for 13 h and 10 min, respectively, and subsequent cooling to 37 °C (Metwalli et al. 1998). Milk temperatures in the present study never exceeded 54 °C, and thus inactivation of plasmin must instead be due to the limited thermal energy increase, combined with some or all of the physical forces experienced by the milk during the process of homogenisation.

Alichanidis et al. (1986) reported that the thermal inactivation kinetics of bovine plasmin and plasminogen in skim milk were similar. Thus, as for plasmin, possible inactivation of plasminogen during HPH-treatment of milk would probably be due to a combination of induced forces and limited temperature increase, with plasmin apparently being less sensitive than its proenzyme.

Heating plasmin in the presence of proteins which have free sulphydryl groups that become exposed on denaturation by heat or other agents (e.g. β-lactoglobulin) can prevent refolding of plasmin by formation, through sulphydryl-disulphide interchange reactions, of a complex between denatured β-lg and the unfolded enzyme, thereby accelerating its thermal inactivation (Alichanidis et al. 1986). Hayes & Kelly (2003) did not observe whey protein denaturation in milk samples HPH-treated at pressures $\leqslant$200 MPa, suggesting that inactivation of plasmin observed in this study was not due to its interactions with denatured β-lg and thus must be due to some other mechanism, possibly the physical forces experienced by the milk during HPH-processing.

Manji et al. (1986) observed decreases in plasminogen-derived activity and increases in plasmin activity in directly- and indirectly-heated UHT milk during 182 d storage. Addition of plasminogen or plasmin to UHT milk can enhance gelation (Kohlmann et al. 1988; Enright et al. 1999). Thus, it has been concluded that plasminogen activators present in milk may be linked to age gelation of UHT milk. The results of this work indicate that there may be advantages in HPH treatment of milk for use in UHT processing as the combination of both processes may inactivate plasminogen-derived activity totally and thus delay age gelation of UHT milk.

In conclusion, plasmin and plasminogen-derived activity in milk are quite susceptible to inactivation by HPH. However, since HPH is a relatively new and emerging technology the consequences of the inactivation of this major indigenous milk protease for milk and dairy products thus treated remains to be studied.

Further investigation of the characteristics of dairy products manufactured from HPH-treated milk is needed to establish applications of this novel processing tool in the dairy industry.

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