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Significance of frictional heating for effects of high pressure homogenisation on milk

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High pressure homogenisation (HPH) is a novel dairy processing tool, which has many effects on enzymes, microbes, fat globules and proteins in milk. The effects of HPH on milk are due to a combination of shear forces and frictional heating of the milk during processing; the relative importance of these different factors is unclear, and was the focus of this study. The effect of milk inlet temperature (in the range 10–50 °C) on residual plasmin, alkaline phosphatase, lactoperoxidase and lipase activities in raw whole bovine milk homogenised at 200 MPa was investigated. HPH caused significant heating of the milk; outlet temperature increased in a linear fashion (0·5887 °C/°C, $R^2 = 0{\cdot}9994$) with increasing inlet temperature. As milk was held for 20 s at the final temperature before cooling, samples of the same milk were heated isothermally in glass capillary tubes for the same time/temperature combinations. Inactivation profiles of alkaline phosphatase in milk were similar for isothermal heating or HPH, indicating that loss of enzyme activity was due to heating alone. Loss of plasmin and lactoperoxidase activity in HPH milk, however, was greater than that in heated milk. Large differences in residual lipase activities in milks subjected to heating or HPH were observed due to the significant increase in lipase activity in homogenised milk. Denaturation of β-lactoglobulin was more extensive following HPH than the equivalent heat treatment. Inactivation of plasmin was correlated with increasing fat/serum interfacial area but was not correlated with denaturation of β-lactoglobulin. Thus, while some effects of HPH on milk are due to thermal effects alone, many are induced by the combination of forces and heating to which the milk is exposed during HPH.

Keywords: High pressure homogenisation, milk enzymes, plasmin, lactoperoxidase, lipase, alkaline phosphatase.

Homogenisation is a very common process in the dairy industry, and indeed many other sectors of the food industry. It is routinely used in preparation of emulsions, as it reduces the size of fat globules by shear, turbulence and cavitation forces caused by the pressure difference across, and high speed collisions within, the homogenising valve. Conventional homogenisation of milk (at 20–50 MPa) thus reduces the size of fat globules, resulting in final globule diameters of <1 μm, with a concomitant increase in surface area (4–10 fold). Two-stage homogenisation, where the primary stage reduces the size of the fat globules and the secondary stage disrupts any clusters that may subsequently form, is most commonly used for milk processing.

High pressure homogenisers operate on the same design principle as conventional homogenisers (i.e., a primary valve with a needle and seat arrangement, and a secondary valve of similar arrangement operating at considerably lower pressures, but utilise far higher pressures (up to 350 MPa). This novel processing tool, increasingly used in the chemical, pharmaceutical and biochemical industries (Floury et al. 2000), is now attracting increasing interest in the food area. The forces encountered during conventional homogenisation are also present in HPH but to far greater extents (Popper & Knorr, 1990; Paquin, 1999), resulting in heating of the homogenised liquid (Floury et al. 2000; Hayes & Kelly, 2003a). The effects of HPH on milk range from microbial inactivation (Wuytack et al. 2002)

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and globule size reduction (Thiebaud et al. 2003) to inactivation of bacteriophages (Moroni et al. 2002) and enzymes (Hayes & Kelly 2003b; Hayes et al. 2004).

Indigenous enzymes in raw milk are of interest due to their use as indicators of severity of heat treatment (Andrews et al. 1987; McKellar et al. 1991), or for detection of mastitis (Kaartinen & Jensen, 1988) or their influence on dairy product flavour (Farkye & Fox, 1992; Farkye & Landkammer, 1992). Enzyme inactivation can be achieved by denaturation (classically due to heating). The objective of this study was to determine the effect of inlet temperature, at a constant homogenization pressure of 200 MPa, on inactivation of the indigenous milk enzymes plasmin, alkaline phosphatase, lactoperoxidase, and lipase during HPH. Levels of residual native β-lactoglobulin (β-lg) and fat globule size distribution of all samples were also determined. Time/temperature conditions to which the milk was exposed during HPH were also applied to milk under controlled isothermal conditions to determine whether enzyme inactivation and denaturation of β-lg during HPH were partly or entirely due to the thermal conditions generated by the process (i.e., to determine whether heat or other forces were the dominant cause of changes observed). The overall goal was to extend the understanding of the manner in which HPH affects the properties of milk, facilitating better evaluation of the potential dairy applications of this novel technology.

Materials and Methods

High pressure homogenisation (HPH) of milk

Raw whole bovine milk was obtained from a local supplier (CMP Dairies, Cork) and subjected to two-stage HPH at 200 MPa (model 'nm-GEN' 7400H, Stansted Fluid Power Ltd., Essex UK) as described by Hayes et al. (2004), at inlet temperatures of 10, 15, 20, 25, 30, 35, 40, 45 or 50 °C. The homogeniser was equilibrated at the relevant temperature by circulating water at that temperature for 30 min through the entire system. Milk samples were rapidly heated in a water bath with stirring to the target inlet temperature and then transferred to the homogeniser without holding at that temperature. Following HPH, samples were rapidly cooled in iced water and sodium azide was added (0·5 g/l) to all samples with the exception of those intended for lactoperoxidase assays, due to its adverse effect on that test. Samples were then frozen at −20 °C until analysis except those intended for fat globule size determination, which were stored at 4 °C until analysis. All results presented are means of data from duplicate analysis of samples from three independent experiments, unless otherwise stated. The temperature increase of milk during processing, as a function of inlet temperature, was ascertained in separate experiments using a digital temperature probe located at the outlet of the homogeniser, before cooling.

Determination of alkaline phosphatase, lactoperoxidase and plasmin activities

Lactoperoxidase (EC 1.11.1.7) activity in milk samples was assayed by the method of Hernández et al. (1990), using the substrate 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and H_2O_2, with modifications; samples were diluted 1 : 2 with 0·12 M-sodium phosphate buffer, pH 6·4, prior to analysis and 0·2 M-H_2O_2 (Sigma-Aldrich Ireland Ltd., Dublin, Ireland) was used. Alkaline phosphatase (EC 3.1.3.1) activity was determined as described by Ludikhuyze et al. (2000), using the substrate p-nitrophenylphosphate (Sigma-Aldrich) and plasmin activity by the method of Richardson & Pearce (1981), which measures cleavage of a non-flourescent substrate, 7-amido-4-methyl-coumarin (Sigma-Aldrich) to yield the fluorescent product 7-amido-4-methyl coumarin.

Determination of lipase activity

Two of the following assay mixtures were prepared in stoppered glass test tubes for each sample and mixed; milk (0·5 ml), 2 M-Tris-HCl, pH 9 (0·2 ml), lipoprotein (2% solution in distilled water; 0·2 ml, Sigma-Aldrich), 0·2 M-calcium chloride (0·2 ml), UHT cream (0·5 ml) (Lakeland Dairies, Killeshandra, Ireland) and distilled water (0·6 ml). One of the test tubes for each sample was then incubated at 37 °C in a shaking water bath for 2 h while the other (T_0) was immediately subjected to the extraction and titration procedure for liberated free fatty acids (FFA) of Deeth et al. (1975). In this case, diluted bromothymol blue indicator (a stock solution of 0·1% bromothymol blue indicator (Sigma-Aldrich) in isopropanol:water [6 : 4] subsequently diluted 1 : 4 with isopropanol (Merck, Darmstadt, Germany)) was used to detect the end-point of titration. FFA titration of incubated samples (T_{2h}) was also performed.

The lipase activity of milk samples, expressed as μequivalents of free fatty acid released per ml of milk per hour, was calculated according to the following equation:

$$\mu equiv/ml/h = \frac{(T_{2h} - T_0)N \times 10^3}{V \times P \times H}$$

where N is the normality of the methanolic KOH, T_0 and T_{2h} are the titration volumes (ml) for the unincubated control and incubated test samples respectively, P is the proportion of the upper layer titrated, V is the volume of milk (ml) and H is the incubation time in hours. Results were then expressed as a percentage of the untreated controls.

Other analyses

Quantification of native β-lg and α-lactalbumin (α-la) was performed as described by Huppertz et al. (2004). The size distribution of fat globules in milk samples was determined by the method of Hayes & Kelly (2003a), using

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Table 1. Typical values (from a single experiment) showing the effect of inlet temperature on outlet temperature of raw whole milk after high pressure homogenisation at 200 MPa

Milk Inlet Temp. (°C)	Milk Outlet Temp. (°C)
Control	—
10	56·4
15	59·2
20	62·2
25	65·4
30	68·3
35	71·1
40	73·6
45	77·1
50	80·0

a polydisperse optical model, a milk fat refractive index of 1·46, an absorbance of 0·00 and a buffer refractive index of 1·33 for calculation of particle size parameters. Compositional analysis of milk samples was determined as described by Hayes et al. (2004); milks from all trials were similar in composition (results not shown).

Isothermal heating experiments

Raw whole milk samples (90–100 µl) were sealed in glass capillary tubes (100 mm × 1 mm) and subjected to isothermal heat treatment at 60, 65, 70, 75 or 80 °C for 26 s. This holding time was chosen as the sum of the equilibration time of 6 s for similar glass capillary tubes (Svensson, 1977) and the estimated holding time of 20 s for the homogenised milk (Hayes et al. 2004) at the highest temperature during HPH. After heating, the tubes were immediately cooled in iced water. Samples sealed in glass capillary tubes and not subjected to isothermal heating (presented in graphs as 20 °C samples) were used as controls. After cooling, the tubes were opened and samples were frozen at −20 °C until analysis. Data presented in the paper are for a single experiment in this regard.

Results and Discussion

Effect of high pressure homogenisation (HPH) on milk temperature

Inlet and outlet temperatures of HPH samples are shown in Table 1. These data were generated by measuring the temperature at the point of exit of milk from the homogeniser, without cooling; these samples were not used for further analysis, but data shown are typical of those measured in repeated experiments. Outlet temperature increased linearly with increasing inlet temperature (0·5887 °C/°C, $R^2 = 0{\cdot}9994$). Linear temperature increases with increasing homogenisation pressure at a constant inlet temperature have previously been reported (Floury et al. 2000, 2003; Hayes & Kelly, 2003a; Hayes et al. 2004; Thiebaud et al. 2003). Enzyme inactivation and protein denaturation will be discussed in relation to HPH outlet temperature throughout this paper, as this was the temperature at which milk was held for 20 s before cooling. However, some uncertainty exists as to whether the milk reached higher temperatures transiently during the homogenisation process, due to a lack of thermocouples within the valve assembly itself.

Comparison of the effects of isothermal heating and HPH on some constituents of bovine milk

(*a*) *Alkaline phosphatase.* Alkaline phosphatase (ALP) activity in milk decreased in an apparent linear manner as HPH outlet temperature increased (Fig. 1a). Due to the range of forces and stresses to which HPH milk is exposed (Moroni et al. 2002), capillary heating experiments were performed to determine whether the observed decrease in ALP activity was partly or entirely due to the heating effect of HPH or if ALP was also susceptible to inactivation by other forces. Inactivation profiles for ALP observed from isothermal heating or HPH processing of milk were almost identical (Fig. 1a), initiation of enzyme inactivation in both cases occurring at ∼60 °C and total enzyme inactivation being achieved at ∼75 °C. This pattern of inactivation is consistent with previously-reported kinetic data for thermal inactivation of ALP (Andrews, 1992; Walstra et al. 1999). The results of this study suggest that inactivation of ALP observed following HPH was entirely due to heating of the milk during processing, as proposed by Hayes et al. (2004).

(*b*) *Lactoperoxidase.* Lactoperoxidase (LPO) activity decreased in a curvilinear manner as the outlet temperature of HPH milk increased from 56 to 68 °C; at higher temperatures, activity decreased in a linear manner (Fig. 1b). The inactivation profile of LPO obtained following isothermal heating was clearly different from that due to HPH; at all temperatures studied, there was considerably higher residual LPO activity following the former treatment compared with the latter. Isothermal inactivation of LPO occurred at >70 °C and complete inactivation was observed at 80 °C, which is consistent with reports by Davis (1963) and Kameni et al. (2002). Barrett et al. (1999) reported total inactivation of LPO on heating at 80 °C for similar time periods but also reported 15–20% loss of LPO activity when milk was heated at 70 °C for 20 s in a high-temperature short-time plate-type pasteurizer. Differences observed in residual LPO activities between those of the latter study and those of this study for isothermal heating of milk may be explained by the different methods of heating; milk in the plate pasteurizer may have had longer come-up and cooling times and thus be exposed to a greater thermal load.

Inactivation of LPO in HPH samples was observed at outlet temperatures >60 °C, with complete inactivation

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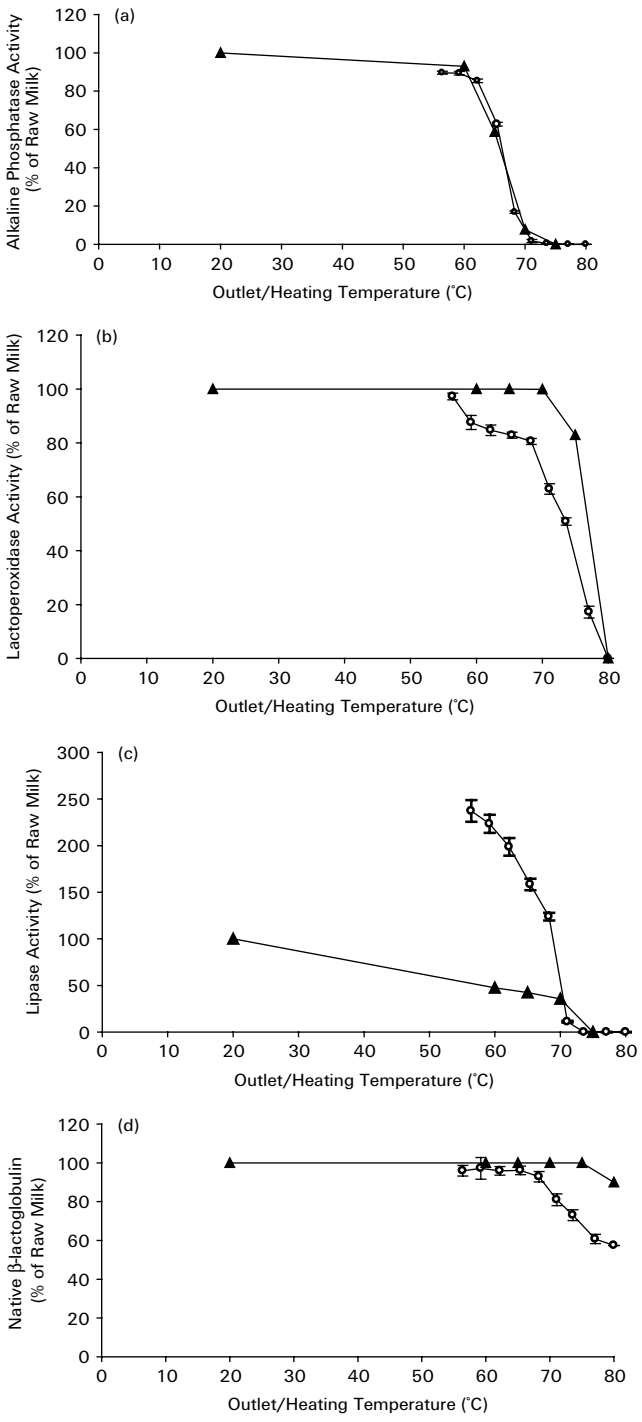

Fig. 1. Residual activities of (a) alkaline phosphatase, (b) lactoperoxidase and (c) lipase and (d) residual levels of native β-lactoglobulin in milk after isothermal heating in glass capillary tubes for 20 s at temperatures of 20–80 °C (▲, representative data from a single experiment) or high pressure homogenization at 200 MPa, with outlet temperatures in the range 56–80 °C (○, means of data from 3 independent experiments, error bars represent standard deviations of data from triplicate trials).

being observed at 80 °C. These results suggest that the many forces experienced by milk during HPH at 200 MPa other than heating partly inactivated LPO. The observed change from a curvilinear to a linear inactivation profile for LPO due to HPH at outlet temperatures of ~70 °C may be due to an increasing significance of thermal inactivation mechanisms.

(*c*) *Lipoprotein Lipase.* Lipases (EC 3.1.1.3) are enzymes which hydrolyse the glycerol esters of fatty acids at oil/water interfaces (Brockman, 1984) and in bovine milk are primarily (~80%) associated with casein micelles (Hohe et al. 1985). The action of lipase on the fat in milk (lipolysis) is undesirable due to resultant rancid flavours. Lipolysis in milk derives from two different enzymatic systems, i.e., natural milk lipase (lipoprotein lipase, LPL, EC 3.1.1.34; synthesised in mammary gland secretory cells) and microbial lipases. All lipase activity reported in milk samples in this study is assumed to be due to LPL and not bacterial lipases, due to the freshness of the milk used.

A higher lipase activity was measured in HPH samples compared with unhomogenised samples, e.g., HPH samples at an outlet temperature of ~57 °C had ~240% of control milk lipase activity (Fig. 1c). A curvilinear profile was obtained for the combined activation/inactivation of milk lipase by HPH, with greater inactivation occurring as outlet temperature increased. Total inactivation of LPL by HPH at 200 MPa was observed at outlet temperatures >71 °C. Isothermal heat treatments, as applied in capillary tubes, resulted in more conventional enzyme inactivation profiles, and were consistent with the results of Andrews et al. (1987), who reported that total inactivation of LPL occurred at 75 °C. However residual lipase activities in milk heated at temperatures between 60 and 70 °C, inclusive, reported by Andrews et al. (1987), were significantly lower than those of the present study; e.g., at 70 °C for 15 s, Andrews et al. (1987) reported 2% residual activity while we recorded a residual activity of 36%. The reason for this difference is not known.

The lipase assays were performed under optimum conditions for measuring the total LPL activity in the milks, i.e., 37 °C, pH 9·0, homogenized milk fat (in the form of [homogenised] UHT cream) as substrate, calcium added to complex the free fatty acids to limit product inhibition and lipoprotein added as a necessary cofactor for expressing the full activity of lipoprotein lipase. Assays were performed on raw milk both without homogenization (untreated control) and after HPH. For all assays, zero time controls were used; therefore, any lipolysis which had occurred in the milk prior to the beginning of the assay was taken into account, and the increase in the amount of free fatty acids during the 2-hour incubation period of the assay was taken as a measure of enzyme activity. In the case of the HPH milk, substantial lipolysis had occurred in some of the

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milk samples before the assay, suggesting very rapid action of lipase either during or immediately after the HPH treatment.

Thus, HPH did not cause more inactivation of milk lipase than the corresponding thermal treatment; on the contrary, it appeared to enhance the activity. An increase in milk lipase activity due to agitation has been previously reported for cow (Deeth & Fitz-Gerald, 1977) and goat (Jandal, 1996) milk. The mechanism for this apparent activation remains to be determined.

(*d*) *β-lactoglobulin.* The conformational state of β-lg, the most abundant whey protein (~3 g/l) in bovine milk, is highly significant, due to its marked influence on the functional properties of milk. There was a significant difference between patterns of denaturation of β-lg by heat or HPH (Fig. 1d).

In general, β-lg was quite resistant to denaturation over the temperature range used in isothermal experiments; isothermal heating in glass capillary tubes at ⩽75 °C did not denature β-lg (Fig. 1d), while heating at 80 °C denatured only ~10% of β-lg. This agrees with the findings of DeWit & Klarenbeek (1984) who reported that denaturation of β-lg occurs at temperatures >78 °C.

On HPH, while β-lg was resistant to denaturation at outlet temperatures up to ~65 °C, above this temperature, however, denaturation increased in a linear fashion with temperature. Differences observed in levels of denaturation of β-lg due to HPH or capillary heating were again possibly due to the effects of the many extreme forces associated with HPH processing. Hayes et al. (2004) also reported higher extents of denaturation of β-lg on HPH of milk than would be expected from published studies of thermal denaturation of that protein in milk.

At outlet temperatures below 65 °C, denaturation was not observed, although the magnitude of non-thermal stress to which the milk was exposed were similar to those at higher outlet temperatures; it may therefore be possible that denaturation of β-lg may be due to a synergistic effect of temperatures and shear forces. Denaturation of α-la was not observed in any of the homogenised or isothermally-heated milk samples (results not shown).

(*e*) *Milk fat globule size.* HPH of milk at 200 MPa with an outlet temperature of 56 °C (i.e., an inlet temperature of 10 °C) reduced mean fat globule diameter from 3·50 to 0·34 μm, with a further linear decrease (from 0·34 to 0·23 μm) with increasing outlet temperatures up to 70 °C; little change was observed at higher outlet temperatures (Fig. 2). Thiebaud et al. (2003) also reported decreasing milk globule sizes with increasing outlet temperature at 200 MPa. This indicates that the state of the fat (liquid or solid, or part-liquid/part-solid) prior to primary homogenisation has a significant influence on the extent of globule size reduction, despite the rapid increase in temperature of the milk at the primary valve. The disruptive forces

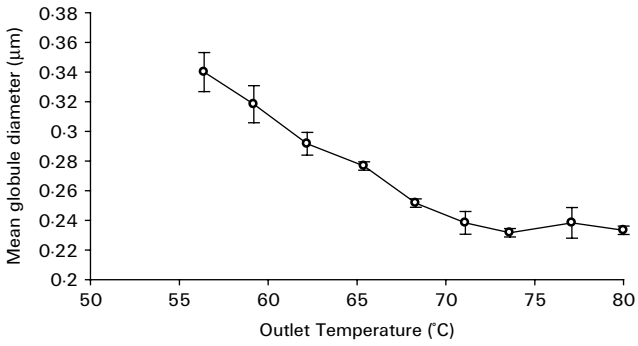

Fig. 2. Effect of HPH of milk at outlet temperatures of 56–80 °C on mean fat globule diameter.

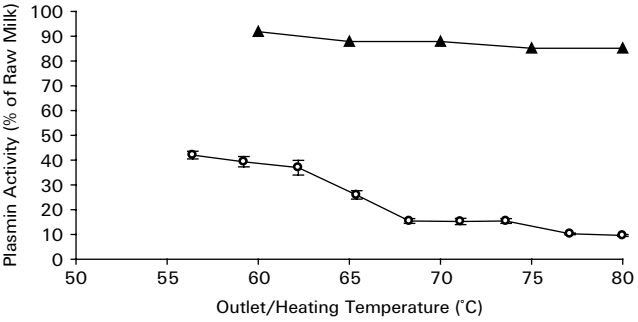

Fig. 3. Residual plasmin activity in milk after isothermal heating in glass capillary tubes for 20 s at 60–80 °C (▲, representative data from a single experiment) or high pressure homogenization at 200 MPa, with outlet temperatures in the range 56–80 °C (○, means of data from 3 independent experiments, error bars represent standard deviations of data from triplicate trials).

applied to droplets in this experimental work by HPH at 200 MPa obviously had the greatest effect on globule size reduction when all the milk fat was in a liquid state. No reduction in size was observed with increasing inlet temperature above 35 °C (resulting outlet temperatures of >70 °C), i.e., beyond the melting point of milk fat. Thus, despite the significant forces experienced by the milk during HPH and the resulting temperature increase, the presence of solid fat, i.e. the inlet temperature of milk, was apparently the key determinant of resulting globule size. Furthermore, there was a smallest globule size that could be produced, which may be due to a lack of sufficient material in milk to cover greater areas of exposed fat-liquid interface (Hayes et al. 2004), or due to the fact that, once the fat is all liquid, further increasing temperature has little additional effect.

(*f*) *Plasmin.* HPH or isothermal heating of milk at similar time/temperature combinations clearly resulted in different extents of inactivation of plasmin in milk, HPH causing much greater inactivation (Fig. 3). Isothermal heating of milk at 65 or 80 °C inactivated 12 or 15% of

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plasmin activity, respectively. However, HPH of milk at 200 MPa at similar outlet temperatures inactivated 74 or 90%, respectively, of the original plasmin activity, with milk that reached temperatures between these two extremes generally showing increased inactivation with increasing temperature. Inactivation of plasmin by HPH has also been observed in other studies (Hayes & Kelly, 2003b; Hayes et al. 2004). The results of the current study confirm, for the first time, that inactivation of this enzyme in HPH milk was largely due to some mechanism other than heating during processing. Inactivation was observed at temperatures lower than those reportedly needed for thermal inactivation of plasmin (Metwalli et al. 1998).

It has been previously proposed that inactivation of plasmin during HPH is linked to the disruption and or absorption of caseins to the oil/serum interface (Hayes & Kelly, 2003b), as it is widely recognised that plasmin in milk is mainly associated with the casein micelles (Bastian & Brown, 1996). The results of this study support this hypothesis. The relatively small decrease in plasmin activity observed in HPH milks at outlet temperatures in the range 68–80 °C may be linked to the fact that little or no further increases in the fat/serum interfacial area occurred in this range, as adjudged from fat globule size measurements (Fig. 2) and hence there would be little further change in the amount of adsorbed caseins. The relationship between fat globule surface area (square of mean globule radius) and residual plasmin activity is shown in Fig. 4a. As the surface area of average globule size decreased (e.g., total surface area increased), plasmin activity decreased; this relationship was approximately linear (R^2=0·8759). Thus, disruption/adsorption of casein micelles in milk during HPH may indeed be the primary reason for inactivation of plasmin.

An established mechanism of plasmin inactivation on heating of milk is sulphydryl-disulphide (SH/SS) interchange reactions between the unfolded enzyme and denatured β-lg (Alichanidis et al. 1986). There was no clear relationship between residual plasmin activity and levels of native β-lg (Fig. 4b); extensive plasmin inactivation occurred while levels of native β-lg were constant and *vice-versa*. Thus, loss of plasmin activity observed in this study due to HPH was not well correlated with denaturation of β-lg, further supporting a different mechanism of inactivation than that believed to occur on thermal treatment.

Overall, it appears that, at the very most, the heating aspect of HPH may have had a small synergistic effect on inactivation of plasmin with the major inactivating mechanism, i.e., adsorption of caseins to the fat/serum interface.

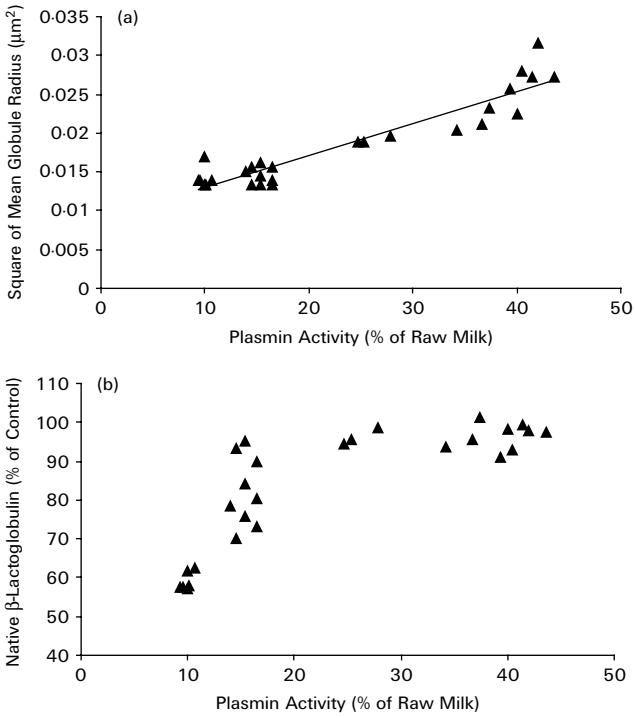

Fig. 4. Relationships between residual plasmin activity and (a) square of mean milk fat globule radius (surface area) and (b) residual levels of native β-lactoglobulin in HPH milk processed at inlet temperatures of 10–50 °C. The line on panel (a) is the best fit line for a linear correlation between the parameters.

Conclusions

Among the 60 or more indigenous enzymes in milk (Farkye & Imafidon, 1995), LPO and ALP are the two enzymes most frequently used as indicators of the thermal history of milk (Sharma et al. 2000; Pellegrino et al. 1996). ALP was inactivated by the thermal conditions generated during HPH alone while both the thermal conditions and other forces inherent in HPH inactivated LPO. Thus, ALP can be used as an effective thermal history indicator of milk processed in such a manner but, since residual LPO activity levels in HPH milks were not equivalent to those resulting from conventional isothermal heating, it is unlikely to be useful as a thermal history indicator for HPH milk. ALP is a dimer of two identical subunits (Shakeel-ur-Rehman et al. 2003) while lactoperoxidase consists of a single polypeptide chain (Pruitt, 2003). One may expect the shear and other stresses associated with HPH to have more of an effect on the more complex structure of ALP. However, the opposite was observed in this study, with the single-chain structure of LPO being more susceptible to inactivation by the extreme forces associated with HPH.

Plasmin inactivation was very extensive in HPH milks; a link with adsorption of caseins to the increasing fat/serum interface is an apparent reason for this phenomenon. Activation/inactivation of LPL by the combination of heat and other stresses experienced by milk during HPH is very complex. Further study is needed in this area to fully understand the mechanisms involved.

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