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Proteolysis of milk heated at high temperatures by native enzymes analysed by trinitrobenzene sulphonic acid (TNBS) method

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Native enzymes play a significant role in proteolysis of milk during storage. This is significant for heat resistant native enzymes. Plasmin is one of the most heat resistant enzymes found in milk. It has been reported to survive several heat treatments, causing spoilage during storage. The aim of this study was to assess susceptibility of high temperature heated milk to proteolysis by native enzymes. The trinitrobenzene sulphonic acid (TNBS) method was used for this purpose. Raw milk was heated at 110, 120, 130,142°C for 2 s and 85°C for 15 s and milk processed at low temperature (85°C /15s) was selected to mimic pasteurisation. TNBS method confirmed that raw milk and milk processed at 85°C /15s were the most proteolysed, whereas treatment of milk at high temperatures (110, 120, 130 and 142°C for 2 s) inactivated the native enzymes. It may thus be concluded that high temperature processing positively affects proteolysis by lowering its susceptibility to spoilage during storage.

Key words: Plasmin, proteolysis, trinitrobenzene sulphonic acid (TNBS), heat treatment, isoelectric precipitation.

INTRODUCTION

Proteolysis in milk may be a positive or negative attribute depending on the processing purposes and conditions (Nielsen, 2002). It is important for cheese ripening through development of desirable changes in flavour and texture, but is undesirable when it results in gelation and bitterness as observed in ultra high temperature (UHT) milk (Datta and Deeth, 2001). It is caused by either bacterial enzymes or naturally occurring enzymes of which plasmin is significant (Grufferty and Fox, 1988). Plasmin has a high heat resistance (Metwali et al., 1998) and can also retain activity in UHT milk (Newstead et al., 2006). Active protease from either source can persist in UHT milk during storage, which can be up to 9 months at ambient temperature (Button et al., 2011). Even low residual or reactivated enzymatic activities can cause serious defects in UHT milk, which is approximately twice as sensitive as pasteurised milk to the action of proteolytic enzymes (McKeller, 1981). Desirable changes taking place during UHT processing of milk such as destruction of microorganisms and inactivation of enzymes occur, while undesirable effects such as browning, loss of nutrients, sedimentation, fat separation,

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cooked flavor also take place. Gelation of UHT milk during storage (age gelation) is a major factor limiting its shelf life (Chavan et al., 2011).

Ultra-high temperature processing refers to heating of a food product at a high temperature for a short time so as to extend its shelf life at room temperature. In milk, various temperature-time combinations are used to achieve this goal by heating either directly such as at 142°C for 4 s (Snoeren et al., 1979) or 138°C for 2 to 5 s (Samel et al*.,* 1971), or directly and indirectly, respectively at 142°C for 5 s and 145°C for 3 s (Manji et al., 1986). These heating temperature and times have influence on casein stability, interactions with whey proteins and hence milk proteolysis. These heating temperature and times have influence on casein stability, interactions with whey proteins and hence milk proteolysis. Generally, heat labile enzymes are inactivated by unfolding followed by molecular scrambling, whereas heat resistant enzymes are inactivated by covalent modification such as cystine cross-links and deamination of asparagines and glutamine residues. Proteinases, lipases and phospholipase (from psychrophic bacteria, mostly *Pseudomonas*) are stable at room temperature but survive pasteurisation and UHT treatment (Chavan et al., 2011).

In a study to investigate the effect of raw milk quality and UHT temperature (145 or 150°C for 4 s) on proteolysis in UHT milk, it was found that proteolysis milk samples increased during storage, mostly from samples produced from low-quality milk at 145°C (Topcu et al., 2006). The same study also found that bitterness in UHT milk processed from low-quality milk at 145°C increased during storage whereas gelation occurred in that milk after 150 days. Bagliniere et al. (2013) found that milk proteolysis leading to milk destabilisation in UHT milk caused by *Pseudomonas fluorescens* was due to the activity of Apr X enzyme.

Homogenisation of milk prior to UHT treatment led to lower levels of enzymes that contributed to proteolysis during storage. However, the same study found that homogenisation induced modifications in proteins that may have played a role in their susceptibility to proteolytic attack- it brought about the attachment of caseins and whey proteins to the fat globule membrane and gave rise to smaller micellar particles not easily sedmentable by centrifugation (Garcia-Risco et al., 2001).

Other researchers have found that a high proportion of β -lactoglobulin and κ -casein (β-κ) complex present at the surface of casein micelles (favoured by high concentrations of denatured β-Lg) and by indirect heating, reduce the degree of proteolysis and gelation by inhibiting the access of proteases to caseins (Garcia-Risco et al., 1999, 2000; Enright and Kelly, 1999).

The aim of the current study was to investigate susceptibility of milk processed at various temperature-time profiles to proteolysis by native enzymes during storage, with raw milk as a control using TNBS method.

MATERIALS AND METHODS

Unless otherwise stated, all materials were from Fisher (Fisher Scientific UK Ltd, Leicestershire, UK.). Raw milk was obtained from the Centre of Dairy Research (CEDAR), University of Reading, UK. It was processed on an APV junior UHT plate heat exchanger (APV, Crawley, UK), with two stages of heating involving hot water (80°C) and steam (112 to 142°C) as described (Browning et al. 2001). A constant flow rate was used, giving a residence time of 2 s in the holding section at 110, 120, 130 and 142°C but 15 s at 85°C. Homogenisation took place between the heating stages at about 170 bars. These temperature-times combinations were selected based on studies of plasmin inactivation. The lowest (85°C/ 15 s) was chosen so as to mimic pasteurisation, whereas others were in a range where inactivation of plasmin could occur and therefore it would be interesting to monitor changes in proteolysis with time.

After cooling, the samples were stored at 2°C for 2 days. The six batches of milk samples were treated with sodium azide (0.05 %) to prevent bacterial contamination. They were then dispensed in sterile bottles in a laminar flow hood cabinet followed by incubation at 37°C for 28 days. Sampling for analysis was done on days 0, 3, 7, 14 and 28. Clarification to obtain pH 4.6 soluble extracts was carried out as detailed below. The soluble extracts were analysed by TNBS method. Clarification procedures were carried out after incubation for all milk samples studied. Prior to clarification, all milk samples were heated and held at 100°C for 10 min to denature the whey proteins. For isoelectric precipitation at pH 4.6, 50 mL warm water (40°C) was added to 5 mL of milk followed by 0.5 mL 10 % (w/v) acetic acid. After standing for 10 min, 0.5 mL 1 M sodium acetate was added and placed in cold water for 10 min before filtration through Whatman no. 41 filter paper and washing and making up to 100 mL. The clear extracts obtained were further filtered by 0.2 µm Millipore filter before being subjected to the TNBS method. The TNBS method was done as described by McKellar (1981). Triplicate samples of pH 4.6 soluble extracts (0.2 mL) were mixed with 2 mL 1 M-potassium borate buffer (pH 9.2) and 0.8 mL 5 mM-TNBS (Sigma-Aldrich, Gillingham, UK). After 30 min incubation in the dark at 25 °C, 0.8 mL freshly prepared 2 M-monobasic Na_2PO_4 [containing 18 mM-Na_2SO_3] and 5 mL distilled water were added. Absorbance was read at 420 nm by spectrophotometer (Cecil CE 1021 1000 series Cambridge, England).

Statistical analysis was carried out by using Statistical Package for Social Sciences (SPSS version 14). General Linear Model of analysis of variance (ANOVA) was used to determine statistical differences between means. Least square differences (LSD) and Duncan's multiple range tests were used to determine values that were statistically different (P <0.05). All analyses were carried out in triplicate, and results were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Effect of storage time (days) on proteolysis of milk heated at high temperatures

The present investigation aimed at examining the effect of various heat processes of milk on proteolysis during storage. Clarified samples of raw milk and milk heated at various temperatures and stored for 28 days were analysed. The results are shown in Figure 1. Generally, the absorbance of all milk samples increased with storage time (Figure 1) and had the significantly higher absorbance value ($p<0.05$) at day 28 indicating that proteolysis

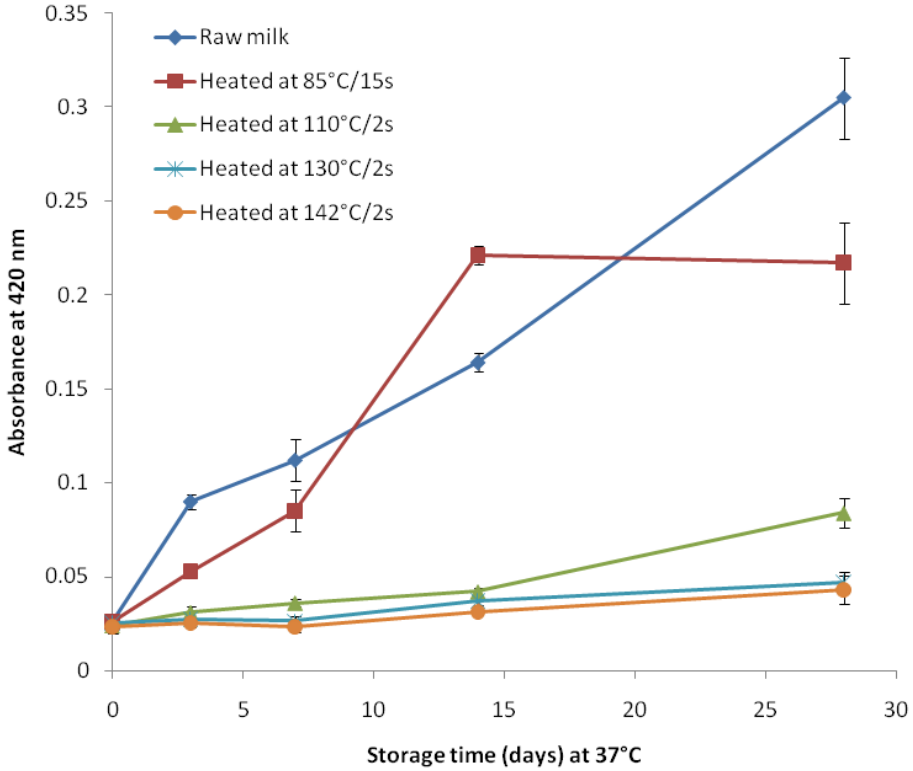

Figure 1. The effect of storage time (days) on proteolysis of raw milk and milk heated at 85°C/15s, 110, 120, 130 and 142°C for 2 s analysed by TNBS at 420 nm. The extent of proteolysis was represented by the absorbance of pH 4.6 soluble extracts at 420 nm measuring the breakdown products of raw milk and milk heated at various processing conditions during storage at 37°C for 28 days.

increased with storage time. Although proteolysis followed an increasing trend with time for all samples, the increasing rates were higher for raw milk and milk heated at 85°C than those of milk samples heated at 110, 120, 130 and 142°C for 2 s (Figure 1). The increase in percentage breakdown proteolysis from days 3 to 28 was as follows: raw milk (22.07%), processed at 85°C (16.84%), 110°C (5.43%), 120°C (3.38%), 130°C (2.05%) and 142°C (1.84%) which shows that it was more than 15% for the first two samples whereas it was less than 6% for the remaining four samples. For milk heated at 110, 120, 130 and 142°C for 2 s, small, steady but significant increase in proteolysis was observed. This is also evident from Table 1, where the increase in percentage protein breakdown decreased with the heating temperature. The increased absorbance with storage time was probably due to prolonged activities of either bacterial (released before incubation) or native enzymes or both. It has been reported that significant proteolysis may occur in the udder prior to milking leading to increased proteose peptones and γ-caseins as a result of plasmin activity on caseins (Schaar, 1985). These would account for proteolysis observed on day 0. Increased proteolysis of proteose peptones from 2.5 to 11.1 mg/ml in whey prepared from raw milk stored for 7 d at 37°C was also observed in previous study (Andrews and Alichanidis, 1983). The same study also revealed that hydrolysis of α_{s1} and β-casein occurred during storage, the peptide fragments of which were not precipitated at pH 4.6 but by TCA from whey where they contributed to the proteose peptones fractions.

It is known that plasmin inhibitors and plasminogen activator inhibitors are heat labile and therefore inactivated at lower temperatures as compared to the other components of the plasmin system (Richardson, 1983). About 81.1 and 35.8% of plasminogen activator inhibitors and plasmin inhibitors respectively were inactivated at 75°C for 15 s (Prado et al., 2006). This in turn increases plasmin activity as more plasminogen is also converted to plasmin (Richardson, 1983) and hence higher proteolysis. Although proteolysis was initially low, it increased significantly by day 28 which was a result of accumulation of breakdown products with storage time. These observations are consistent with the results reported by Gillis et al. (1985) who stated that although enzymatic activity may be decreased by UHT heat treatment, it is not

Table 1. Absorbance of pH 4.6 soluble extracts of raw milk and milk processed under various temperature-time conditions and incubated at 37°C for 28 days to examine the effect of proteolysis on storage time by the TNBS method at 420 nm.

Incubation time (days)	Treatments	Absorbance of pH 4.6 soluble extracts of UHT skim milk at 420 nm	Protein breakdown (%)
Day 0	Raw milk	0.026 ± 0.0023^{aA}	*
	Heated at 85°C	0.026 ± 0.0014^{aE}	*
	Heated at 110°C	0.024 ± 0.0008^{aI}	*
	Heated at 120°C	0.025 ± 0.0021^{aM}	*
	Heated at 130°C	0.025 ± 0.0020^{aU}	*
	Heated at 142°C	0.023 ± 0.0035^{aV}	*
Day 3	Raw milk	0.090 ± 0.0105^{bB}	6.57
	Heated at 85°C	0.053 ± 0.0040^{cF}	2.77
	Heated at 110°C	0.031 ± 0.0034^{dJ}	0.72
	Heated at 120°C	0.027 ± 0.0031^{dN}	0.21
	Heated at 130°C	0.027 ± 0.0015^{dU}	0.21
	Heated at 142°C	0.025 ± 0.0023^{dV}	0.20
Day 7	Raw milk	0.112 ± 0.0250^{eB}	8.83
	Heated at 85°C	0.085 ± 0.0111^{fF}	6.06
	Heated at 110°C	0.036 ± 0.0018^{gJK}	1.23
	Heated at 120°C	0.033 ± 0.0025^{gOP}	0.82
	Heated at 130°C	0.026 ± 0.0029^{gU}	0.10
	Heated at 142°C	0.023 ± 0.0028^{gV}	0.00
Day 14	Raw milk	0.164 ± 0.0060^{hC}	14.17
	Heated at 85°C	0.221 ± 0.0050^{iG}	20.02
	Heated at 110°C	0.042 ± 0.0017^{jK}	1.84
	Heated at 120°C	0.038 ± 0.0025^{kjP}	1.33
	Heated at 130°C	0.037 ± 0.0021^{kjS}	1.23
	Heated at 142°C	0.031 ± 0.0012^{kV}	0.82
Day 28	Raw milk	0.305 ± 0.0087^{lD}	28.64
	Heated at 85°C	0.217 ± 0.0216^{mH}	19.61
	Heated at 110°C	0.084 ± 0.0078^{nL}	6.15
	Heated at 120°C	0.060 ± 0.0091^{oQ}	3.59
	Heated at 130°C	0.047 ± 0.0056^{oT}	2.26
	Heated at 142°C	0.043 ± 0.0074^{oW}	2.05

Different lower case letters on the same column show significant differences ($p<0.05$) per treatment per day whereas different uppercase letters on the same column show significant differences ($p<0.05$) for the same treatment on different days; The experiment was replicated 3 times (N = 9); The pH 4.6 soluble extracts were diluted (20x). *Day 0 was used as a reference. Breakdown products (from days 3 to 28) were calculated with reference to this day (day 0).

inactivated.

The small but significant differences in each of the treatments could be due to inactivation of plasmin, plasminogen and plasminogen activators which are the main actors responsible for increased proteolysis in the samples. It may thus be concluded that lower activities in the heated samples was a result of higher temperature (>85°C) used which may have caused reduced activities of enzymes responsible for proteolysis. It has been reported that during severe heat treatments such as UHT pro-

cessing, decreased plasmin activity is due to thiol-disulphide interactions between disulphide groups in plasmin and reactive SH groups of β-lactoglobulin during the unfolding and denaturation that occurs at high temperatures (Grufferty and Fox, 1986; Kelly and Foley, 1997). Other changes that were described in association with severe heat treatments include denaturation of whey proteins especially β-lactoglobulin, leading to the formation of β-lactoglobulin-κ-casein complex example through interaction with κ-casein (Datta and Deeth, 2003; McMahon, 1996).

Degradation of caseins throughout storage of direct and indirect UHT whole and skim milk was also observed in previous study where directly and indirectly processed whole and skim UHT milks were observed for 11 weeks (Lopez-Fandino et al., 1993). In their study, the researchers found that after 11 weeks, significant proteolysis occurred in skim milk as compared to whole milk as analysed by the TNBS method.

Effect of heating temperatures on the proteolysis in milk stored at 37°C

Table 1 shows the absorbance (at 420 nm) of soluble extracts of raw milk and milk processed under various temperature-time conditions. Samples with similar treatments were compared per day of storage as shown in Table 1. At day 0 of storage, the level of proteolysis of raw milk did not significantly differ ($p>0.05$) from milk samples heated at 85°C /15 s, 110, 120, 130 and 142°C for 2 s. This means that although there was some proteolysis probably occurring from udder, none of the samples had undergone any significant increase in proteolysis. On days 3, 7, 14 and 28 raw milk and milk heated at 85°C/15 s significantly differed ($p<0.05$) from the rest of the samples heated at 110, 120, 130 and 142°C for 2 s (Table 1). Although, a study by Andrews and Alichanidis (1983) concluded that proteolysis progressed faster in pasteurised milk than in raw milk, it was not exactly the case in the samples studied here. Raw milk showed higher proteolysis than milk heated at 85°C in the current study. Reasons for the difference in results is not clear as lower temperature was used in the previous study (72°C/18 s), as compared to 85°C/15 s in this study. It has been reported that plasmin levels in raw milk vary, so probably higher levels were already present in the raw milk samples used by the previous researchers, which increased further upon pasteurisation due to inactivation of inhibitors. Another possibility is the presence of bacterial enzymes in raw milk which could have resulted in increased proteolysis in raw milk. It was reported that a milk sample that has a high population of psychrotrophic bacteria could have a reduced shelf-life even after the viable bacteria have been inactivated (Adams et al., 1975). The former reason is more likely as the raw milk was of good bacterial quality (10^3 cfu/mL psychrotrophic count– results not shown). It is therefore suggested that higher proteolysis in raw milk was most likely due to native enzymes.

Moreover, these two samples (raw milk and milk heated at 85°C) showed the highest levels of proteolysis among all treatments analysed most probably due to activity of native enzymes. The rest of the heated samples (at 110, 120, 130 and 142°C) had very low proteolysis on days 3, 7, 14 and 28 as compared to raw milk and milk heated at 85°C for 15 s signifying possible inactivation of plasmin and its inhibitors. It is obvious from the trend that the increase in the rate of proteolysis was inversely proportional to the heat treatment implying that the lower the temperature employed, the higher the enzyme activity and the higher the proteolysis. It has been documented that variability of heat stability of UHT milk during storage results from multiple and complex reactions which occur in milk such as Maillard reactions, lactose degradation and other biochemical changes such as polymerisation by disulphide bridges and dephosphorylation (Gaucher et al., 2008). It is well established that radicals that are generated during the Maillard reaction can promote a selective attack on the protein backbone which would break specific bonds (Guinot-Thomas et al., 1995). However, it is unlikely that the heat treatment of the milk led to the Maillard reaction, as proteolysis was still low in the samples.

It may thus be concluded that raw milk and milk heated at 85°C have shown the highest proteolysis as compared to the rest of the samples due to the presence and activity of native enzymes. Lower proteolysis in milk processed at higher temperatures is associated with the destruction of these enzymes.

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