Gel Electrophoresis and Fluorescamine Methods for the Detection of Proteolysis of Milk Heated at High Temperatures

L.M. Chove[1], A.S. Grandison[2] and M.J. Lewis [2]

[1] Department of Food Science and Technology, Sokoine University of Agriculture, P. O. Box 3006, Morogoro, Tanzania

[2] Department of Food and Nutritional Sciences, University of Reading, P. O. Box 226, Reading RG6 6AP, UK

Abstract

This study describes the effect of high temperature processing of milk and its susceptibility to proteolysis during storage. Raw milk was subjected to various Temperature-time combinations (110, 120, 130 and 142 ˚C for 2s) to assess its susceptibility to proteolysis by native enzymes during storage at 37 ˚C for 28 days. Raw milk and processed milk at low temperature (85/15 s) were also used as benchmarks for the native state and mild heat treatment. Gel electrophoresis (for qualitative analysis) and fluorescamine (for quantitative analysis) methods were used to detect proteolysis in high temperature heated milk caused by native enzymes. For the fluorescamine method, clarification was achieved by isoelectric precipitation and precipitation with acid to obtain pH 4.6 and 6% TCA soluble extracts respectively. Non-clarified samples were used for gel electrophoresis. Both methods confirmed that raw milk and milk processed at 85/15s were the most proteolysed, indicating that the high temperatures (110, 120, 130 and 142 ˚C for 2s) lowered proteolysis through inactivation of heat resistant native enzymes possibly plasmin and hence decreased milk's susceptibility to spoilage.

Key words: proteolysis, fluorescamine, gel electrophoresis, plasmin, isoelectric precipitation

Introduction

Thermal processing of milk is important because it increases of shelf life of milk. In addition, it also ensures safety through the destruction of microorganisms, enzymes inactivation, and changing the chemical composition (Lehmann and Buckin, 2005). However, during storage a number of biochemical changes may occur even in high temperature treated milk. Proteolysis and lipolysis are the two most important biochemical changes responsible for the degradation of ultra high temperature (UHT) heated milk during storage. Native enzymes play a crucial role in milk proteolysis (Nielsen, 2002). Plasmin is one of the most heat resistant enzymes found in milk. The milk plasmin system is composed of five main components: plasmin (EC 3.4.21.7), plasminogen, plasminogen activators, plasmin inhibitors and plasminogen activator inhibitors (Grufferty and Fox, 1988; Crudden and Kelly, 2003). The first three components are associated with casein whereas the last two are found in the serum. Unlike the two inhibitors which are heat labile, the rest of the components are highly resistant to heat (Datta and Deeth, 2001). Plasmin is an alkaline serine proteinase with an optimum pH of 7.5 at 37 ˚C, which readily hydrolyses β-casein, α_{s2}-casein and (more slowly) α_{s2}-casein. It is also able to reactivate during storage, thereby reducing shelf life of milk and milk products (Bastian and Brown, 1996). It may survive pasteurisation and many UHT processes. Plasmin causes breakdown of milk proteins in a variety of dairy products, which results in the change of texture and flavour in these products (Ma and Barbano, 2003; Chavan *et al.*, 2011). The inhibitors present in fresh milk are heat labile whereas the activators are known to be heat stable (Richardson, 1983). The plasmin activity is therefore driven by a complex system of activators and inhibitors with different heat stabilities. Protease activity that is insignificant during short storage

may become important during extended storage. Plasmin cannot be totally eliminated by the thermal processing technologies that we have today without rendering the milk unpalatable. The best approach to minimize the impact of proteolysis is to ensure high quality raw milk.

The current study is focused on understanding proteolysis of high temperature heated milk during storage.

Materials and methods

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—142 °C) as described by 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 represent pasteurisation, whereas other temperature profiles were in a range where inactivation of plasmin could occur and therefore would reveal corresponding changes in proteolysis with time.

After cooling to 5°C, 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 growth in the milk. 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 6% TCA and pH 4.6 soluble extracts was carried out as detailed below. The soluble extracts were analysed by the fluorescamine method only whereas for gel electrophoresis, non-clarified samples were used.

Prior to clarification, milk samples for fluorescamine method were heated and held at 100 °C for 10 min to denature the whey proteins. Clarification to obtain pH 4.6 soluble extracts was done by adding 50 mL warm water (40 °C) to 5 mL of milk followed by 0.5 mL 10 % (w/v) acetic acid. After standing for 10 minutes, 0.5 mL 1M sodium acetate was added and placed in cold water for 10 minutes 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 fluorescamine method. The 6% TCA soluble extracts were obtained as follows: Five mL of 12% (w/v) trichloroacetic acid (TCA) was added to an equal volume of the milk samples. The test tubes were vortexed for 2-3 minutes and left at room temperature for 1h. The solutions were vortexed again for 2-3 minutes followed by filtration through Whatman no 41. The filter paper was washed with water and the volume of the supernatant made up to 10 mL with distilled water. Filtration was further carried out by 0.20 µm Millipore filter.

The micro-method for fluorescamine (Castel *et al.*, 1979) was adopted in this study, as small quantities of reagents were required. Duplicates of pH 4.6 soluble extracts (0.1 ml) were placed in a sterilin bottle, followed by the addition of 0.1 ml potassium borate buffer (pH 8.5) and finally 0.1 ml fluorescamine (0.03 % in acetone). The reaction was instantaneous and after 1 min, 3.5 ml potassium borate buffer (pH 8.5) was added. Fluorescence was determined with a spectrofluorimeter (Perkin Elmer LS-5 Luminescence spectrometer, Beaconsfield, Bucks, UK) after excitation at 390 nm and emission at 475 nm. For gel electrophoresis, a protocol based on Laemli (1970) with slight modifications was used.

Urea-PAGE electrophoresis

Reagents and solutions

Acrylamide-bisacrylamide stock solution (40% T): (Sigma-Aldrich Gillingham, UK): The solution was used directly from the bottle.

Separating gel buffer (0.5 M Tris, pH 8.8): Tris base (12.86 g) and 77.14 g urea (77.14 g) was dissolved in 200 mL distilled water and pH adjusted to 8.8 with 5M HCl. The solution was stored at 4 ˚C.

Stacking gel buffer (0.07 M Tris, pH 7.6): Tris base (1.66 g) was dissolved in 200 mL of distilled water. pH was adjusted to 7.6 with 5M HCl and stored at 4 ˚C.

Electrode buffer: Tris base (1.5 g) and glycine (7.3 g) were dissolved in 1000 mL of distilled water.

No pH adjustment was made (the pH was 8.3). The solution was freshly prepared and cooled to 4 ˚C for at least 3 hours before use.

Reducing sample buffer: Tris base (0.75 g) and urea (49 g) were dissolved in 100 mL distilled water and pH adjusted to 7.6 with 5M HCl. β-mercaptoethanol (0.7 mL) and 0.9 g of bromophenol blue were added. The solution was stored at 4 ˚C.

Ammonium persulphate (APS): This solution was freshly prepared by dissolving 0.1 g of APS in 1 mL of distilled water.

Sodium Dodecyl Sulphate (SDS): This was made by dissolving 10 g of SDS in 100 mL distilled water and stored at 25 ˚C.

N,N,N',N'-Tetramethylenediamide-TEMED (Sigma-Aldrich Gillingham, UK): This was used directly from the bottle.

Staining solution: Coomasie blue G250 (1 g) was dissolved in 400 mL methanol, 100 mL acetic acid and the volume made up to 1000 mL with distilled water. It was stored at 25 ˚C.

Destaining solution: To 400 mL of methanol, 100 mL of acetic acid was added and the volume made up to 1000 mL with distilled water

Preparation of the gel

Reagents	12% Separating Gel	4% Stacking Gel
Separating Gel buffer (mL)	7.0	-
Stacking Gel buffer (mL)	-	4.50
40% acrylamide solution (mL)	3.00	0.5
10% SDS (µL)	100.0	-
TEMED (µL)	10.0	5.0
10% APS (µL)	50.0	25.0
Total volume (mL)	10.160	5.030

Samples and markers preparations

Samples: 100 µL of samples were mixed with 400 µL of reducing sample buffer. These were allowed to stand in boiling water for 2 min and allowed to cool to 25 ˚C before loading into the gel.

Markers: Low molecular weight markers ranging between 6.5 - 66 kDa (Sigma-Aldrich Gillingham, UK) were prepared as follows: A vial containing low molecular weight markers was reconstituted with 100 µL of deionised water. To ensure complete dissolution of the chemical, the vial was vortexed for a few seconds. The markers were dispensed in Eppendorf vials in aliquots and any unused portion was stored at -18 ˚C until required. The proteins in the low molecular weight marker were as follows: albumin, bovine serum (66 kDa); ovalbumin from chicken egg (45 kDa); glyceradehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa); carbonic anhydrase from bovine erythrocytes (29 kDa); trypsinogen from bovine pancrease (24 kDa); trypsin inhibitor from soybean (20 kDa); α-lactalbumin from bovine milk (14.2 kDa) and aprotinin from bovine lung (6.5 kDa)

Statistical analysis was carried out by using Statistical Package for Social Sciences (SPSS version 16). General Linear Model of analysis of variance (ANOVA) was used to determine statistical differences between means. LSD (Least Square Differences) 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 are expressed as mean ± standard deviation (SD).

Results and Discussion

Proteolysis by the fluorescamine method

Fluorescamine reacts with free amino acids to form fluorescent derivatives which are measured by the spectrofluorimeter.

Table 1 indicates that on day 0, only pH 4.6 soluble extracts of samples heated at 85°C and of those heated at 142°C were significantly different ($p<0.05$) in proteolysis from each other whereas the rest were not statistically different from either of them. However, from day 3 to day 28 statistically significant differences in proteolysis ($p<0.05$) were observed between pH 4.6 soluble extracts of three

samples (raw milk, those processed at 85/15s and 110°C/2s) with the rest of heat processed samples (heated at 120, 130 and 142°C for 2s). The latter samples were not statistically different from each other for the stated period of time (days 3 to day 28). The same table also clearly shows that pH 4.6 soluble extracts of raw milk and that of milk processed at 85 °C had higher fluorescence readings than the rest of the samples for all the days of incubation whereas samples processed at 120, 130 and 142°C had lower proteolysis than that of raw milk and of milk processed at 85 and 110 °C. Similar results were obtained from proteolysis of high temperature treated milk study analysed by the TNBS method (Chove *et al.*, 2013). It may thus be concluded that processing at higher temperature lowers proteolysis and hence susceptibility of UHT milk to spoilage. This is probably through denaturation of enzymes responsible for proteolysis, hence decreased proteolysis. Some authors have reported that severe heating results in the denaturation of whey proteins, with β-lactoglobulin unfolding and interacting via its reactive thiol group, with protein molecules comprising of disulphide bonds (Enright and Kelly, 1999; Datta and Deeth, 2003). Hence high heat treatment may have caused decreased proteolytic activity due to inactivation of plasmin by thiol-disulphide interchange reaction (Kennedy and Kelly, 1997). B-lg/κ-casein interactions seem to be the main complex formed during heat treatment of milk due to the intermolecular disulphide bond (Raikos, 2010).

Table 2 shows that on day 0, 6% TCA soluble extracts of raw milk and of milk processed at 85 ˚C had the highest value which was statistically different from all other samples. On day 3 however, the first three samples (raw milk, milk heated at 85˚C /15s and 110˚C /2s) were statistically different ($p<0.05$) in proteolysis from the other heat treated samples (120, 130 and 142 °C for 2s). From day 14 to day 28, two samples (raw milk and milk heated at 85 ˚C were statistically different in proteolysis from the rest of heat treated samples (110,120,130, and 142˚C/2s). Again, this confirms the earlier observation that heat treatment at higher temperatures lowers proteolysis in milk through reduced proteolytic activity upon processing at higher temperatures.

Table 1: Relative fluorescence 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 fluorescamine method at 475 nm

Incubation time (days)	Treatments [Temperature (˚C) /time (s)]	Relative fluorescence of pH 4.6 soluble extracts of skim milk at 475 nm
day 0	CONTROL (Raw milk)	105± 2.0 ab A
	85/15	114± 2.8 a F
	110/2	106± 7.0 ab J
	120/2	108± 5.4 ab O
	130/2	105± 4.0 ab S
	142/2	100 ± 4.4 b W
day 3	CONTROL (Raw milk)	345± 44.0 c B
	85/15	301± 9.8 d G
	110/2	125± 5.0 e K
	120/2	120± 3.1 f P
	130/2	118± 1.4 f T
	142/2	118± 2.8 f X
day 7	CONTROL (Raw milk)	487± 45.5 g C
	85/15	322± 32.1 h G
	110/2	203± 4.5 i L
	120/2	138± 2.8 j Q
	130/2	132± 1.8 j U
	142/2	121± 3.1 j XY
day 14	CONTROL (Raw milk)	600± 20.1 k D
	85/15	563± 25.3 l H
	110/2	214± 4.1 m M
	120/2	143± 2.6 n Q
	130/2	136± 2.2 n U
	142/2	131± 3.8 n Y
day 28	CONTROL (Raw milk)	1100 * o E
	85/15	1100 * o I
	110/2	1100 * o N
	120/2	861± 6.0 p R
	130/2	723± 4.9 p V
	142/2	642± 10.7 p Z

Different lower case letters on the same column show significant differences ($p<0.05$) per day of analysis whereas different uppercase letters on the same column show significant differences ($p<0.05$) per samples; The experiment was replicated 3 times (N=9); *Detection limit of the equipment.

Table 2: Relative fluorescence of 6% TCA 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 fluorescamine method at 475 nm

Incubation time (days)	Treatments [Temperature (˚C) /time (s)]	Relative fluorescence 6% TCA soluble extracts of skim milk at 475 nm	
day 0	CONTROL (Raw milk)	48± 8.5	a A
	85/15	34± 2.9	b F
	110/2	31± 2.1	b J
	120/2	31± 5.2	b N
	130/2	30± 5.5	b S
	142/2	31 ± 3.6	b W
day 3	CONTROL (Raw milk)	176± 8.6	c B
	85/15	103± 6.0	d G
	110/2	64± 4.0	e K
	120/2	53± 3.5	f O
	130/2	51± 2.1	f T
	142/2	46± 5.3	f X
day 7	CONTROL (Raw milk)	287± 57.4	g C
	85/15	141± 44.6	h G
	110/2	115± 21.5	ih L
	120/2	85± 12.7	ih P
	130/2	65± 10.4	i T
	142/2	54± 10.7	i XY
day 14	CONTROL (Raw milk)	418± 23.0	j D
	85/15	334± 21.4	k H
	110/2	127± 4.4	l L
	120/2	104±10.7	l Q
	130/2	102± 7.4	l U
	142/2	67± 9.2	m Y
day 28	CONTROL (Raw milk)	1100 *	n E
	85/15	1100 *	n I
	110/2	422± 9.1	o M
	120/2	215± 4.5	p R
	130/2	202± 11.2	q V
	142/2	184± 5.3	r Z

Different lower case letters on the same column show significant differences ($p<0.05$) per day of analysis whereas different uppercase letters on the same column show significant differences ($p<0.05$) per sample; The experiment was replicated 3 times (N=9); The pH 4.6 soluble extracts were diluted (x20) whereas the 6% TCA soluble extracts were diluted (x2); *Detection limit of the equipment.

Although the trend for proteolysis in these soluble extracts appears to be similar, some unexpected results were observed. On day 28, the pH 4.6 soluble extracts of milk heated at 110, 120, 130 and 142 ˚C had higher fluorescence readings than its corresponding 6% TCA soluble extracts (Table 2). Moreover, the increase in fluorescence between day 14 and 28 was very high for both pH 4.6 and 6% TCA soluble extracts of raw milk samples and of samples heated at 85 ˚C (Table 2). The pH 4.6 soluble extracts of milk heated at 110 ˚C reached a detection limit on day 28 unlike its corresponding 6% TCA soluble extract which was less than half the value.

Unlike TCA soluble extracts in which TCA precipitates large peptides, pH 4.6 soluble extracts solubilise these large peptides, hence their presence in the extracts. It has been documented that TCA precipitates large peptides as those formed by plasmin (Datta and Deeth, 2003) and hence TCA soluble extracts would consist of only small peptides (as those formed by microbial proteases). Another study revealed that it was impossible to determine precipitation threshold in relation to peptide size as peptides containing 7-30 residues may be soluble, insoluble or partially soluble at the various TCA concentrations (Yvon *et al.*, 1989). The same authors suggested that interactions between TCA and the peptides induce an increase of the hydrophobicity of the peptides which leads to aggregation through hydrophobic interactions. It is likely that the larger peptides had undergone proteolysis during storage and leading to the formation of smaller peptides and amino acids which were soluble in TCA.

Proteolysis by gel electrophoresis

To study proteolysis of milk samples processed at various temperatures-time conditions, breakdown products were monitored by gel electrophoresis following incubation for 28 days at 37˚C. The information highlighted the association of these treatments with the rate of protein breakdown which is useful for shelf life studies.

Figure 1 shows an electrophoretogram of day 0 samples of raw milk and milk processed at various temperature-time conditions (lanes 1-6). With the exception of lane 3, whose α and β-casein were faint, samples of raw milk and of other heat processed

milks had intense α and β-caseins bands indicating low or no activity on day 0. However, the γ-caseins bands appearing could be a result of proteolysis by native enzymes in the udder (Andrews and Alichanidis, 1983). A study whereby direct heating at 142 ˚C for 4 s was employed, revealed that these treatments were insufficient to inactivate native and bacterial proteases (Snoeren *et al.*, 1979). Although milk samples in the current study were indirectly heated, it is still reasonable to consider the presence and activity of these enzymes which increase with incubation time.

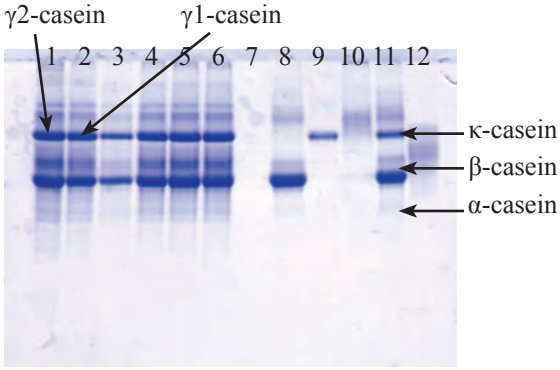

Fig. 1: Urea–PAGE **electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures on day 0. *Lane 1:* Raw milk control; *Lane 2:* 110 ˚C for 2s; *Lane 3:* Milk processed at 85 ˚C for 15s; *Lane 4:* 120 ˚C for 2s; *Lane 5:* 130 ˚C for 2s *Lane 6:* 142 ˚C for 2s; *Lane 7:* Empty lane; *Lane 8:* α- casein; *Lane 9:* β-casein; *Lane 10:* κ- casein; *Lane 11:* A Mix of α β κ- caseins. *Lane 12:* B-lactoglobulin**

Figure 2 shows electrophoretogram of day 3 samples of raw milk and milk processed at various temperature-time combinations (lanes 1-6). It is evident that unlike samples on lanes 1 and 2 whose α and β-caseins were faint indicating proteolytic activity, other heat processed samples had intense α and β-caseins indicating low/no activity after 3 days of incubation. The faint bands in raw milk and milk processed at 85 ˚C suggest the role of native enzyme, probably plasmin whose activity on α and β-caseins results in the formation of γ-caseins. It was stated by Andrews and Alichanidis (1983) that the cleavage sites for plasmin in β-caseins were Lys28-Lys29 whose derivative peptide is γ1-caseins (f29-209); Lys 105-His106 to yield γ2-caseins (f106-209) and finally Lys107-Glu209 yielding γ3-caseins (f108-209).

The disappearance of β-caseins is accompanied by the appearance of γ-caseins and hence plasmin activity was implicated in these samples. The activity was highest at 85 ˚C which is in agreement with the finding by Andrews and Alichanidis (1983) who reported increased activity as being due to destruction of the plasmin and plasminogen activator inhibitors. The inhibitors are more heat labile than plasmin and plasminogen. As discussed earlier, the increased activity in raw milk could be due to proteolysis by native or bacterial enzymes. Small amounts of γ-caseins appear in lanes 3-6, probably due to proteolysis by plasmin in the udder. Low proteolytic activities in these samples were due to the higher temperatures used which inactivated plasmin and lowered proteolysis.

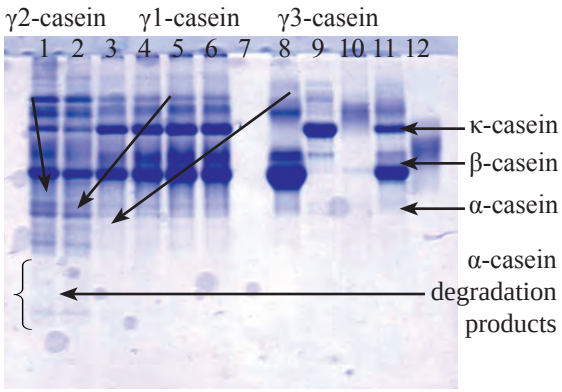

Fig. 2: Urea–PAGE **electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures and incubated at 37 ˚C for 3 days. *Lane 1:* Raw milk control; *Lane 2:* 85 ˚C for 15s; *Lane 3:* Milk processed at 110 ˚C for 2s; *Lane 4:* 120 ˚C for 2s; *Lane 5:* 130 ˚C for 2s; *Lane 6:* 142 ˚C for 2s; *Lane 7:* Empty lane; *Lane 8:* α- casein; *Lane 9:* β-casein; *Lane 10:* κ-casein; *Lane 11:* A Mix of α β κ- caseins; *Lane 12:* B-lactoglobulin**

It is obvious from Figure 3 that after one week of

incubation, there was progressive degradation of α and β-caseins in the order previously observed (milk processed at 85 ˚C > raw milk > milk processed at 110 ˚C > at 120 ˚C > at 130 ˚C > at 142 ˚C). Complete disappearance of α and β-caseins on lane 2 was obvious, confirming highest proteolysis in this sample more than any other. Lower proteolysis in samples processed at higher temperatures (lanes 3-6) were due to inactivation of plasmin and its activators as previously described. Similar trends of proteolysis as on day 7 were observed for samples incubated for 14 days as shown in Figure 4. In addition, the β-casein band in the raw milk sample had disappeared. Samples heated at higher temperatures (lanes 3-6) were more proteolysed as α and β-caseins appeared to be fainter than on day 7, indicating progressive proteolysis with time. It was evident that β - casein was degraded more rapidly than α- casein as observed from faint bands in the samples (Figure 4). Similar results were reported previously (Enright *et al.,* 1999; Kelly and Foley, 1997; Snoeren *et al.,* 1979).

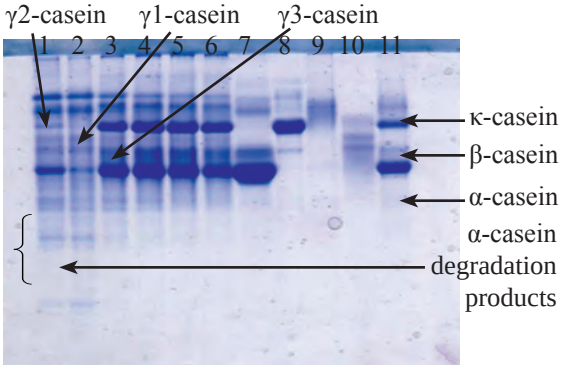

Fig. 3: Urea–PAGE **electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures and incubated at 37 ˚C for 7 days. *Lane 1:* Raw milk control; *Lane 2:* Milk processed at 85 ˚C for 15s; *Lane 3:* 110 ˚C for 2s; *Lane 4:* 120 ˚C for 2s; *Lane 5:* 130 ˚C for 2s; *Lane 6:* 142 ˚C for 2s; *Lane 7:* α- casein; *Lane 8:* β-casein; *Lane 9:* κ- casein; *Lane 10:* β –lactoglobulin; *Lane 11:* A Mix of α β κ- caseins.**

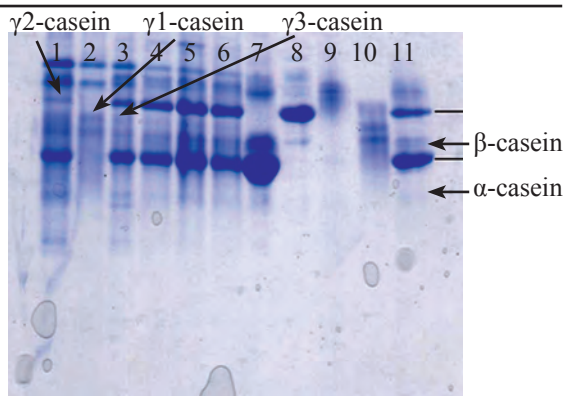

Fig. 4: Urea–PAGE **electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures and incubated at 37˚C for 14 days. *Lane 1:* Raw milk control; *Lane 2:* Milk processed at 85 ˚C for 15s; *Lane 3:* 110 ˚C for 2s; *Lane 4:* 120 ˚C for 2s; *Lane 5:* 130 ˚C for 2s; *Lane 6:* 142 ˚C for 2s; *Lane 7:* α- casein; *Lane 8:* β-casein; *Lane 9:* κ-casein; *Lane 10:* β lactoglobulin; *Lane 11:* A Mix of α β κ- caseins.**

After 28 days of incubation, disappearance of β-casein was apparent from milk processed at 110˚C which was accompanied by the formation of γ-casein as shown in Figure 5. This band had been present as a faint band from day 14 but intensified on day 28 probably as a result of plasmin activity on β-casein. The rest of lanes (4-6) still showed low activities. Similar results were observed where UHT milk processed at 138˚C for 2.4 s showed lower extent of proteolysis than raw milk with little change in the major casein bands (Enright *et al.,* 1999).

The final gel (Figure 6) compares the two most proteolytic samples i.e. raw milk and milk samples heated at 85˚C. Caseins, mostly α and β had been hydrolysed progressively from day 0 to day 28. On day 0, both batches of milk samples had low proteolysis. However, after 3 days of incubation, although both α and β-caseins appeared fainter than on day 0, the rate of degradation especially for β-caseins was higher for the heat treated sample than the raw milk. Complete disappearance of β –caseins occurred in both samples on day 7, but on days 14 and 28 only the heat treated sample showed complete disappearance of α -caseins confirming increased proteolytic products in the latter sample

than the former.

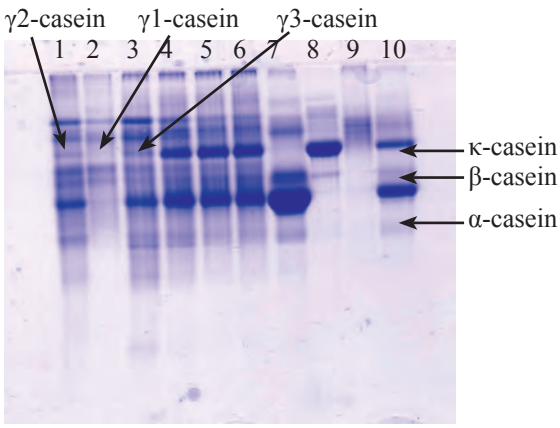

Fig. 5: Urea–PAGE **electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures and incubated at 37 ˚C for 28 days: *Lane 1:* Raw milk; *Lane 2:* Milk heated at 85 ˚C for 15s; *Lane 3:* Milk heated at 110 ˚C for 2s; *Lane 4:* Milk heated at 120 ˚C for 2s; *Lane 5:* Milk heated at 130 ˚C for 2s; *Lane 6:* Milk heated at 142 ˚C for 2s; *Lane 7:* α-casein; *Lane 8:* β-casein; *Lane 9:* κ -casein and *Lane 10:* A mix of α β κ –caseins.**

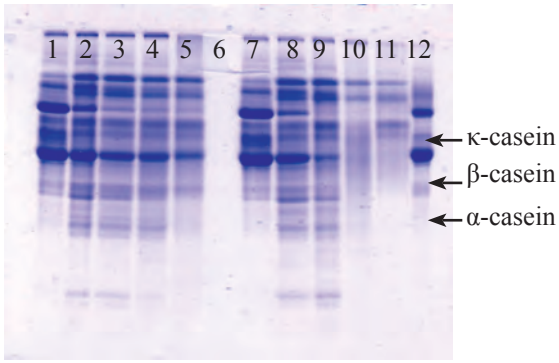

Fig 6: Urea-PAGE gel electrophoretograms **(pH 8.9, T=12.% T, C=4%) of raw milk and milk heated at 85 ˚C /15s and incubated for 0-28 days: *Lanes 1-5:* Raw milk incubated for 0,3,7,14 and 28 days; *Lane 6:* Empty lane; *Lane 7-11:* Milk heated at 85 ˚C for 2s and incubated on 0,3,7,14 and 28 days; *Lane 12:* A mix of α β κ –caseins.**

Conclusion

Both methods-fluorescamine and gel electrophoresis revealed that raw milk and milk heated at 85 °C for 15s had the highest proteolytic products concentration. It is unlikely that high proteolysis could be due to microbial enzymes since raw milk was of good quality (10^4 cfu/ mL-results not shown). In addition, sodium azide had been added to prevent microbial activity. It may thus be concluded that these two milk samples were the most susceptible to proteolysis by native enzymes during storage, possibly by plasmin. Thus, the higher the temperature employed for heating the milk, the less susceptible it is to proteolysis. The two methods –fluorescamine (for quantitative analysis) and gel electrophoresis (for qualitative analysis) are useful methods for the detection of proteolysis in milk.

Acknowledgements

The authors are grateful to the Association of Commonwealth Universities (ACU) for financial support that enabled this study to be conducted.

References

Andrews, A.T. and Alichanidis, E. (1983) Proteolysis of caseins and the proteose-peptone fraction of bovine milk. *Journal of Dairy Research*, 50, 275-290

Bastian, E. D. and Brown, R. J. (1996) Plasmin in milk and dairy products: an update. *International Dairy Journal*, 6, 435-457.

Browning, E., Lewis, M.J. and Macdougall, D. (2001) Predicting safety and quality parameters for UHT -processed milks. *International Journal of Dairy Technology*, 54, 111-120.

Castel, J.V., Cervere M. and Marco R. (1979) A convenient micromethod for the assay of primary amines and proteins with fluorescamine. A re-examination of the conditions of reaction. Journal of Analytical Biochemistry 99, 379-391

Chavan, R.S., Khedka C.D. and Jana A.H. (2011). UHT Milk Processing and Effect of Plasmin. Comprihensive review. *Food Sci. Food Saf.* 10 (5): 251-268. Retrieved from http://onlinelibrary.wiley.com/journal/10.1111/

(ISSN)1541-4337/ accessed on 13/12/13 at 6 pm

Chove, L.M., Issa-Zacharia, A., Grandison ,A.S and Lewis, M.J. (2013). Proteolysis of milk heated at high temperatures by native enzymes analysed by trinitrobenzene sulphonic acid (TNBS) method. *African Journal of Food Science,* 7 (8), 232-237

Crudden, A. and Kelly, A.L. (2003). Studies of plasmin activity in whey. *International Dairy Journal,* 13, 987-993.

Datta, N. and Deeth, H.C. (2001). Age gelation of UHT milk. *Food and Bio-product processing,* 79, 197-210

Datta, N. and Deeth, H.C. (2003). Diagnosing the cause of proteolysis in UHT milk. *Lebensmittel-Wissenschaft und -Technologie*, 36, 173-182.

Enright, E. and Kelly, A. (1999). The influence of heat treatment of milk on susceptibility of casein to proteolytic attack by plasmin. Milchwissenschaft, 54, 491-493.

Enright, E., Bland, A.P., Needs, E.C. and Kelly, A. L. (1999). Proteolysis and physicochemical changes in milk on storage as affected by UHT treatment, plasmin activity and KIO3 addition. *International Dairy Journal*, 9, 581-591.

Grufferty, M. B. and Fox, P. F. (1988). Milk alkaline proteinase. *Journal of Dairy Research,* 55, 609-630.

Kelly, A.L. and Foley, J.(1997). Proteolysis and storage stability of UHT milk as influenced by milk plasmin activity, plasmin/[beta]-lactoglobulin complexation, plasminogen activation and somatic cell count. *International Dairy Journal*, 7, 411-420.

Kennedy, A. and Kelly, A. (1997). The Influence of Somatic Cell Count on the Heat Stability of Bovine Milk Plasmin Activity. International dairy Journal, 7, 717-721.

Laemli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature*, 227, 680-685.

Lehmann, L and Buckin, V. (2005). Determination of the heat stability profiles of concentrated milk and milk ingredients using high resolution Ultrasonic Spectroscopy. *Journal of Dairy Science,* 88, 3121-3129.

Ma, Y. and Barbano, D. (2003). Effect of CO_2 addition to raw milk as affected by the action of plasmin. *Journal of Dairy Science,* 88, 1616-1631.

Nielsen, S.S. (2002). Plasmin system: characteristic, roles, and relationships. *Journal of Agriculture and Food Chemistry,* 50, 6628-6634.

Raikos, V. (2010). Effect of heat treatment on milk protein functionality at emulsion interfaces. A review. *Food Hydrocolloids*, 24, 259-265.

Richardson, B.C. (1983). The proteinases of bovine milk and the effect of pasteurisation on their activity. *New Zealand Journal of Dairy Science and Technology,* 18, 233-245.

Snoeren, T.H.M., Speck, M.L., Dekker, R. and Both P (1979). Proteolysis during the storage of UHT-sterilised whole milk. 1. Experiments with milk heated by direct system for 4 seconds at 142^0C. *Netherlands Milk Dairy Journal*, 33, 31-39.

Yvon, M., Chabanet, C. and Pelissiner, J.P. (1989). Solubility of peptides in TCA solutions. *International Journal of Peptide Protein Research,* 34, 166-176.