Macrothink Institute™

Journal of Food Studies
ISSN 2166-1073
2018, Vol. 7, No. 1

Preliminary Study on the Role of Ionic Calcium in Gelation and Proteolysis of UHT Milk

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Received: July 9, 2017 Accepted: December 26, 2017 Published: December 28, 2017

doi:10.5296/jfs.v7i1.12081 URL: https://doi.org/10.5296/jfs.v7i1.12081

Abstract

A study was conducted to determine the influence of ionic calcium on gelation and proteolysis in milk. Raw milk was heated to 142 °C for 2s followed by cooling to 5 °C to make UHT milk. Two sequestering agents, Trisodium citrate (TSC) and Sodium hexametaphosphate (SHMP) were added at 0.04 - 0.08% (w/v) to the processed UHT milk to reduce the ionic calcium levels. Proteolysis and gelation were induced by addition of trypsin (248 BAEE units), 0.03% (v/v) chymosin and 10^6 cfu/mL *Pseudomonas fluorescens* NCIMB 702085 (*Ps. fl.* 416) to UHT milk. Samples were stored at 25 °C for 2½ weeks to monitor gelation and Ca^{2+} and some were incubated at 37 °C for 2h to monitor proteolysis. SHMP reduced more Ca^{2+} than TSC. Ca^{2+} reduction was accompanied by an increase in pH, most evident with TSC at 25 °C. Gelation was not observed in samples inoculated with *Ps. fl. 416* (with sequestering agents) even after 9 days of storage, suggesting the importance of calcium in gelation. Chymosin treated samples gelled on day 0, whereas other samples gelled after 4 days. Trypsin increased Ca^{2+} to levels higher than originally present in control UHT skim milk. Although in the current study, proteolysis was higher in samples inoculated with *Pseudomonas fluorescens* 416, no clear relationship was established between proteolysis and gelation in UHT milk. This observation

implies that proteolytic activity is not influenced by Ca^{2+}. However, longer time study using sodium azide to prevent bacterial contamination would be required to confirm these findings.

Keywords: Chymosin, Gelation, Ionic Calcium, Proteolysis, *Pseudomonas fluorescens,* Sodium hexametaphosphate (SHMP), Trypsin, Trisodium citrate (TSC)

1. Introduction

Calcium in milk is significant for both its nutritive value and its key role in many functional properties of milk and milk products. Manipulating its concentration, particularly of the ionic form, alters the properties of the products and facilitates or hinders certain processing operations. Stability to thermal treatment is the major property affected, but several others such as gelation, coagulation and foaming are influenced by either adding or removing calcium (Deeth & Lewis, 2015; Faka et al., 2009). Calcium in milk is partitioned between the serum (30%) and colloidal phase of milk (70%). Total calcium in milk is around 30 mM, of which about 2 mM is ionic calcium (Holt et al., 1981). Though ionic calcium in milk is low, it is essential for stability of casein micelles and influences clotting of milk and precipitation of colloidal calcium phosphate (CCP). It is essential for gelation of milk by rennet during cheese manufacture (Tsioulpas, 2005; Hyslop, 2003). In addition, a minimum concentration of ionic calcium is necessary for aggregation of casein micelles (Manji et al., 1986; Manji & Kakuda, 1988; Van Hooydonk et al., 1986). Gelation in UHT milk is one of the most common problems encountered in the dairy industry (Datta & Deeth, 2001). A gel is a three dimensional protein matrix formed during heating by the β-lactoglobulin interacting with κ-casein to form β-κ complexes. Proteases cleave the peptide bond which anchors κ-casein to the casein micelle, facilitating the release of β-κ complexes from the micelle. Aggregation of β-κ complexes forms a three-dimensional network of cross-linked proteins which produce a gel when a critical concentration is reached (Datta & Deeth, 2003). A study revealed that removal of calcium caused prolonged coagulation and production of a weaker gel which was accompanied by ionic calcium reduction (Sharma & Sindhu, 2001). To examine the role of ionic calcium in gelation and proteolysis, it was reduced in heated milk by two sequestering agents. The effect of ionic calcium reduction and its effect on gelation and proteolysis were studied in milk samples with added trypsin, chymosin and *Pseudomonas fluorescens* 416.

2. Materials and Methods

2.1 Processing of UHT Milk

Raw milk was processed on an APV junior UHT plate heat exchanger with two stages of heating involving hot water (80 °C) and steam (112-144 °C) . A constant flow rate was used, giving a residence time of 2 s in the holding tube as described in detail by Browning et al. (2001). Homogenisation took place between the heating stages at about 170 bar. Raw milk was heated at 142 °C for 2 s. This was followed by cooling and storage at 2 °C prior to laboratory analysis.

In a preliminary experiment, two sequestering agents TSC and SHMP were initially each added at 0.5%, 1%, 1.5% and 2% (w/v) to UHT milk. These levels corresponded to TSC (1.36, 2.72, 3.4, 6.8 and 1.02 mM) and SHMP (0.65, 1.31, 1.63, 3.27 and 4.9 mM). Samples were thoroughly mixed using magnetic stirrers for 30 min. Preliminary results indicated that more

than 40% calcium had been reduced and hence lower levels of sequestering agents (0.04 - 0.08%) were proposed and used.

2.2 Samples Inoculation

The mechanism of gelation by trypsin (10,100 units/ mg protein; Sigma-Aldrich St Louis, USA), chymosin (chymax Plus batch 071101, Hansen, Denmark) and *Pseudomonas fluorescens* NCIMB 702085 (416) were obtained from the departmental stock culture, maintained at -80 °C were studied. This was done by adding 8.705 x 10^{-3} Units of trypsin equivalent to 248 BAEE units, 0.03% (v/v) chymosin and 10^{6} cfu/mL *Pseudomonas fluorescens* 416 to heated milk. Samples were stored at 25 °C for 2½ weeks to monitor gelation and ionic calcium concentration and some were incubated at 37°C for 2 h to study proteolysis.

2.3 Ionic Calcium Monitoring

Ionic calcium levels were measured was carried out at room temperature (20±0.5 °C) using a minimum of 35 µL sample before inoculation of the agents and after incubation (except gelled samples and samples inoculated with *Pseudomonas fluorescens* 416) by a Ciba corning 634 ISE Ca^{2+}/ pH analyser (Bayer Ltd., Newbury,UK. The electrodes were washed with a deproteinising solution (Siemens Medical solutions Diagnotics Tarrytown, New York, USA) containing active pepsin diluted in a solution containing NaCl, KCl, $CaCl_2$, LiCl and HCl and a conditioning solution (Siemens Medical solutions Diagnotics Tarrytown, New York, USA) consisting mainly of NaCl. Standardisation by Ca^{2+} standard solution (1.25 mM) was automatically performed daily. A calibration curve was constructed by using known calcium standards of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM. Three readings (in mV) were taken, averaged and plotted against log (Ca) mM. The concentration of calcium was obtained from the equation of the calibration curve. Results from the standards are given in Appendix 1.

2.4 Gelation Monitoring

To study the effect of ionic calcium reduction on gelation, samples with added trypsin (248 BAEE units), chymosin (0.03%) and inoculated with *Pseudomonas fluorescens* 416 (10^{6} cfu per mL) were monitored and recorded daily. Gelation was indicated by the high resistance of milk to flow when poured out from the container**.**

2.5 Proteolysis Monitoring

2.5.1 The Effect of Ionic Calcium Reduction on Proteolysis

Proteolysis of reduced ionic calcium sample was induced by incubating them at 37 °C for 2 h followed by clarification to obtain 6% Trichloroacetic acid (TCA) and pH 4.6 soluble extracts. Fifty 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 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 RP-HPLC method to determine breakdown products. All samples were analysed in triplicates.

2.5.2 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Analysis by HPLC was performed on a Dionex chromeleon equipment consisting of a P580 pump (Dionex corporation, Munchen, Germany) with a photodiode array detector (Dionex PDA-100, Munchen, Germany), an automated sample injector a STH 585 version 2.5 HPLC

column compartment with 150 × 4 mm reverse phase column (SGE 150 GL4-C-P-8/5, Australia) at 40 °C. Data analysis was computed by the Chromeleon Datasystem software v. 6.50 SP4 Build 1000. The flow rate was 0.75 mL/min and detection was by a UV/Vis detector at 210 nm. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC grade water whereas Solvent B was 0.1% (v/v) TFA in HPLC-grade acetonitrile. The volume injected was 50 µL. During the first 25 minutes, solvent B was increased from 15% to 35%. After 5 min, it was increased to 100% B and held for 10 min. The column was then equilibrated at 15% B for 10 min in readiness for the next sample injection.

2.6 Statistical Data Analysis

Data was–analysed using excel program for Analysis Of Variance (ANOVA) and simple regression analysis. All analyses were carried out in triplicates, and results were expressed as mean ± standard deviation (SD).

3. Results

3.1 Effect of Sequestering Agents on Calcium Reduction

Table 1, 2 and 3 show results of preliminary trials to assess the optima concentration of agents in reducing ionic calcium. It shows that there was more than 50% reduction of calcium (76.9% and 53.7%) respectively by 8.17 mM SHMP and 17 mM TSC respectively as shown on Table 1. The reduction levels were high and hence lower levels were proposed to cause not more than 20% reduction of ionic calcium.

A second trial with reduced agents concentration showed respective reduction of 44.4% by 3.40 mM TSC and 53.8% by 1.63 mM SHMP (Table 2) leading to further attempt use lower concentrations of sequestering agents so as to establish the lowest ionic calcium reduction that would promote gelation.

Further concentration reduction to 1.36 mM for TSC and 0.65 mM for SHMP caused 17.36 and 19.6% reduction of ionic calcium respectively. It was obvious that SHMP caused more reduction in ionic Calcium than TSC.

Table 1. The effect of added TSC and SHMP on ionic calcium reduction in milk at 25°C

Sequestering agent	**ConcentrationmM)**	***Free ionic Ca (mM)**
TSC	0.00	1.336± 0.92
	0.68	1.198± 0.63
	1.36	1.104± 0.81
	1.70	0.933± 0.69
SHMP	0.00	1.336± 0.87
	0.33	1.165± 0.93
	0.65	1.074± 0.64
	0.82	0.956± 0.79

Values are expressed as mean±SD (n=3).

3.2 Effect of Sequestering Agents on Calcium Reduction

Table 4 indicates that 1.36 mM and 2.72 mM TSC reduced ionic calcium by 8.6 and 13.9 %, respectively, from control milk (without trypsin addition). However, at the same level of TSC in trypsin added sample, the ionic calcium was reduced by 10.4 and 17.0%, respectively indicating lower Ca reduction. Addition of 0.65 mM and 1.01 mM SHMP, resulted in ionic calcium reduction by 17 and 31% from the control milk whereas 11 and 23% was reduced from trypsin treated samples. Based on these results, it is notable that addition of trypsin generally increased the ionic calcium level in milk.

Table 2. The effect of ionic calcium reduction by TSC and SHMP stored at 25 °C

Samples	*Free ionic Ca (mM)
UHT Milk (No sequesters)	1.309± 0.57
UHT Milk with 1.36 mM TSC	1.196± 0.92
UHT Milk with 2.72 mM TSC	1.127± 0.46
UHT Milk with 0.65 mM SHMP	1.083± 0.68
UHT Milk with 1.31 mM SHMP	0.904± 0.05
UHT milk with trypsin (No sequesters)	1.704± 0.86
UHT Milk with trypsin and 1.36 mM TSC	1.526± 0.72
UHT Milk with trypsin and 2.72 mM TSC	1.416± 0.81
UHT Milk with trypsin and 0.65 mM SHMP	1.520± 0.61
UHT Milk with trypsin and 1.31 mM SHMP	1.319± 0.59

Values are expressed as mean±SD (n=3).

3.3 Effect of Sequestering Agent on Gelation

Table 5 shows the gelation times of various UHT treated samples at 25 °C. It was interesting to note that only one sample inoculated with *Pseudomonas fluorescens* 416 (no sequesters) gelled on day 2, but none of the other samples inoculated with *Pseudomonas fluorescens* 416 with added sequestering agents gelled.

Table 3. Gelation of UHT treated milk samples with sequestering agent stored at 25° C

Sample	Gelation day
Ps. fluorescens 416 with 3.40 mM TSC	NG
Ps. fluorescens 416 with 6.80 mM TSC	NG
Ps. fluorescens 416 with 10.20 mM TSC	NG
Ps. fluorescens 416 with 1.63 mM SHMP	NG
Ps. fluorescens 416 with 3.27 mM SHMP	NG
Ps. fluorescens 416 with 4.90 mM SHMP	NG
Ps. fluorescens 416 (NO SEQUESTERS)	2
UHT with 3.40 mM TSC	5
UHT with 6.80 mM TSC	9
UHT with 10.20 mM TSC	9
UHT with 1.63 mM SHMP	9
UHT with 3.27 mM SHMP	9
UHT with 4.90 mM SHMP	12
UHT (NO SEQUESTERS)	5
Chymosin UHT with 3.40 mM TSC	4
Chymosin UHT with 1.63mM SHMP	5
Chymosin UHT (NO SEQUESTERS)	0

*NG- no gelation observed; * all samples were observed in triplicates.*

3.4 The Effect of pH Reduction on Gelation of UHT Milk at 25 °C

Figure 1 shows the pH of the various samples stored for 9 days. It is obvious that the pH was decreasing with time, with inoculated samples showing highest levels of decrease as compared to non-inoculated samples.

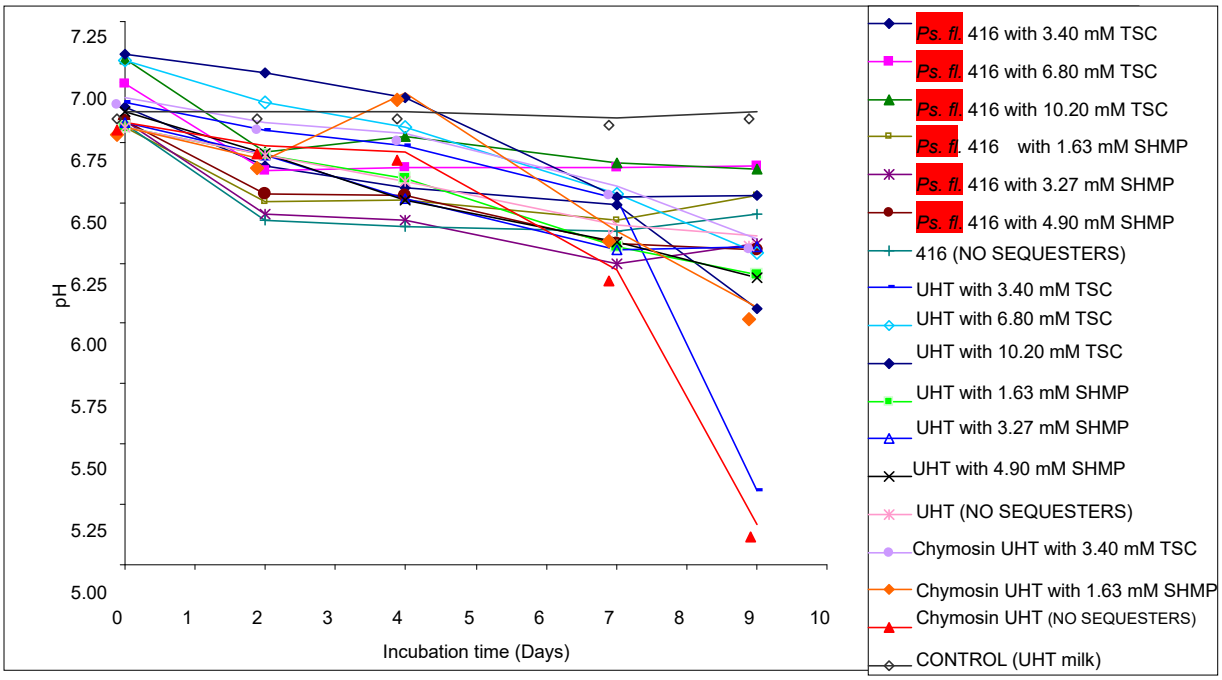

Figure 1. pH of UHT milk samples incubated with sequestering agents and *Pseudomonas fluorescens 416* at 25 °C for 9 days *(* all samples were observed in triplicates)*

3.5 Effect of Sequestering Agent on Proteolysis

The effect of sequestering on proteolysis of milk samples are shown in Figures 2 and 3. With a few exceptions the degree of proteolysis increased with time for all samples except the control sample which remained constantly low throughout the incubation time. Proteolysis in latter samples was low (less than 140 mAU*min), possibly from activities of native enzymes in milk (Datta & Deeth, 2003; Nielsen, 2002). There was no clear relationship between the effect of proteolysis with either the type or quantity of sequestering agent added in non-inoculated samples. As both sequesters showed increased proteolysis with time, this suggests that in the current study sequestering agents did not influence proteolysis.

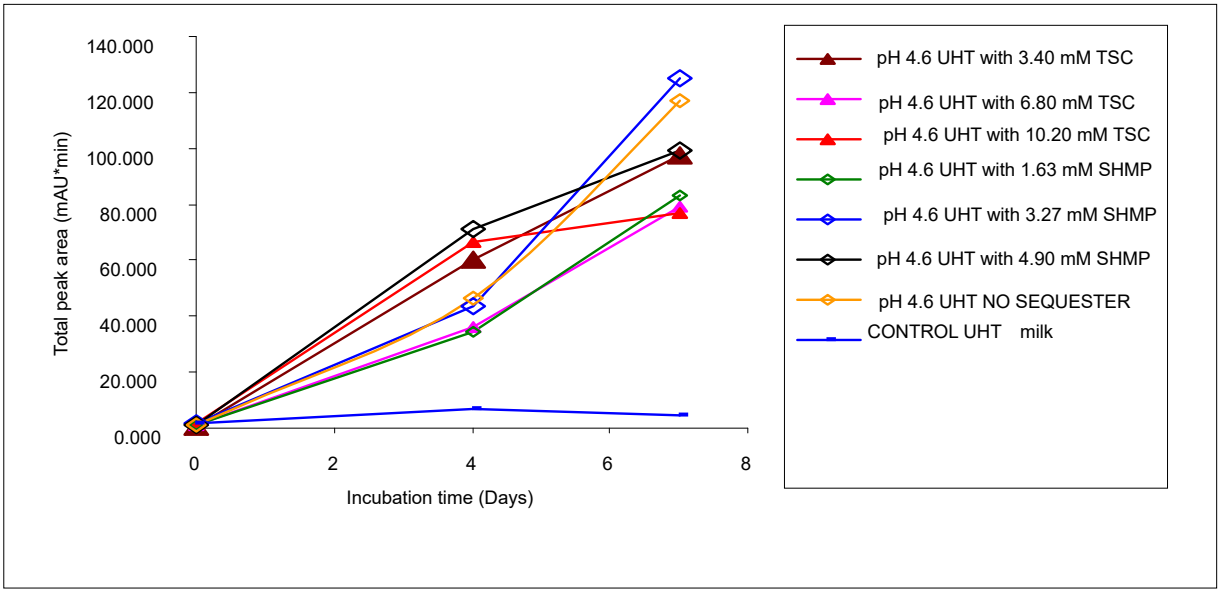

Figure 2. Proteolysis of pH 4.6 soluble extracts of UHT skim milk with added sequestering agents analysed by the RP-HPLC method *(* all samples were observed in triplicates)*

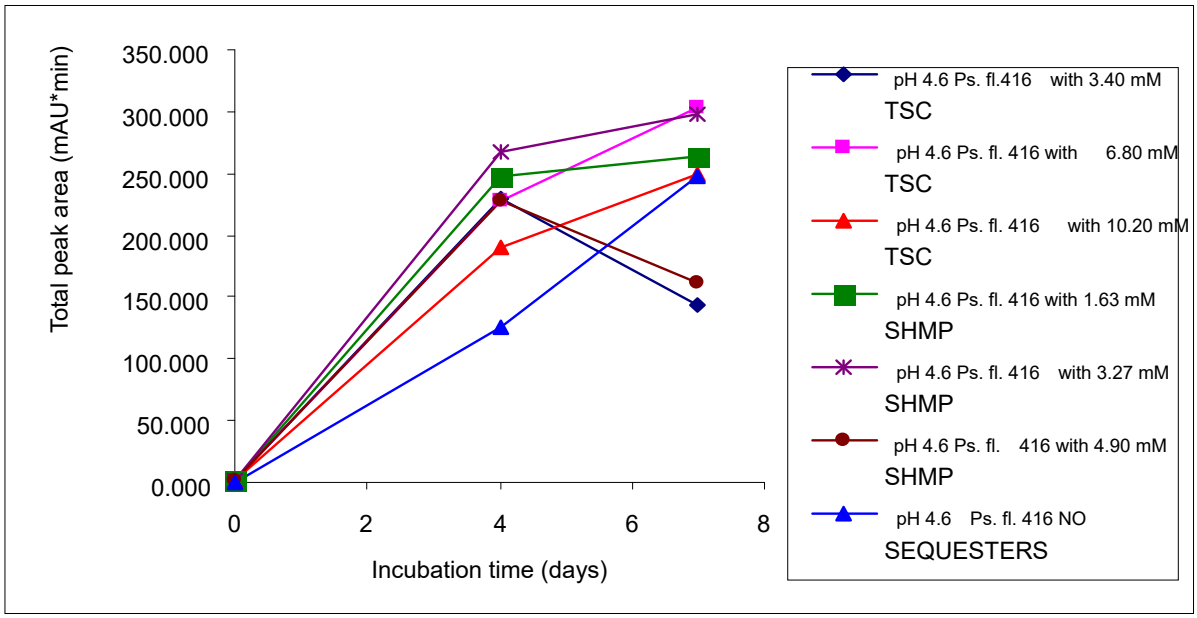

Figure 3. Proteolysis of pH 4.6 soluble extracts of UHT skim milk inoculated with *Pseudomonas fluorescens* 416 (with sequestering agents) analysed by the RP-HPLC method (**all samples were observed in triplicates*)

4. Discussion

4.1 The Effect of Ionic Calcium Reduction by Sequestering Agents on Gelation

TSC was less effective in lowering the level of ionic calcium as compared to SHMP, which caused higher reduction in ionic calcium. A study on soymilk revealed that increasing SHMP concentration reduced Ca ion concentration (Faka et al., 2009; Pathomrungsiyounggul et al.,

2007). Similar results were also observed from the work of Tsioulpas (2005) who stated that SHMP had a strong chelating effect in milk. In one study it was revealed that TSC treated samples gelled more rapidly than either controls or samples with added SHMP (Kocak & Zadow, 1985). The researchers concluded that TSC accelerated age-gelation, although they did not explain the mechanism. It was probably due to its lower affinity for calcium ions in comparison to SHMP.

4.2 The Effect of pH Reduction on Gelation of UHT Milk at 25 °C

The observed finding that pH decreased steadily with incubation time was also reported previously (Kocak & Zadow, 1985). The only exceptions were the two samples whose pH was below 5.5 on the 9th day, since most samples had a pH of above 6.25±0.02 during incubation. As the two samples with the lowest pH values (UHT milk with 3.40 mM TSC and 0.03% chymosin treated UHT milk without sequesters) did not show any relationship in terms of treatments, it is suggested that the pH drop was most probably due to bacterial contamination as sodium azide was not used. The control sample however, was constant (6.77±0.02) throughout storage. It was observed that TSC increased the pH of the samples on day 0 and the increament was direct proportional to concentration (Figure 1). However, the pH eventually decreased with time.

It has been previously reported that TSC increases the pH of samples (Faka et al., 2009; Tsioulpos, 2005). This is achieved because the reduction in ionic calcium caused by TSC lowers the ionic calcium in the serum and this will increases the pH to maintain equilibrium as shown in the equation suggested by (Horne, 2003)

$$3Ca^{2+} + 2HPO_4^{2-} \leftrightarrow Ca_3(PO_4)_2 + 2H^+$$

The same mechanism was described during acidification. In the serum, calcium exists bound with either phosphate or citrate, or as free calcium ions, whereas in the colloidal phase it exists as colloidal calcium phosphate (CCP). Calcium and phosphorus migrate from the micelle and calcium phosphate becomes more soluble. This results in increase of free ionic calcium, causing liberation of hydrogen ions and decrease in pH (Augustin & Clarke, 1990). The distribution of caseins and calcium between the serum and micellar phase is pH and temperature dependent. Furthermore, upon acidification casein micelles lose their colloidal stability and start to aggregate and gel. Vasbiner et al. (2003) studied two methods of acid-induced gelation: acidification of milk at temperatures of 20 to 50 °C (T-pH) and decreasing the pH at 20 °C to just above the gelation pH and subsequently inducing gelation by increasing the temperature (pH-T). It was revealed that gelation kinetics and the properties of the final gels obtained are affected by the gelation route applied. The pH-T milks gelled at higher pH and lower temperature and the gels formed are stronger and show less susceptibility to syneresis

4.3 The Effect of Ionic Calcium Reduction in UHT Milk on Gelation at 25 °C

Trypsin, chymosin and *Pseudomonas fluorescens* 416 were used so as to accelerate proteolysis and gelation and to determine the lowest level of ionic calcium responsible for gelation.

It is obvious form Table 4 that addition of trypsin increased the ionic calcium level in milk. The possible explanation is that when trypsin was added to milk, it disrupted the casein micelles. This would cause shift in equilibrium of ionic calcium between the soluble and colloidal phase.

The colloidal calcium phosphate precipitates causing calcium ion liberation in the serum (Lin et al., 2006). This is an important finding which needs further investigation. Lack of gelation observed in samples inoculated with *Pseudomonas fluorescens 416* even after 9 days of storage gelation on day 2 with *Pseudomonas fluorescens* without sequesters indicates the role of the bacterial proteases to gelation. Investigation into the gelation of milk by *Pseudomonas fluorescens* proteinases confirmed that a gel formed depending on the activity of the organism before heat treatment (Costa et al., 2002; Law, 1979; Law et al., 1977) ,. It was also revealed that proteases from psychrotrophic bacteria are capable of causing gelation of UHT milk (Cogan, 1977). Therefore, it may be concluded that enzymes from the *Pseudomonas fluorescens* 416 initiated proteolysis causing gelation in the sample without sequestering agents. Furthermore, lack of gelation for samples with added sequestering agents could be due to low levels of calcium found in these samples. This is an interesting finding which indicates the possible role of ionic calcium to gelation. It may thus be concluded that proteolysis caused by *Pseudomonas fluorescens 416* in this study was not able to cause gelation. Calcium is critical for milk gelation. It is possible that sequestering agents inhibited bacterial activity. This needs further investigation. Research has shown that proteinases from psychrophic bacteria, mostly *Pseudomonas spp* are stable at room temperature but survive pasteurisation and UHT treatment (Chavan et al., 2011). Further study on this is significant because it has an implication on milk that might be required for yoghourt or other fermented milk products. It would be interesting to observe the influence of higher levels of proteinases on milk with reduced ionic calcium.

It is also obvious from the table that gelation was delayed in UHT milk samples with sequesters, whereby the lower the concentration of the sequestering agents, the longer the time for gelation and vice-versa. It was also obvious that milk without sequestering agents gelled earlier than the rest because of the role of ionic calcium on gelation. However, some researchers did not find any correlation between ionic calcium with onset of gelation (Kocak & Zadow, 1985). The sample that gelled on day 12 however was from the highest SHMP used. The delayed gelation observed in SHMP is in accordance with previous findings by Datta and Deeth (2001).

The chymosin treated sample (without sequesters) gelled on the same day of incubation compared to samples with sequestering agents which gelled on the 4th and 5th day. This confirms that although chymosin is crucial for proteolysis and gelation, a critical concentration of ionic calcium is required to initiate this process. This finding is of high significance for cheese manufacture. It was revealed that rennet coagulation time was lower for milk with a higher Ca^{2+} concentration, but curd firmness was not related to Ca^{2+} concentration (Datta & Lewis, 2015). There was a poor correlation between the pH reduction caused by acid addition and that resulting from increasing temperature. Sediment formation was related to pH change at high temperature (Lewis, 2011). It was concluded from one study that the nature of the calcium- and heat-induced gelation or coagulation of milk is influenced, and can be manipulated, by a combination of factors including ionic calcium concentration, temperature, milk pH and milk solids level (Deeth & Lewis, 2015). The technology of manufacturing calcium-induced milk coagulums and gels has potential for the development of novel dairy products (Lakshmi, 2013).

4.4 Effect of Proteolysis of UHT Milk at 25 °C

The information should highlight the relationship between ionic calcium reduction by sequestering agents and bacterial activity with proteolysis which will confirm the role of ionic calcium reduction to gelation and proteolysis. UHT milk was used because it was concluded from previous studies that processing at higher temperature lowered proteolysis and hence susceptibility of UHT milk to spoilage (Chove et al., 2013, 2014). This is probably through denaturation of enzymes responsible for proteolysis, hence decreased proteolysis.

This trend is unclear but it may be due to complexing of SHMP or TSC with the breakdown products formed during proteolysis.

Although inoculated samples had higher rates of proteolysis than non-inoculated samples, no clear relationship could be established between proteolysis and gelation at the levels of sequestering agents used in the current study. Neither did the ionic calcium reduction in this study confirm the relationship with proteolysis. Although samples inoculated with *Pseudomonas fluorescens* without added sequesters gelled on day 2 (as explained above), Figure 3 shows that they had the lowest rate of proteolysis. Studies regarding the link between proteolysis and gelation are inconclusive. It has been reported that addition of SHMP (0.1%) did not influence the rate of proteolysis, but inhibited the aggregation process (Kocak & Zadow, 1985). It was confirmed in a study that the stabilising effect of SHMP was independent of proteolysis in unconcentrated UHT milk (Snoeren et al., 1979). Datta and Deeth (2001) stated that although SHMP inhibited gelation, it did not affect proteolysis. It was documented that addition of 0.15 mg/L plasmin caused gelation of UHT milk in 90 days (Kohlmann et al., 1991), indicating the significance of minor proteolysis by plasmin prior to gelation (Chove et al., 2011). It was also revealed that neither gelation nor proteolysis (9 months at 20 °C) was observed in a study where serine protease inhibitors were added to UHT skim milk to inhibit plasmin activity, implicating the role of plasmin in proteolysis preceding gelation (De Koning et al., 1985). Longer observation time at 20-25 °C would be more useful to study the effect of storage on proteolysis and gelation.

5. Conclusion

This paper highlighted the role of sequestering agents in ionic calcium reduction and how this influenced gelation and/or proteolysis. Results demonstrated that addition of sequestering agents reduced ionic calcium in the samples through the formation of complexes with the ions. SHMP reduced more ionic calcium than TSC in UHT milk as it has more chelating power. Ionic calcium reduction was in most cases accompanied by an increase in pH which was most evident with TSC at 25 °C. Gelation of non-inoculated samples on day 2 indicates the role of Ca in gelation.

Trypsin, chymosin and *Pseudomonas fluorescens* 416 were used to accelerate proteolysis. Increased ionic calcium in trypsin treated samples requires more investigation. Although proteolysis was higher in samples inoculated with *Pseudomonas fluorescens* 416, no clear relationship was established between proteolysis and gelation in UHT milk.

This study has shown some indication of the role of ionic calcium levels to proteolysis and gelation. However, longer time study using sodium azide to prevent bacterial contamination

would be required to confirm these findings and give more comprehensive results and conclusion.

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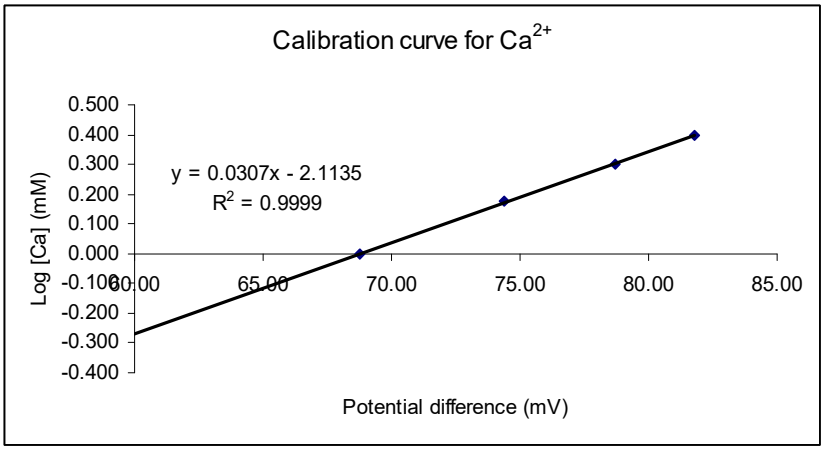
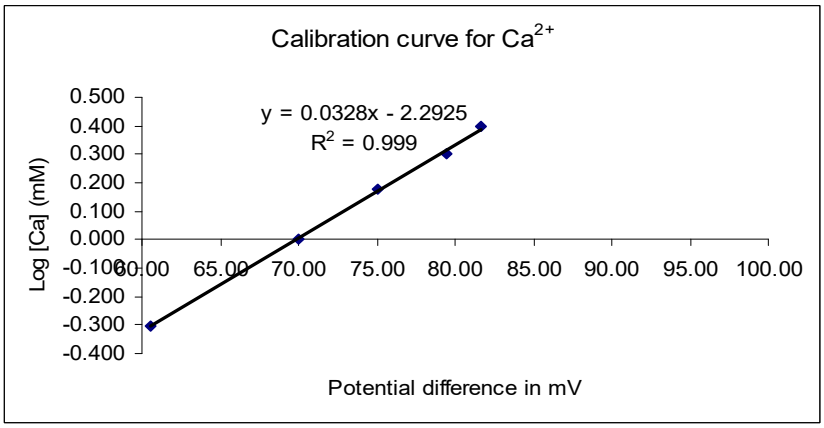
Appendix 1

Calibration curve for free ionic calcium as analysed by the ionic calcium analyser



Calibration curve for free ionic calcium as analysed by the ionic calcium analyser



Calibration curve for free ionic calcium as analysed by the ionic calcium analyser