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Detection of proteolysis in milk by four selected methods

A thesis submitted by

Lucy Mlipano Chove

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Department of Food Biosciences

School of Chemistry, Food Biosciences, Pharmacy

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DEDICATION

To my one and only, Ben and our Berkshire born precious little angels Twilumba Sophie and Lupyana Harry...and to my lovely and really wonderful mum, Sophie Mwambenja (Mama Mlipano), to my brothers and sisters Ben, Juliet, Sr Angela, Sam and Caroline

.....and above all.....

This is for you my beloved Dad, Sefrine Emmanuel Mlipano Mwachotamasege

This has always been for you, only you....for no one wanted it more than you, not even myself!!! How I wish that you were here to see this day!!!You would be the proudest dad in the whole wide world! Everyone would know you have a doctor daughter!

From the first day you drove me to Ngarenaro nursery school in Arusha- you surely had a big dream for your little girl..... then to Oysterbay primary school in Dar es Salaam, then to Kifungilo, then to Jangwani High School and how you carried my suitcase all the way to my room at Sokoine University in Morogoro when I joined the university for my Bachelors degree... And you were there at the airport when I left for Belgium to undertake my Masters degee.....and again in 2005 when I left for Reading to pursue my PhD.... You were always there. . .to see me off and to receive me back again.....It is hard to believe that this time you will not be at the airport...How is life going to be without you dad?I cant imagine! We pray for Gods strength to see us through this difficult time..... Dad, you never missed any milestones in my life. You were always in the front seat, giving all the support and encouragement that I needed. Cheered me all along the way!! And how you liked to tell and listen to stories...I was really looking forward to sharing the stories of my PhD journey and I can almost see you asking....."Aha, say that again, what was it like-what was the most difficult question in your viva and how did you answer that?" I can not imagine that all the jokes, laughter, stories will only remain in our memories.....at least we have such great memories to cherish forever!

Oh dad, I wish you could have waited. .....just a year..... and you would witness it all! Although I can not see you, I am sure that you are watching me from somewhere up there smiling very proudly saying "Once again you have done it my dear, well done my Lucy!" Thank you so much dad for making me what I am. I owe it all to you! You brought so much joy and happiness to my life, I hope I can share it with my family and everyone I meet!!!

You are the best dad ever! Thank you dad!

.....And now, this is for you dad.....

"And he carried me away in the Spirit to a mountain great and high, and showed me the Holy City, Jerusalem, coming down out of heaven from God"

~~~~~~~~~~Revelation 21:10~~~~~~~~~~

LOOK FOR ME (FOR I WILL BE THERE TOO)

"When you finally make your entrance to that city,
Of jasper walls and bright golden avenues,
As you behold all of its beauty and its splendor,
Remember, there's just one request I make of you.

Look for me, for I will be there too,
I realize when you arrive there'll be so much to view;
After you've been there ten thousand years,
A million, maybe two, look for me for I will be there too.

As you go down your list of firsts, there's no question,
You'll want to see your precious loved ones waiting there for you;
Oh, when you've finally shared your story with the last one,
That wants to hear you tell just how you made it through;

Look for me, I'd like to hear it too,
I realize when you arrive there'll be so much to view;
After you've been there ten thousand years,
A million, maybe two, look for me for I will be there too.
Look for me for I will be there too "

.....I love you so much dad and I really miss you.....
.....Until we meet again, you rest in peace daddy! .....
~~~~~~~~~~

DECLARATION

I confirm that this is my own work and the use of all materials from other sources has been properly and fully acknowledged.

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ABSTRACT

Sensitive methods that are currently used to monitor proteolysis are limited due to their high cost and lack of standardisation for quality assurance in the various dairy laboratories. In this study, four methods, trinitrobenzene sulphonic acid (TNBS), reverse phase high pressure liquid chromatography (RP-HPLC), gel electrophoresis and fluorescamine, were selected to determine their suitability for the detection of proteolysis in milk by added trypsin, plasmin, *Pseudomonas fluorescens* NCIMB 701274 (414) and *Pseudomonas fluorescens* NCIMB 702085 (416) and *Bacillus licheniformis* enzymes. Raw, pasteurised and UHT milk used to study proteolysis were analysed by the four methods. *Pseudomonas fluorescens* enzyme was extracted in this study since commercial enzymes are not available. All four methods confirmed that *Pseudomonas fluorescens* 416 was more proteolytic than *Pseudomonas fluorescens* 414. Dialysis was effective in the purification of *Pseudomonas fluorescens (Ps. fl.)* enzymes increasing the detection limit for the fluorescamine method, which had a low upper detection limit. Prominent peaks by RP-HPLC were shown to occur between 20-30 min for *Pseudomonas fluorescens* but 20-25 min for *Bacillus licheniformis*. RP-HPLC confirmed a peptide peak at 35 min in pH 4.6 soluble extract, which was absent in 6% TCA soluble extract, was from plasmin. Casein breakdown profiles by gel electrophoresis confirmed preference for β-casein degradation over α and κ-caseins by both *Ps. fl.* and *B. licheniformis*.

Comparison of raw and pasteurised milk (72, 85 and 90^{0}C for 15 s) revealed that pasteurisation was insufficient to inactivate plasmin inhibitors. The pH 4.6 and 6% TCA soluble extracts of UHT skim milk with added trypsin or plasmin showed high correlations ($R^2 > 0.93$) by the TNBS, fluorescamine and RP-HPLC methods, confirming increased proteolysis during storage. Gel electrophoresis showed that breakdown products from trypsin were similar to plasmin although the former caused more extensive proteolysis than the latter due to higher enzyme activity. γ-caseins, formed as a result of β-casein degradation disappeared (1484 and 742 BAEE units of added trypsin on days 3 and 7) due to extensive proteolysis. This finding had not previously been reported. Milk processed at high temperatures (110, 120, 130 and 142^{0}C for 2 s) had lower proteolytic activities than raw milk and milk heated at 85^{0}C implying inactivation of plasmin at temperatures of 110^{0}C and above. This was observed in all the methods assessed.

The TNBS method was recommended on the basis of its accuracy, reliability, simplicity and cost.

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CHAPTER 1 INTRODUCTION

1.1 Rationale of the study

Total world production of milk is 560 x 10^6 tonnes per annum, of which 85% is from cows, 11% buffalo, 2% by sheep and 2% by goats (Fox, 2001). In Tanzania, total annual milk production is 1.47 x 10^6 tonnes per annum from 18.8 million dairy cattle in the country (Njombe and Msanga, 2007). High quality raw milk is essential for the production of high quality milk products. The dairy sector in Tanzania and many developing countries is hampered by, among other factors, by high production cost, poor infrastructure from collection and processing, poor raw milk quality due to poor handling practises, lack of cooling facilities during transportation and lack of collection centres. It was reported that quality assurance systems are either weak or absent and hence processing plants face stiff competition from imported milk (Kurwijila and Boki, 2004).

Most of the problems mentioned above result in prolonged storage between production and processing which affects the quality of raw milk and results in losses through spoilage by native or bacterial enzymes or both. Proteolysis and lipolysis are the two most important biochemical changes responsible for the degradation of milk during storage. The current study is focused on proteolysis of milk.

Proteolysis in milk is brought about either by bacterial or native proteinases naturally found in milk (Nielsen, 2002). Plasmin is the major proteinase occurring in milk of most mammals (Deharveng and Nielsen, 1991). It is part of a complex system consisting of plasminogen (PG), plasminogen activators (PA), plasminogen activator inhibitors (PAI), plasmin (PL) and plasmin inhibitors (PI) (Crudden and Kelly, 2003). It is an alkaline serine proteinase with an optimum pH of 7.5 at 37^{0}C, which readily hydrolyses β-casein, α_{s2}-casein and (more slowly) α_{s1}-casein (Bastian and Brown, 1996). It 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). The hydrolysis may have positive or negative effects depending on the product and the level of activity (Nielsen, 2005). It plays a positive role in cheese ripening for many varieties of cheese such as Swiss, Gouda and Romano (Borda *et al.*, 2004). The negative effects of plasmin include development of age-gelation in UHT milk (Datta and Deeth, 2001), bitterness and other off-flavours in dairy products (Ma *et al.*, 2003) and reduction in coagulation properties of milk and cheese (Mara *et al.*, 1998). Plasmin and its complex system is highly resistant to heat treatment and is also able to reactivate during storage, thereby reducing shelf life of milk and milk products (Bastian and Brown, 1996). It survives pasteurisation and many UHT processes. The inhibitors present in fresh milk are heat labile whereas the activators are known to be heat stable (Richardson, 1983b). 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.

Bacterial contamination is usually caused by *Pseudomonas fluorescens*. Although the level may be low initially, it may increase to significant numbers under favourable conditions to produce heat resistant exo-enzymes which survive high heat processing and cause spoilage to milk.

Several methods have been used to determine proteolytic activities in milk and milk products. These methods are based on monitoring either main proteolytic products, measuring enzymatic activity or directly quantifying the amount of components of plasmin system. The currently used sensitive fluorimetric and spectrophotometric

methods use specific substrates to monitor plasmin activity. The major problem associated with monitoring plasmin activity in milk is the lack of standardisation of assay methodologies. It was stated that multiple assay methods are used by different research groups and therefore the results from each type of analysis yielded different kind of information, and this makes comparison of the methods difficult (Kelly and Fox, 2006b). Moreover, sample preparation prior to analysis varies as some methods eliminate the inhibitors while others do not. Some activities were measured directly in milk whereas others were determined in buffer after plasmin isolation from milk. However, to date, no single method is ideal for this purpose (Le *et al.*, 2006). Knowledge of the factors that produce biochemical changes leading to proteolysis is important so as to help processors and manufacturers find appropriate conditions of treatment and storage (Pereda *et al.*, 2008).

Due to economic constraints in the developing countries, the sensitive methods that are currently used to monitor proteolysis as mentioned above are too expensive and can not be affordable for routine analysis in the dairy laboratories. However, even in developed countries, there is no routine test that is recommended for quality assurance to be able to pinpoint raw milk with high proteolytic activity, in order to prevent it being used for products where it might cause problems such as UHT milk and milk powders. In view of this, there is a great need to assess the commonly used methods and recommend a method to be used for such purposes.

Since residual levels of plasmin can cause defects in milk and milk products, there is a great need to develop a simple, rapid and accurate method for the detection of low levels of protease activity in milk.

This research aims to assess some of the commonly used methods in the dairy industry for the determination of proteolysis (with focus on plasmin and bacterial enzymes) in raw, pasteurised and UHT milk. Based on the results obtained, a simple, rapid and accurate method will be proposed as a standard method to be used in the dairy industry.

1.2 Aims and Objectives

From the above, it is clear that there is an urgent technical need for the assessment of methods used for the detection of proteolysis in milk. The main aim was to assess the current methods used in the dairy industry and suggest the best method to be used as a routine method in the laboratory to detect proteolytic activity in milk. Raw and processed milk, with or without bacterial and/or native enzymes, was examined for their proteolytic activities. Exogenous enzymes were also added to observe their effect on proteolysis at low and higher concentrations.

In view of the various factors that affect proteolysis in milk, the following study was carried out:

- To study the effect of plasmin induced proteolysis in milk
- To study the effect of bacterial proteolysis in milk
- To investigate the susceptibility of raw, pasteurised and high temperature processed milk to proteolysis during storage
- To study the relationship between proteolysis and gelation as caused by bacterial enzymes and plasmin, and the role of ionic calcium in gelation
- To suggest reliable and accurate methods for evaluating proteolysis in milk

CHAPTER 2 LITERATURE REVIEW

2.1 Milk proteins

Milk and dairy products are important components of the human diet in many parts of the world. They serve both nutritional and physiological functions. Bovine milk contains 4.0% fat, 4.8% lactose, 0.8% ash, and 3.4% protein and 87% water (O'Connor, 1994). The physiological functions include the roles of enzymes, enzyme inhibitors, immunoglobulins, growth factors, hormones and anti-bacterial agents (Fox, 2003). Fig 2-1 shows the distribution of various proteins in milk.

2.1.1 Casein

About 80% of proteins in milk are present in casein micelles which are large spherical complexes containing 92% protein and 8% low molecular mass inorganic salts, the predominant one being calcium phosphate (Schmidt, 1980). The whey proteins make up the remaining 20% of the total milk proteins. Casein exists in four major forms αs_1, αs_2, β, and κ -casein, with αs_1casein being the predominant casein (12-15 g/L). Several genetic variants of these caseins occur, some more often than others as shown in Figure 2-1. The lack of tertiary structure accounts for the stability of caseins against heat denaturation because there is very little structure to unfold.

Techniques used to separate caseins from the whey proteins include ultracentrifugation at 100,000xg for 1h, salting out with $(NH_4)_2SO_4$ at 260 g/L, microfiltration, gel filtration, rennet coagulation, precipitation by 40% ethanol, cryoprecipitation and isoelectric precipitation. Although several techniques are available to separate caseins from whey proteins, the most commonly used method is isoelectric precipitation (Fox, 2003). This is achieved by lowering the pH of milk to 4.6 whereby the caseins aggregate to form a coagulum while whey proteins remain soluble. Although HCl is widely used for precipitation, acetic acid and lactic acid may be used (Fox, 2003). Dilution of milk with

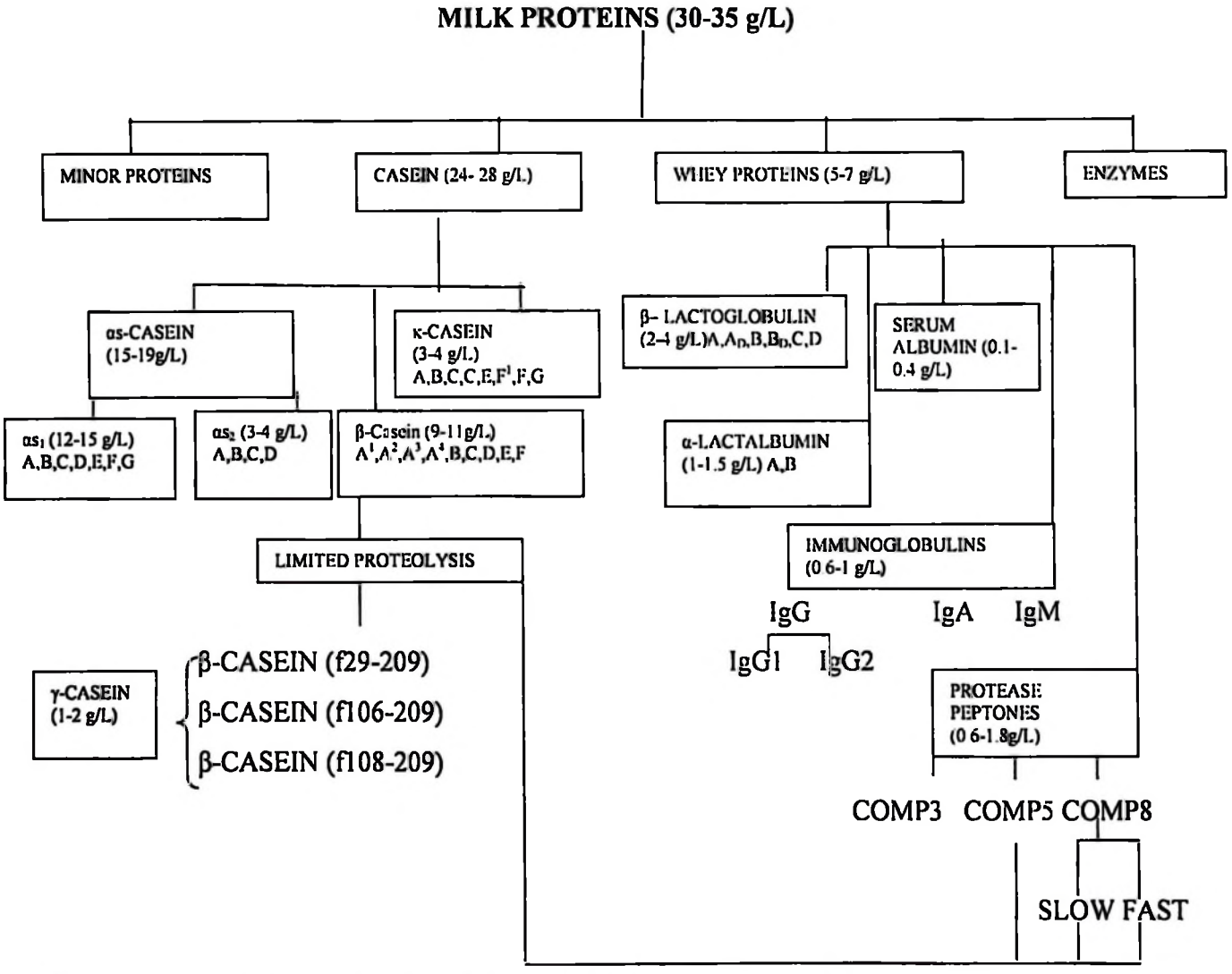


Figure 2-1 Distribution and concentration of proteins in bovine milk (Swaisgood, 2003)

water is usually carried out so as to obtain a finer precipitate, with less inclusion of other compounds.

2.1.1.1 α_{s1} casein

α_{s1}- casein is the principal casein with 12-15 g/L and six variants (Fig 2-1). It has a molecular weight 23 kDa and has a high proline content. The high number of proline residues in caseins causes particular bending of the protein chain and inhibits the formation of close-packed, ordered secondary structures. It is hydrolysed by plasmin (Table 2-1).

2.1.1.2 α_{s2} -casein

α_{s2}- casein has a molecular weight of 25 kDa. There are 4 variants of α_{s2} casein (Fig 2-1). It is hydrolysed by plasmin as shown on Table 2-1.

2.1.1.3 β-casein

β-casein has a molecular weight of 24 kDa. Eight variants of β casein occur. It has a highly charged N-terminal region and a hydrophobic C-terminal region. It is hydrolysed by plasmin.

2.1.1.4 κ-casein

κ-casein has a molecular weight of 19 kDa and proline residues. It has 8 variants (Fig 2-1). Its hydrolysis by plasmin is controversial (Table 2-1).

2.1.2 Whey proteins

The major whey proteins in milk are β-lactoglobulin (50% of total whey proteins), α-lactalbumin (20% of total whey proteins), bovine serum albumin (BSA: 0.3 -1.0% of total N), immunoglobulins (3% of total N in mature milk; 10% of total N in colostrum) and proteose peptones representing 0.6-1.8 g/L (Fig 2-1).

2.1.2.1 β-lactoglobulin (β-lg)

β-lg is the principal whey protein and represents 12% of the total protein in milk. Some genetic variants have been identified in milk (Figure 2-1). Its molecular mass is ~ 18 kDa, with an isoelectric point of ~ 5.2. It is rich in sulphur amino acids especially cysteine. Following heat denaturation, cysteine reacts with the intermolecular disulphide of κ-casein and significantly affects rennet coagulation and heat stability properties of milk (Fox, 2003). It is very resistant to proteolysis in its native state due to high levels of secondary and tertiary structure.

2.1.2.2 α-lactalbumin (α-la)

α-la represents 3.5% of total milk protein, with a molecular mass of 14 kDa. Two genetic variants exist, A and B (Figure 2-1). α-la has intramolecular disulphide bonds and methionine, but no cysteine (Fox, 2003).

2.1.2.3 Bovine Serum Albumin (BSA)

BSA level in milk is low (0.1-0.4 g/L) and also due to leakage from blood. The molecular weight is ~ 66 kDa (Fox, 2003).

2.1.2.4 Immunoglobulins (Ig)

Although colostrums contains 10% Ig, the level decreases to 0.6-1 g/L in mature milk. The physiological function of Ig is to provide immunity to neonates (Fox, 2003). The variants include IgG1, IgG2, IgM and IgA (Fig 2-1).

2.1.2.5 Proteose peptones

Proteose peptone fraction is defined as the 12% TCA insoluble proteins in acid (pH 4.6) whey prepared from milk heated at 90^0C for 30 min (Rowland, 1938). The principal whey

proteins are denatured under these conditions and hence co-precipitate with the caseins on acidification.

The principal peaks observed on free boundary electrophoresis of milk are 3, 5 and 8 (PP 3, PP 5 and PP 8) respectively. PP 8 was shown to contain 2 peptides (PP 8 f and PP 8 s) by SGE and PAGE. It was confirmed that PP 3 was present only in the whey acid fraction whereas PP 5, PP 8f and PP 8s were found between the casein and whey fractions (Andrews, 1978a; Andrews, 1978b).

2.2 Proteolytic enzymes in milk

Proteolytic enzymes are of great importance to the dairy industry because they are responsible for imparting desirable or undesirable properties to dairy products through changes in flavour and texture.

Proteolysis refers to protein breakdown as a result of protease action. A protease is an enzyme which catalyses the hydrolysis of peptide linkages of protein to produce smaller protein fragments i.e. peptides, peptones, amino acids the extent of which depends largely on the degree of hydrolysis. Proteolytic enzymes that cause problems in milk and dairy products are of two major types, milk proteinases which naturally occur in all milk and bacterial proteinase produced by contaminating bacteria (Nielsen, 2002). The section below will focus on proteolytic enzymes in milk comprising of indigenous, endogenous and exogenous enzymes as categorised by Fox (1981).

2.2.1 Indigenous proteolytic enzymes

There are about 70 indigenous enzymes in milk, 20 of which have been well isolated and characterised (Fox and Kelly, 2006). The first enzyme to be reported was lactoperoxidase in 1881, followed by 7 more enzymes nine years later (Kelly *et al.*, 2006a). These enzymes originate from: animal's blood plasma, leucocytes (somatic cells) and the epithelium or cytoplasm of the secretory cells (Grufferty and Fox, 1988b) .

Indigenous enzymes play a key role in regulation of lactogenesis and are essential components of antioxidants and innate immune system of milk (Silanikove *et al.*, 2006). Since the physico-chemical properties of milk are sensitive to proteolysis, proteinases play a significant role in dairy technology more than any other group of enzymes (Fox, 1981a).

Milk contains a number of indigenous proteases such as plasmin, cathepsins, collagenase, elastase, and thrombin (Kelly *et al.*, 2006a). Non plasmin native enzymes originate from the lysosomes of milk somatic cells, the principal one being cathepsin D (Andrews, 1983 a). These enzymes are important when somatic cells reach > 2.5 x 10^5 cells/mL whereby almost 40% of activity could be due to non-plasmin enzymes (Le Roux *et al.*, 1995). A few of these enzymes will be discussed below.

2.2.1.1 Plasmin and its system

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, 1988b). 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).

The presence of a natural proteolytic enzyme in milk was first reported in 1897 (Babcock and Russel, 1897). This enzyme is known as "plasmin" since it originates from blood plasma. In 1945, Warner and Polis reported similar findings but were unsure whether the proteinase was of indigenous or bacterial origin (Grufferty and Fox, 1988b). In 1960 however, other researchers confirmed that milk did contain a low amount of indigenous proteinase(s) (Harper *et al.*, 1960). It was concluded that Milk Alkaline Protease (MAP) and plasmin were identical because they both had similar optimal pH as well as stabilities to pH and heat and sensitivities to various inhibitors (Kaminogawa *et al.*, 1972). Since then several researches have confirmed that the enzyme is present in milk (Korycka-Dahl *et al.*, 1983; Reimerdes *et al.*, 1981; Harper *et al.*, 1960; Rham and Andrews, 1982 a).

Plasmin (fibrolysin, fibrinase, EC 3.4.21.7) is a trypsin like serine proteinase found in milk and blood and has optimal activity at pH 7.5 and 37^0C (Kelly and McSweeney, 2003). The physiological role of plasmin is to dissolve blood clots, and promote wound healing.

Plasmin structure is composed of amino-terminal heavy/regulatory chain (~60 kDa) with five intramolecular disulphide-linked kringles (Figure 2-2) which are responsible for its proteolytic activity (Fox and McSweeney, 2003; Kelly and McSweeney, 2003; Novokhatny, 2008). The C-terminal light chain of plasmin (~25 kDa) contains the catalytic triad, composed of Ser 195, His 57 and Asp 102. The molecular weight of plasmin was found to be 100 kDa on Sephadex G-200 (Dulley, 1972), 48 kDa on Sephadex G-100 (Kaminogawa *et al.*, 1972) and 81 kDa by amino acid sequencing (Castellino and Powell, 1981). From these variations, it was suggested that the enzyme existed as a dimer under certain conditions (Fox, 1981a).

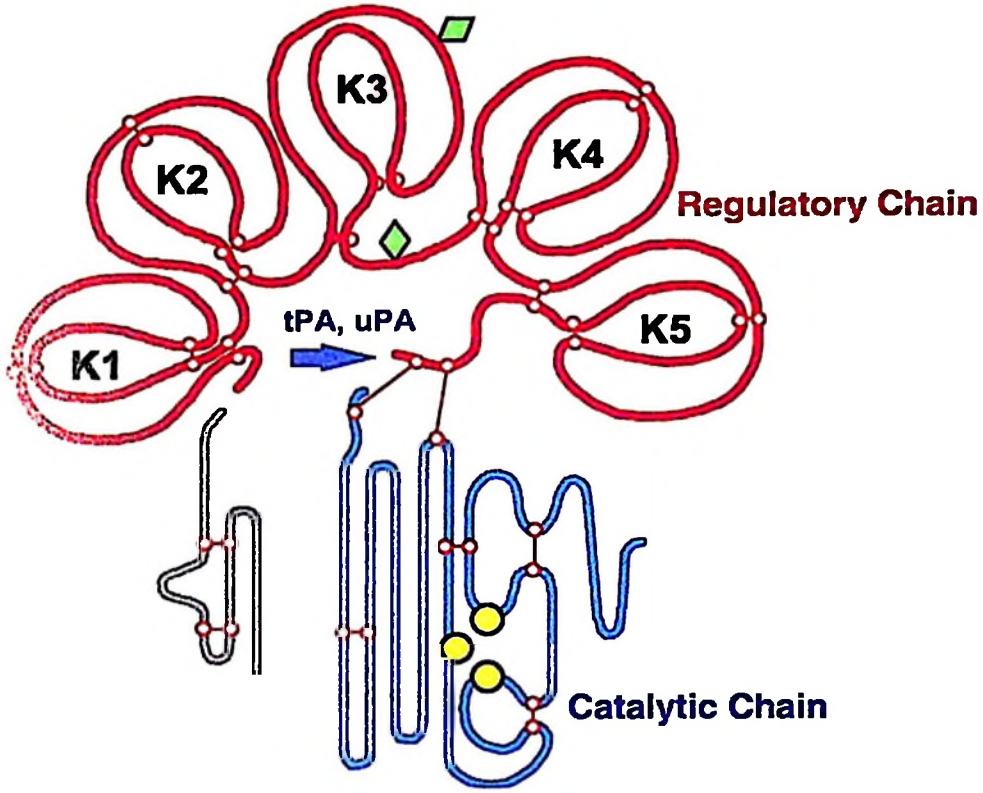


Figure 2-2 The structure of plasmin/plasminogen. The light (catalytic) chain is shown in blue, the catalytic triad in yellow and the heavy (regulatory) chain in red. A detached piece of the N-terminal part of the plasminogen depicted in grey is cleaved by plasmin and not presnt in plasmin preparations.
(Novokhatny, 2008)

2.2.1.1.1 Plasminogen

About 85-90% of plasmin in milk exists as plasminogen. Plasminogen is the zymogen or precursor for plasmin containing 786 amino acid residues with a molecular mass of 88 kDa (Bastian and Brown, 1996). In bovine milk 0.83-1.59 µg/ mL plasminogen is present (Benslimane *et al.*, 1990). The plasminogen molecule consists of an amino-terminal preactivation peptide (Benfeldt *et al.*, 1995) followed by five characteristic triple-loop structure kringles (Figure 2-2), each containing a lysine binding site (Bastian and Brown, 1996). The kringle domains are responsible for many properties of plasminogen such as interaction with activation inhibitors and activation stimulators. It is now generally

accepted that plasminogen is synthesized in the liver (not in the mammary gland) and secreted into the plasma and serves as unlimited supply of proteolytic activity (Politis *et al.*, 1995). Plasminogen activation is achieved through the cleavage of the peptide bond Arg_{557}-Ile_{558} by plasminogen activators as well as cleavage of Lys 77- Arg 78 releasing the PAP fragment (Benfeldt *et al.*, 1995).

2.2.1.1.2 Plasminogen activators

These are specific serine proteases which convert the inactive protein plasminogen to plasmin. These are divided into two main groups: indigenous activators or animal plasminogen activators and exogenous activators or bacterial plasminogen activators. Two major types of animal plasminogen activators are the tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) found in the casein fraction and somatic cells respectively (Lu and Nielsen, 1993). Bovine u-PA is a serine protease with a molecular weight of 54 kDa and composed of 2 subunits linked by a disulphide bond (Grufferty and Fox, 1988b; Christman and Silverstein, 1977). The active site of u-PA is located on the carboxyl terminal of the u-PA molecule. Although both u-PA and t-PA are distinct, they act on the same peptide bond in plasminogen (Politis, 1996). The formation of midi-plasmin with a molecular mass of 50 kDa is achieved through proteolytic cleavage in the Arg_{557}-Ile_{558} peptide bond of its molecule (Benfeldt *et al.*, 1995). Urokinase is most commonly used in plasminogen activation studies and the most characterised plasminogen activator (Christman and Silverstein, 1977).

Bacterial plasminogen activators are enzymatically inert. However, they serve as cofactors with plasminogen to form a functional activator complex. The complex formed brings about a conformational change in the plasminogen molecule, which in turn exposes the active site to activate a second plasminogen molecule (Ward *et al.*, 2004). Some pathogenic micro-organisms such as mastitis pathogens that infect the mammary gland

are able to synthesize and secrete plasminogen activators. *Streptococcus uberis* secrets streptokinase whereas *Staphylococcus aureus* secretes staphylokinase (Ward *et al.*, 2004).

2.2.1.1.3 Plasminogen activator inhibitors

PAI-1 is one of the three plasminogen activator inhibitors occurring in milk serum. It is a single chain glycoprotein with a molecular mass of 55 kDa. It forms complexes with either u-PA or t-PA and thereby controls the milk plasmin system (Politis, 1996).

2.2.1.1.4 Plasmin inhibitors

Plasmin inhibitors are heat labile, being inactivated at pasteurisation temperatures. The most common endogenous inhibitors of plasmin are found in milk serum and consist of trypsin inhibitor, α_1-proteinase inhibitor, α_2-Antiplasmin, α_2-macroglobulin and inter- α-trypsin inhibitors (Christensen *et al.*, 1995). The inhibitors possess plasmin inhibitory activity and thus prevent the degradation of casein. α_2- antiplasmin and α_2-macroglobulin contribute 10% of the total plasma proteins and are the fast acting inhibitors of plasmin. The former is a specific plasmin inhibitor whereas the latter is non-specific capable of reacting with almost all known proteases (Politis, 1996).

Thus, the final plasmin activity in milk is determined not only by plasminogen and plasminogen activator but also on the quantity of inhibitors (Korycka-Dahl *et al.*, 1983).

Fig 2-3 shows the relationships within the plasmin system in milk.

Some chemical and synthetic inhibitors inhibit plasmin activity. Most inhibitors of trypsin are also known to inhibit plasmin activity such as Cu, Zn, Hg, benzamidine and its derivatives, aromatic amidines, tetra-amidines, leupeptin and its synthetic analogs (Grufferty and Fox, 1988b). Lysine and its analogues inhibit plasmin activity at higher concentration by competing for the lysine-binding site in the kringles of plasmin (Richardson, 1983a). Protein inhibitors such as serpin (serine protease inhibitor), soy-bean, Kunitz type, limabean trypsin inhibitors also inhibit plasmin (Grufferty and Fox,

1988b). Plasmin is not inhibited by EDTA or iodoacetate, but is partially inhibited by Mg and Ca (Grufferty and Fox, 1988b).

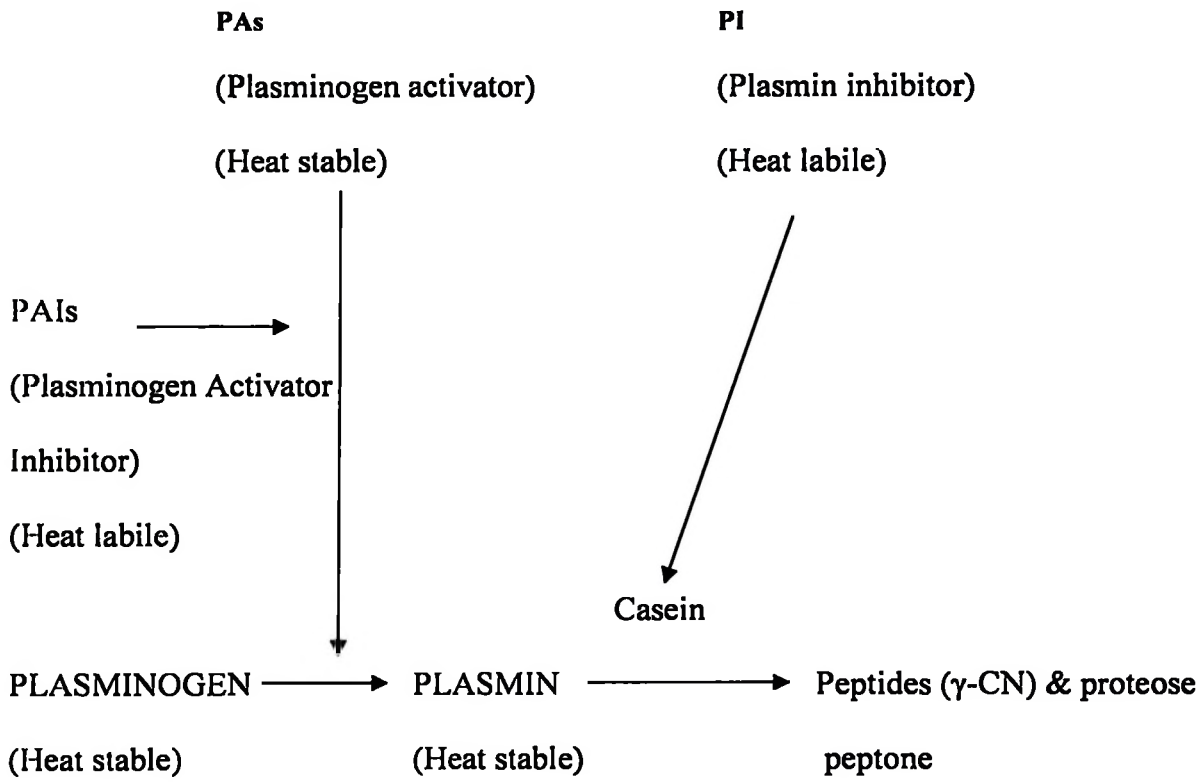


Figure 2-3 The plasmin-plasminogen system (Richardson, 1983a)

Hydrolysis of caseins by plasmin

Plasmin preferentially hydrolyses β -casein, which is found in the hydrophobic core of the casein micelle (Bastian and Brown, 1996). Plasmin is specific for peptide bonds to which lysine or arginine contribute to the carboxyl group (Kelly and McSweeney, 2003) . The cleavage sites in β -casein are Lys_{28}-Lys_{29}, Lys_{105}-$Hist_{106}$ and Lys_{107}-Glu_{108} (Table 2-1). The products of β-casein degradation are γ-caseins (3% of total casein) which represent the C-terminal segments, while the proteose peptones (PP) components PP 5, PP 8 s and PP 8 f s fractions represent the N-terminal segments (Andrews, 1978a). However, only 25 of the 38 components of proteose peptone fractions are due to proteolysis of caseins by plasmin (Grufferty and Fox, 1988a) suggesting the presence of other proteolytic enzymes. α_{s2}-casein is hydrolysed by plasmin to yield about 14 peptides, three of which are

potentially bitter (Table 2-1). α_{s1}-casein is less sensitive to hydrolysis by plasmin as compared to β -casein and α_{s2}-casein. Upon degradation, α_{s1}-casein forms λ-casein (McSweeney *et al.*, 1993). Hydrolysis of κ-casein by plasmin is controversial. Some authors (Le Roux *et al.*, 1995; Kaminogawa *et al.*, 1972; Eigel, 1977; Diaz *et al.*, 1996) reported that it was not hydrolysed at all whereas others (Trujillo *et al.*, 1998b; Aslam and Hurley, 1997; Andrews and Alichanidis, 1983 b) reported hydrolysis of κ-casein by plasmin. It was suggested that plasmin was unlikely to hydrolyse κ -casein due to the presence of the carbohydrate moieties attached to κ-casein as well as high levels of secondary and tertiary structures (Fox and McSweeney, 1998). κ-casein contains galactose, N-acetylgalactosamine and N-acetylneuraminic acid, which occur as tri or tetra-saccharides. These differences in results are due to different methods used as well as variations in experimental conditions.

Table 2-1 Specifity of plasmin in buffered solutions (Touch and Deeth, 2005)

Substrate	**Specificity**	**Derivative peptides**	***References***
β -casein	Lys_{28}-Lys_{29}	γ_1-casein (f-29-209)	(Andrew & Alichinidis, 1983)
	Lys_{105}-His_{106}	γ_2-casein (f106-209)	
	Lys_{107}-Glu_{108}	γ_3-casein (f108-209)	
	Lys_{113}-Tyr_{114}	f 1-105/7	
	Arg_{183}–Asp_{184}	f29 -105/7 ; f1-28	
αs_2 - casein	Lys_{21} –Gln_{22}	14 peptides (3 are bitter)	(Le Bar & Gripon, 1989)
	Lys_{24}-Asn_{25}		(Visser *et. al*, 1989)
	Arg_{114}- Asn_{115}		
	Lys_{149}-Lys_{150}		
	Lys_{150}-Thr_{151}		
	Lys_{181} –Thr_{182}		
	Lys_{188}-Ala_{189}		
	Lys_{197} –Thr_{198}		
αs_1 - casein	Arg_{22}- Phe_{23}	λ-caseins	(McSweeney *et al.*, 1993)
	Arg_{90}- Tyr_{91}		
	Lys_{102}-Lys_{103}		
	Lys_{103}-Tyr_{104}		
	Lys_{105}-Val_{106}		
	Lys_{124}-Glu_{125}		

Isolation of Plasmin

Isolation and purification of milk plasmin is given in detail in Fox, (1981a). Plasmin was first isolated from a solution of commercial casein by using 15-23% $(NH_4)_2SO_4$ and adjusting the pH to 4.6 (Warner and Polis, 1945). The specific activity was 150 times higher than the original protein mixture. Another attempt was made by concentrating plasmin activity 20 fold using 40% $(NH_4)_2SO_4$ from pH 3.5 extract of casein (Zittle, 1965). This preparation was further purified by Dulley (1972) by chromatography on CM cellulose and sephadex G-200 which resulted in a 629 increase in plasmin activity (Grufferty and Fox, 1988b).

2.2.1.2 Other naturally occurring proteases

2.2.1.2.1 Cathepsins D

An indigenous acid proteinase, with a pH optimum of 4.0, temperature optimum of 50^{0}C and a molecular weight of 36 kDa was reported (Kaminogawa and Yamauchi, 1972). The enzyme was not inhibited by EDTA, iodoacetic acid or DFP (di-isopropyfluorophosphate). It had more activity on α_{s1}-casein than β-casein, with very little activity on κ-casein. The authors concluded that the enzyme was similar to lysosomal proteinase, cathepsin D.

It is synthesized on the rough endoplasmic reticulum and found in the whey fraction of milk (Larsen *et al.*, 1996). It is secreted as the inactive precursor, procathepsin D and is activated by a series of proteolytic cleavages. Although the enzyme level is correlated to high somatic cell count, it is not known whether this is due to increased production of cathepsin D or activation of indigenous procathepsin D (Hurley *et al.*, 2000). Somatic cells in milk consist of neutrophils (also called polymorphonuclear leucocytes), macrophages, leucocytes and a few epithelial cells. During mastitis, neutrophil levels increase with concomitant increase in serine proteases, cathepsins G and elastase (Wedholm *et al.*, 2008).

Most lysosomal enzymes are cystein (thiol) proteinases which include cathepsin B, L, and H (Fox and McSweeney, 2003). It was reported that somatic cells containing more than 400,000 cells/ mL indicate udder inflammation (Akers, 2002). Milk containing somatic cells of 20,000 and 980,000 cells/ mL may degrade proteins and cause increase in peptides from 0.03 mg/ mL to 0.27 mg/ mL (Lindmark-Mansson *et al.*, 2005). It was therefore suggested that proteolysis in milk with low somatic cell count was due to plasmin whereas at high cell count it was due to cathepsins and elastase (Kelly and Fox, 2006b; Larsen *et al.*, 2004).

2.2.1.2.2 Thrombin

Thrombin is a serine protease (EC 3.4.21.5) that converts soluble fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation-related reactions. It was shown to hydrolyse peptide bonds containing arginine (Reimerdes, 1983). However, since it showed a high substrate specificity, it was concluded that the enzyme would not be expected to cause significant proteolysis in milk (Christman and Silverstein, 1977).

2.2.1.2.3 Elastase

It is one of the principal polymorphonuclear leucocytes (PMN) serine proteases with a molecular weigh of 24-30 kDa. It was found to cleave several sites of β-casein (Considine *et al.*, 1999) and hence could be significant in proteolysis of milk with high SCC.

2.2.2 Endogenous proteolytic enzymes

These are enzymes secreted by the microorganisms. Milk contains a number of micro-organisms which are capable of secreting these enzymes causing changes in flavour and texture of milk and milk products. Many psychrotrophs are present in raw milk, such as Pseudomonads with *Pseudomonas fluorescens* being the predominant species. These microbes are capable of producing heat stable enzymes which survive pasteurisation and UHT treatments (Fox, 1981a). The resulting proteases produced are metalloproteins which require divalent ions such as Ca^{2+}, Zn^{2+} for activity and stability (Nielsen, 2002; Larson *et al.*, 2006; Fairbairn and Law, 1986). Pasteurised milk may be spoiled by Gram positive bacteria which survive pasteurisation. Fox (1981) documented the important microorganisms as being *Streptococcus spp*, *Lactobacillus spp*, and sporeforming *Bacillus spp* which is the predominant genus. It was reported that about one third of pasteurised milk spoilage was

spoiled by G positive bacteria, out of which 77% was due to *Bacillus polymyxa* and *Bacillus cereus* (Ternstrom *et al.*, 1993) .

Microbial and native proteinases differ in their mode of casein hydrolysis and the nature of curd formed. Unlike plasmin, bacterial proteases such secreted by *Pseudomonas fluorescens spp* cause extensive protein breakdown of κ -casein to para- κ -casein in a manner similar to rennet, followed by extensive non-specific hydrolysis. This causes minimal destruction of the casein structure resulting in a soft curd whereas plasmin attacks the β-casein buried in the core of the casein micelle causing destruction of the casein structure and leading to the formation of a hard curd (Datta and Deeth, 2001). The preference for *Ps. fluorescens* to break down κ -casein is due to the presence of calcium (Ca). Ca as well as Zn, is essential for stability and activity of these proteases (Mitchell *et al.*, 1986). These proteases are detected when microbiological counts reach 10^7 cfu/ mL (Kohlmann *et al.*, 1991a).

The current trend in the dairy industry is to keep refrigerated milk for two or more days prior to processing in order to minimise the frequency of milk collection. During this time, the psychotropic population could reach 10^3 to 10^6 cfu/ mL, a level high enough to cause deterioration. It was also reported that in the USA, the total age of milk from farm to supermarkets was 21 days under refrigeration (Cousin, 1982). It is estimated that about 70-90% of refrigerated milk samples contain psychrotrophs capable of producing heat stable proteinases (Shah, 1994).

The heat stability of *Pseudomonas fluorescens* proteinases was evident in a study where 20-40% of their activity was retained after exposure to 140^0C for 5 s (Griffiths *et al.*, 1981). Thus, under normal UHT processing conditions, raw milk contaminated with *Pseudomonas fluorescens* will have significant levels of these proteinases. Proteinase activity is observed when cells are in late exponential to early stationary phase when bacterial counts are about

10^7-10^8 cfu/mL (Matselis and Roussis, 1998). The same researchers reported that maximum proteinase activities were observed at 10^8-10^9 cfu/mL However, in a different study it was revealed that although the average times for psychrotrophs to reach 10^7 cfu/ml (the level used to indicate spoilage) at 2^0C, 4^0C, and 7^0C was 9, 7 and 4 days respectively, there was little correlation between bacterial count and either proteolysis or proteinase level(Haryani *et al.*, 2003). This was due to the fact that although the enzyme was present, it was not active at such low temperatures. This was contradictory to other studies where increased proteolysis and proteinase production during storage of raw milk was reported. In addition the previous researchers analysed ewes' milk whereas bovine milk was analysed by the latter (Haryani *et al.*, 2003). There could be slight differences in the species. However, Hankin and Shields (1983) explained that the most important factor was the strain of the psychrotrophs and not their number. Thus, raw milk with higher psychrotroph count did not necessarily have higher activity than those with lower counts. However, minimum bacterial counts at which bacterial proteinases can be produced must be monitored (Haryani *et al.*, 2003).

Studies on reconstituted non- fat dry milk show that growth of *Pseudomonas fluorescens* causes a shift of plasmin location through the disruption of casein structure releasing plasmin enzyme into the serum (Fajardo-Lira and Nielsen, 1998). As a result, there is increased activity in whey and a concomitant decreased activity in casein.

Although the protease can cause dissociation of plasmin from casein, some authors found that under normal cheese making conditions, *Pseudomonas fluorescens* protease triggered plasminogen activation rather than plasmin dissociation from casein (Frohbieter *et al.*, 2005). It was also observed that under normal cheese making conditions, there was less loss of plasmin from curd to whey. Both human and bovine plasminogen activators were stimulated

by *Pseudomonas fluorescens* M 3/6 as observed by increased activity by 4.5 and 2.5 fold respectively (Frohbieter *et al.*, 2005).

Bacillus polymyxa protease (BPP) was able to activate plasminogen and hence increase plasmin like activity in milk and buffer systems during storage. BPP can also hydrolyse casein (Larson *et al.*, 2006). The protease in interaction with plasmin already present in milk and in the presence of plasminogen may bring about extracellular proteolysis and cause detrimental effects upon storage of milk (Larson *et al.*, 2006). However, BPP is an approved coagulant which may be added to cheese under controlled conditions to shorten ripening time.

Table 2.2 gives a summary of inhibitors, activators, thermal stability, optimum pH and temperature of some *Pseudomonas* proteases. *Pseudomonas putrefaciens* and *Pseudomonas fluorescens* are both active at 37^{0}C, pH 7.0 to 8.0 but heat labile at 60^{0}C for 40 s. *Ps. aeruginosa* however had optimum temperature of 37-60^{0}C, which was higher compared to the rest of the proteases (Drohse and Foltmann, 1989).

Table 2-2 Some characteristics of *Pseudomonas* proteinases

Organism	Mass	pH opt.	Temp. Opt.	Inhibitors					Activators	Thermal stability	Reference
				EDTA	Thiol	DFP	Trypsin inhibitors	Heavy metals			
P. putrefaciens	-	7.0-8.0	37	-	-	-	-	Slight	Mn_2 Mg	60°C,3min	
P. fluorescens	-	7.0-8.0	37	-	-	-	-	Yes	Fe^{2+}	60°C,40s	(Hurley *et al.*, 1963)
P. myxogens	77	-	-	No	-	-	-	Yes	-	50°C,10min in PO4 buffer; stabilised by buffer; more stable at 72°C than 64°C	
P. aeruginosa	40,48	6.5,8.5,10	37-60	Yes	No	No	No	Yes	Ca, Mg, Zn, Fe	60-70°C, 15min	(Morihara *et al.*, 1965)
P. fragi	40-50	6.5-8.0	40	Yes	No	-	No	-	Zn, Ca, Mn, Co	50°C,10min	(Porzio and Pearson, 1975)
P. maltophilia	35	10	37	Yes	No	yes	-	-	Ca, Sr, Ba, Co	60°C,10min	(Boethling, 1975)
P. fluorescens MC23	23	-	-	-	-	-	-	-	-	121°C,9min	(Mayerhofe rm *et al.*, 1973)
Pseudomonas MC60	48	7-8	45	Yes	-	-	-	-	Ca, Zn	D149=90s;Z=32.5°C ,unstable at 55°C	(Barach *et al.*, 1978)
Pseudomonas AR11	38	6.5	35	No	yes	-	No	Yes	-	t1/2 150C=8.5s in PO4, pH 6.5	(Alichanidi s and Andrews, 1977)
Pseudomonas		7-8.5,6-10	-	Yes	-	-	-	-	-	40-90% inactivation at 149°C,30s	(Richardso n and Te Whaiti, 1978)
Pseudomonas	46	6.5-7.5	45	Yes	-	-	-	-	-	t1/2 150C=8.5s;D150=8s ;Z=32; unstable at 55°C but some stabilised preheating at 140°C, 10s	(Stepaniac, Dally and Fox, unpublishe d)

(Fox, 1981a)

2.2.3 Exogenous enzymes

These are enzymes that are intentionally added to milk to impart desirable texture and flavour. Several fermented products such as cheese, yoghurt are produced by the action of exogenous enzymes.

Chymosin (EC 3.4.23.4) is an aspartic protease that specifically hydrolyses the peptide bond in Phe_{105}-Met_{106} of κ-casein and is considered to be the most efficient protease for the cheese-making industry. Rennet coagulation occurs in two steps:

In the 1st step, κ -casein fraction is hydrolysed as shown below

Casein $\xrightarrow{\text{rennet}}$ para κ-casein + glycomacropeptide

In step 2, para κ-casein in association with the Ca ions, produce a lattice structure in milk. The coagulum then contracts and whey is gradually exuded (O'Connor, 1994). Other microbial proteases that can be used include *Mucor miehei*, *Mucor pusillus* and *Endothia parasitica* (Fox, 1981a). There are also other milk-clotting proteases that, being able to cleave the Phe_{105}-Met_{106} bond in the κ-casein molecule, also cleave other peptide bonds in other caseins, such as those produced by *Cynara cardunculus* (Silva and Malcata, 1999; Silva and Malcata, 2005). This allows the manufacture of different cheeses with a variety of rheological and organoleptic properties.

This thesis however is focused on indigenous and endogenous enzymes as these have been documented to be the major cause of proteolysis in milk (Nielsen, 2005).

2.3 Heat stability of plasmin system

Temperature and pH influence plasmin activity. Its activity is reduced and inactivated at low pH values. It was reported that although it was not clear at what pH the release of plasmin

from caseins occurred, most of the activity was dissociated at pH 4.6-4.7 (Grufferty and Fox, 1988a).

Plasminogen activators are more heat resistant than plasminogen and plasmin with D-values of 109 min at 70^{0}C and 32 s at 140^{0}C (Lu and Nielsen, 1993).

The D-values of plasmin at various temperatures were reported by Driessen and van der Waals (1978) as being 55.6 min at 67.5^{0}C, 47.6 min at 70^{0}C, 35.7 min at 72.7^{0}C, 18.2 min at 75^{0}C, 8.6 min at 77.5^{0}C, 6.4 min at 85^{0}C, 2.9 min at 95^{0}C, 0.42 min at 110^{0}C, 0.25 min at 120^{0}C and 0.12 min at 142^{0}C.

2.3.1 Factors affecting the heat stability of plasmin

Heating medium, pH and type of equipment for milk processing affect the measured heat stability of plasmin. Plasmin activity is more heat stable in milk or micellar casein dispersions than in aqueous solution. As stated above, casein tends to protect the enzyme from inactivation during heating (Metwalli *et al.*, 1998; Alichidinis *et al.*, 1986). Thermal inactivation of plasmin is accelerated by the presence of sulphydryl compounds or free SH containing proteins. Alichanidis *et al.* (1986) and Metwalli *et al.* (1988) reported that the sulphydryl compounds participate in irreversible thiol-disulphide interchange reactions with plasmin. The SH groups in β-lactoglobulin and disulphide bond of plasmin are buried inside the folded molecules, hence inactivation of plasmin can occur at temperatures leading to denaturation or unfolding of proteins (Metwalli *et al.*, 1998).

Addition of oxidising agents enhances plasmin activity. Addition of potassium iodate (KIO_3) to milk before heat treatment protects the enzyme against inactivation due to the inhibitory effect on the thiol-disulphide interchange reactions with whey proteins (Kelly and Foley, 1997).

Plasmin is less stable to heat at alkaline pH than at acid and neutral pH because the thiol-dilsulphide interchange reactions proceed more rapidly at more alkaline pH (Alichidinis *et al.*, 1986).

Milk processed by indirect and direct UHT heating systems have different levels of plasmin. The major difference between the two systems is the time-temperature profiles which reflect a higher heat load or increased severity of heating for the indirect heat treatment (Datta and Deeth, 2001). UHT milk processed by indirect steam injection contains more denatured whey proteins attached to caseins than directly heat treated milk. As a result, indirect UHT milk has a lower level of plasmin than directly processed UHT milk due to higher heat inactivation on the enzyme and higher content of denatured β - Lactoglobulin (Manji *et al.*, 1986; Grufferty and Fox, 1988b; Datta and Deeth, 2001) . The higher level of denatured β–lactoglobulin undergoes thiol-disulphide interchange reactions with the plasmin molecule and also interacts with casein micelles, reducing interactions between plasmin and casein (Enright *et al.*, 1999).

2.4 Effects of plasmin system in milk

2.4.1 Positive effects of plasmin levels in milk

Cheese ripening is a long, slow, and expensive process. Accelerated ripening by the use of exogenous plasmin enzyme is a promising alternative (Nielsen, 2002). The contribution of plasmin in cheese ripening has been studied by eliminating other proteolytic agents from cheese; reducing plasmin activity by using specific inhibitors; adding plasmin and by increasing plasmin activation through plasminogen activators (Kelly and McSweeney, 2003). A study where rennet free (RF- rennet inactivated after its action on κ-casein), starter free (SF- chemically acidified using gluconic acid–δ-lactone) and rennet and starter free (RSF) cheeses were manufactured, concluded that plasmin contributed to proteolysis in cheese but

produced very low levels of small peptides (Visser, 1977). Farkye and Fox (1991) used 6-amino hexanoic acid (a plasmin inhibitor) to study the contribution of plasmin to the ripening of Cheddar and Gouda cheeses. Lower rates of proteolysis were found in these cheeses. In another study, exogenous plasmin that was added to cheese milk accelerated cheese ripening. Plasmin was found to be more suitable for the acceleration of cheese ripening as compared to other proteases because it linked itself to the casein and hence was included in the curd, unlike other proteases which were lost in the whey (Kelly and McSweeney, 2003). Addition of urokinase (plasminogen activator) to cheese milk was shown to accelerate proteolysis in Cheddar cheese with increased levels of γ-caseins and concomitant decreased β-casein (Barrett *et al.*, 1999).

2.4.2 Negative effects of plasmin enzyme

2.4.2.1 Bitter off-flavour

The presence of low molecular weight hydrophobic peptides is responsible for bitterness in milk. Caseins, in particular αs_2 and β, contain a number of hydrophobic amino acids such as valine, leucine, isoleucine, phenylalanine, tyrosine and tryptophan which are buried in the interior of the molecule.

UHT milk is more prone to bitterness than raw and pasteurised milk and it is highly related to proteolysis (McKellar *et al.*, 1984). This is caused by the high heat applied during UHT treatment which induces changes in the casein micelle structure and also due to inactivation of plasmin inhibitors (McKellar, 1981; Enright *et al.*, 1999). Bitterness in pasteurised milk was more evident during mastitis when the levels of plasmin activity were high. Studies have indicated that bitterness was not detected in milk with low somatic cell count for the entire

shelf life whereas it was detected in milk with high SCC between 2 and 3 weeks (Ma *et al.*, 2000).

Bitterness in cheese is also a problem in the dairy industry. However, the role of plasmin in inducing bitterness is still questionable because it has been shown that plasmin added to cheese accelerated proteolysis without imparting bitterness but improved the overall quality (Nielsen, 2005; Farkye and Fox, 1992).

It was also revealed that bitterness was a difficult parameter to measure because the bitter peptides being hydrophobic are associated with the fat phase thereby reducing the bitterness intensity. Moreover, the presence of other flavour compounds reduces or masks the bitterness intensity.

2.4.2.2 Reduction in curd firmness, curd yield and rennet coagulation time

Studies have indicated that plasmin may be responsible for the increase in coagulation time or poor coagulation properties of cheese during cheese processing (Table 2-3). An increase in curd firming time, reduced curd yield and firmness with increased levels of plasmin (0.1-10 mg/ L) was reported (Mara *et al.*, 1998). The same authors also confirmed that rennet clotting time increased at higher than 5 mg plasmin/ L . However, Bastian *et al.* (1991) did not find any relationship between plasmin and milk clotting properties. Other factors were probably involved such as milk pH, protein content, season and cow breed and these had greater influence in determining the milk clotting times than plasmin. In addition, the former authors used higher levels of plasmin ~10 mg/ L as compared to the latter study where < 5 mg/ L was used.

2.4.2.3 Astringent off-flavour

This was evident in raw, pasteurised and UHT milk (Harwalkar *et al.*, 1993). Astringent off-flavour has been linked to the production of γ-caseins, which are specific C-terminal breakdown products of β-casein's reaction with plasmin (Harwalkar *et al.*, 1993). The same author indicated that some psychrotrophic proteases gave rise to astringent off-flavour in milk.

Table 2-3 Negative effects of plasmin in milk (Touch and Deeth, 2005)

Effect	Product	References
Bitter off-flavour	Pasteurised milk, UHT milk and cheese	(Ma *et al.*, 2000; Ma and Barbano, 2003)
Astringent off-flavour	Raw, Pasteurised and UHT milk	
Age-gelation	UHT milk	(Crudden *et al.*, 2005b; Kohlmann *et al.*, 1988; Kelly and Foley, 1997; Enright *et al.*, 1999)
Sediments	UHT milk	(Enright *et al.*, 1999)
Reduction in coagulation properties	Milk	(Mara *et al.*, 1998)
Reduction in curd firmness and yield	Cheese	(Mara *et al.*, 1998)
Reduction in viscosity	UHT-sterilised custard	(Driessen *et al.*, 1981)

2.4.2.4 Age gelation

Age gelation of UHT milk is a major problem in the dairy industry because it limits shelf life and market potential of milk (Datta and Deeth, 2003). It is thought to be related to proteolysis of caseins either by plasmin or bacterial enzymes or both (Datta and Deeth, 2001). The amount of enzyme required and the degree of proteolysis necessary for gelation have not been determined (Kohlmann *et al.*, 1991b; Kohlmann *et al.*, 1991a). Bacterial enzymes produced by psychrotrophs such as *Pseudomonas fluorescens* produce extracellular proteases which survive pasteurisation and UHT treatment (Kohlmann *et al.*, 1988). Some investigators

have shown that milk samples containing plasmin gelled faster than milk without added enzymes (Kelly and Foley, 1997; Enright *et al.*, 1999; Crudden and Kelly, 2003). It was reported that low levels of plasmin added to commercially processed UHT milk caused gelation in 3 months (Kohlmann *et al.*, 1988). UHT milk with 1 ng bacterial proteinase/ mL may have a shelf life of three months only (Richardson and Newstead, 1979).

Although the exact mechanism of age gelation is unknown, two theories have been proposed (Datta and Deeth, 2001). The first theory involves the action of protease enzyme on milk proteins and their subsequent rearrangement. The second theory refers to the physico-chemical effects which result in the rearrangement of casein micelles and β-lactoglobulin. However some authors agree that it is a combination of the two mechanisms whereby proteinases hydrolyse caseins which result in the physical chemical process of gelation (Datta and Deeth, 2003). It was explained that during heating, hydrolysis of casein occurs which leads to the release of β-lactoglobulin-κ-casein (β-κ complex) from the micelle (Datta and Deeth, 2003). Plasmin and bacterial proteinase hydrolyse the proteins that attach the β-κ complex to the micelles and hence the β-κ complex is released from the micelle. The released complexes subsequently accumulate and aggregates during storage to form a three dimensional network of cross-linked proteins which causes gelation (McMahon, 1996). The gel formed appears to be thick and white but weak and fragile (Kelly and Foley, 1997).

2.4.2.4.1 Factors affecting age gelation:

Several factors affect age gelation of UHT milk. The factors according to Datta and Deeth (2001) are:

2.4.2.4.1.1 Mode and severity of heat treatment

UHT milk processed by direct steam injection has been shown to gel sooner than the indirect method. This is because the indirect method is more severe having a higher heat load and hence a larger portion of the enzyme is destroyed (Andrews, 1982; Corradini and Pecchini, 1981). In addition, severe heat causes more denaturation of β-lactoglobulin (Manji and Kakuda, 1988), which interacts with plasmin via disulphide bridges as well as ionic and hydrophobic interactions, and modifies the properties of casein micelles which increases its resistance to gelation (Datta and Deeth, 2001).

2.4.2.4.1.2 Non-enzymatic reactions

These reactions have been suggested to be responsible for the modification of surface properties of casein micelles. Thus, the interaction at high temperatures between lactose and NH_2-lysine residues (Maillard type of reactions) prevented gelation due to the blockage of the reactive side chains. These prevented the micelles from interacting and gelling (Samel *et al.*, 1971).

2.4.2.4.1.3 Storage temperature

Although it was stated by Datta and Deeth (2001), that gelation occurred more readily at room temperatures (20-25^{0}C) than at low (4^{0}C) and high (35-40^{0}C), other authors reported that no gelation occurred in samples stored at 37^{0}C or 22^{0}C to 25^{0}C, although samples at 37^{0}C were more proteolysed than those at 22^{0}C and 25^{0}C (Manji *et al.*, 1986). The lack of gelation at 37^{0}C may have been due to the high activity of the enzyme at this temperature causing a high degree of proteolysis. The highly degraded proteins were then unable to form

a gel matrix (Manji *et al.*, 1986). In addition, continuous proteolysis could be responsible for the degradation of the gel formed.

2.4.2.4.1.4 Fat content

Age gelation was more evident in UHT skim milk than whole milk as observed by RP-HPLC and PAGE (Lopez-Fandino *et al.*, 1993 b). The same authors suggested a possible protective effect of fat for protein against enzymic attack.

2.4.2.4.1.5 Milk production factors

The microbial quality of raw milk affected age gelation. Raw milk with high numbers of psychrotrophs such as *Pseudomonas fluorescens* are capable of producing heat stable enzymes which may survive UHT processing and cause age gelation (Datta and Deeth, 2001).

Season may also influence age gelation. It was reported that winter milk had higher somatic cell count than summer milk and thus was more susceptible to age gelation (Farkye and Fox, 1992).

Other factors such as age of the cow, mastitis and stage of lactation increase susceptibility of milk to age gelation by increased plasmin activity as discussed under 2.4.3.

2.4.2.4.2 Proteolysis

Three factors play an important role in the gelation phenomenon: heat treatment, storage temperature, and the extent of proteolysis (Manji and Kakuda, 1988). It was reported that gelation of UHT milk during storage was caused by proteolysis of casein by milk proteinase (Grufferty and Fox, 1988b). Addition of 0.15 mg plasmin / L to UHT milk formed a gel at 90 days (Kohlmann *et al.*, 1991c). In studies where plasmin inhibitors have been added, no

proteolysis or gelation was observed after 9 months at 20^{0}C (De Koning *et al.*, 1985). This shows that limited proteolysis is necessary for gelation to occur.

As limited proteolysis is a prerequisite for gelation which is influenced by minerals in milk, the effect of these minerals in milk on gelation and proteolysis in relation to microbial enzymes and plasmin is important but has not been established. Calcium is one of the important minerals essential for gelation by rennet during cheese manufacture. Removal of ionic calcium resulted in the increased coagulation time and formation of a weaker gel (Sharma and Sindhu, 2001). The level of calcium necessary for gelation, induced by proteolytic enzymes in milk, has not been established (Kohlmann *et al.*, 1991a). The current study will look into this aspect.

2.4.3 Factors affecting plasmin level in milk

Plasmin concentrations in pasteurised milk ranges between 0.14-0.73 μg/ mL (Richardson and Pearce, 1981) whereas it is about 0.15-0.37 μg/ mL in raw milk (Richardson, 1983a). However, the level of plasmin and plasminogen in milk is variable depending on the factors that affect the permeability of mammary gland membranes, especially mastitis and stage of lactation.

2.4.3.1 Mastitis

During mastitis, plasmin activity increases which suggests increased transport of active enzyme across the mammalian epithelium (Politis *et al.*, 1989). Proteinases from leucocytes are present in mastitic milk. These leucocytes contain plasminogen activators which are responsible for the increase of plasmin activity in mastitic milk (Grufferty and Fox, 1988b). Thus, higher plasmin activity in mastitic milk is due to proteolytic enzymes and/or

plasminogen activators that occur in somatic cells. It was reported that somatic cells obtained from mastitis quarters had higher plasmin activity than those from healthy quarters (Kelly and McSweeney, 2003).

2.4.3.2 Stage of lactation

Plasmin activity is higher during late lactation as measured by the decrease of β-casein and α_s-casein and the concomitant increase of γ-casein (Richardson, 1983a; Bastian *et al.*, 1991b; Politis *et al.*, 1989). This is attributed to both physiological changes and inflammation of the mammary gland (Bastian and Brown, 1996). Although it was documented that the ratio of plasminogen to plasmin at the end of lactation decreased signifying plasminogen activation (Politis et al., 1989), it was suggested that increased plasmin activity was due to leakage into the mammary gland and not plasminogen activation (Richardson, 1983a). Other authors found that plasmin as a percent of total enzyme (plasmin and plasminogen) was constant during lactation but increased dramatically in the last 3 months (Bastian and Brown, 1996). These findings suggest that in early lactation the increased plasmin activity is due to flow of the enzyme from blood to milk but in late lactation is mainly due to plasminogen activation. This seems to be a plausible explanation as decreased plasminogen activity is observed in late lactation (Bastian and Brown, 1996).

2.4.3.3 Age

Higher plasmin activities have been reported from milk of older cows (Bastian *et al.*, 1991a). Plasmin activity was also increasing with lactation number. Older cows showed an increase in γ-casein and decrease in β-casein during late lactation (Grufferty and Fox, 1988b) which is indicative of casein hydrolysis by plasmin.

2.4.3.4 Cows breed

Plasmin concentration of Holstein-Friesian was found to be 0.27-0.53 mg/ L while it was 0.15-0.37 mg/ L from of Jersey cows (Richardson, 1983a). Higher plasmin concentration was also reported for Swedish Friesian than Jersey cows (Schaar, 1985).

2.5 Methods for determining proteolysis in milk

Several methods are available for the detection of proteolytic activities in milk. Plasmin activities are measured by synthetic substrates because casein and fibrin, the natural substrates of plasmin are not chromogenic.

Determination of proteolysis by plasmin is based on:

i. monitoring the main proteolytic products: proteose peptones, γ-casein, amino acids and peptides also known as markers of plasmin activity

ii. measuring the enzymatic activity

iii. directly quantifying the amount of components of the plasmin system

2.5.1 Monitoring proteolytic products

Solubility of nitrogen under defined conditions is widely used as criteria of proteolysis (Kuchroo *et al.*, 1983). Several methods have been used such as solubility in 2%, 2.5%, 5%, 10%, 12%, 24% trichloroacetic acid (Le *et al.*, 2006; Trujillo *et al.*, 2002); sodium acetate at pH 4.6 (Lopez-Fandino *et al.*, 1993 b; Datta and Deeth, 2003; Trujillo *et al.*, 2002); 30-70% ethanol (Rohm *et al.*, 1996), 70% ethanol (Piraino *et al.*, 2007; Pripp *et al.*, 2006); sodium citrate pH 4.4 (Gorostiza *et al.*, 2004); phosphotungstic acid (Michaelidou *et al.*, 2003). Different kinds of extracts are obtained with each method, therefore the kind of peptides in the extract will differ (Kuchroo *et al.*, 1983). The pH 4.6 soluble extract method

has been used by several researchers because it can distinguish between large and small/medium peptides (Kuchroo *et al.*, 1983).

Plasmin activity is measured after incubation with a protein substrate to yield degradation products such as proteose peptones and γ-caseins from β-casein breakdown. The degradation products which are soluble in trichloroacetic acid and at pH 4.6 are good markers of plasmin activity. Some of the common methods used are discussed below.

2.5.1.1 Trinitrobenzene Sulphonic acid (TNBS)

In basic conditions, TNBS reacts with amino groups (both α and ε) to give a yellow trinitrophenylated derivative which is measurable by molecular absorption spectrophotometry. The TNBS-reactive group is produced by the cleavage of a peptide bond and thereby produces a simple, sensitive and direct method of monitoring proteolysis (Wallace and Fox, 1998). The reaction is shown on Figure 2-3. The TNBS method has been widely used to measure primary amines and to determine amount and reactivity of amino groups in peptides and proteins (Spadaro *et al.*, 1979).

The TNBS procedure was first introduced by Satake *et. al.* (1960) who used ~1 mM TNBS at temperatures from 20 to 40°C.

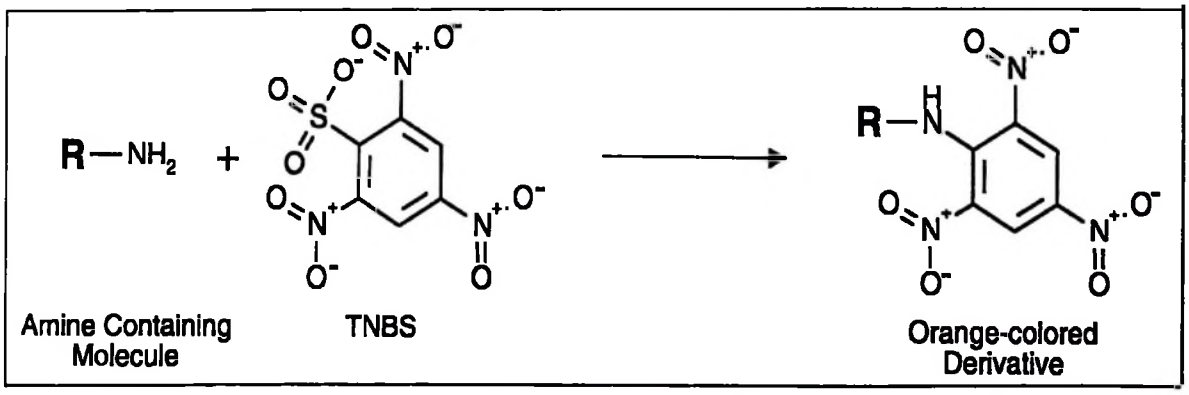


Figure 2-4 Reaction of TNBS with a primary amine

The reaction is stopped by neutralising the pH of the reaction mixture after the trinitrophenylation step. Sulphite, which increases the absorbance at 420 nm, forms a reversible complex with trinitrophenylated amino acids. A reaction time of 5 min at room temperature with 21 or 36 mM TNBS at pH 9.5 was used (Fields, 1971). As high concentrations of TNBS were used, precise control of reaction time was essential. Spadaro *et al* (1979) modified the procedure by reducing the concentration of TNBS to 1mM and the pH to 9.2 and increasing the reaction time to 30-50 min which is sufficient for mixtures having 5 to 100 nM of amino acids. The absorption of the sulphite-TNBS- amino group complex was measured at 420 nm, thereby avoiding the region of maximum absorption of TNBS itself (335-340 nm).

The TNBS method was found to be more sensitive to pH 4.6 or 12% TCA soluble extract and was recommended for routine assessment of proteolysis of cheese during ripening (Kuchroo *et al.*, 1983). Barlow *et al.* (1986) recommended the TNBS method by Fields (1971) as simple routine method for determination of soluble nitrogen in cheese.

2.5.1.2 Fluorescamine method

A synthetic compound "fluorescamine" was developed in attempt to detect amino groups of TCA-soluble peptides and amino acids from milk. Although the products are fluorescent, the reagent is non-fluorescent (Weigele, 1972). Fluorescamine reacts with free amino groups at alkaline pH. Peptides have maximum fluorescence at pH 7.0 whereas amino acids have a maximum fluorescence at pH 9.0 (Wallace and Fox, 1998). At pH below 6, fluorescamine reacts only with α- amino groups. It was reported that the loss of fluorescence at pH lower than 6.0 was due to cyclation of the lactone ring in the molecule (Castel *et al.*, 1979) and also by the decrease in reactivity of ε-amino groups (Beeby, 1980).

2.5.1.3 o-Phthaldialdehyde (oPA) method

In alkaline media containing a reducing agent (eg. mercaptoethanol), OPA reacts with α amino acids and small peptides forming a fluorescent product which can be measured at 340 nm (Church *et al.*, 1983). It is rapid, simple and sensitive. Refer to Figure 2-4.

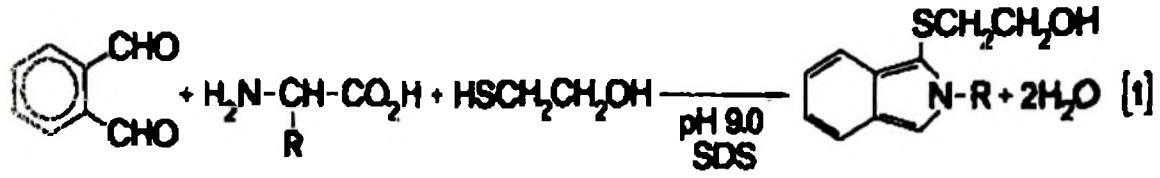
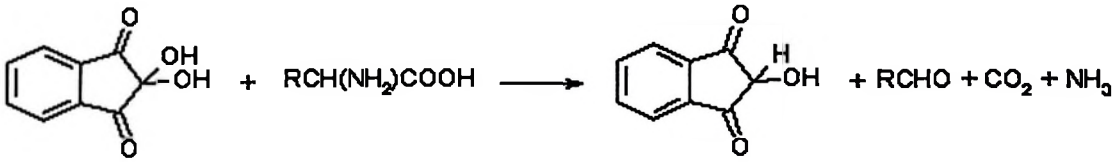


Figure 2-5 Reaction of o-phthaldialdehyde with a primary amine

2.5.1.4 Ninhydrin method

Ninhydrin reacts with free amino groups to form a purple chromophore whose absorbance is read at 570 nm. It is a recommended method for monitoring proteolysis in cheese (Wallace and Fox, 1998).

Ninhydrin degrades amino acids into aldehydes, ammonia, and CO_2 through a series of reactions; the net result is ninhydrin in a partially reduced form hydrindantin:



Ninhydrin then condenses with ammonia and hydrindantin to produce an intensely blue or purple pigment, sometimes called Ruhemann's purple:

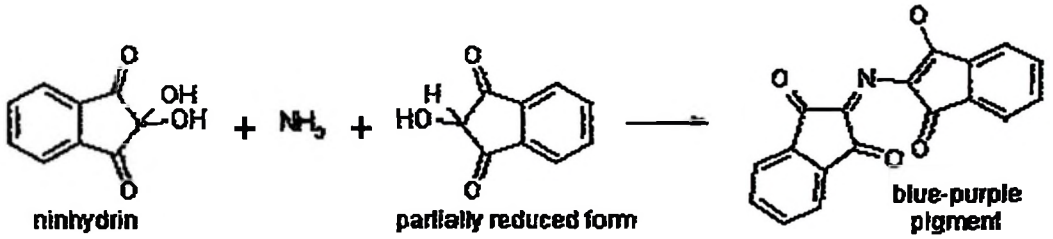


Figure 2-6 Reaction of ninhydrin with ammonia and hydrindantin to form Ruhemann's purple

The colour varies slightly from acid to acid, probably because unreacted acids complex with the pigment.

2.5.1.5 UV absorbance at 280 nm

This method is based on the presence of the aromatic amino acids tryptophan, phenylalanine and tyrosine which absorb maximally at 280 nm (Wallace and Fox, 1998). This method can be used to measure plasmin activity because the soluble tryptophan and tyrosine have been shown to be related to peptides produced from β-casein. This method however, is not recommended for monitoring proteolysis because significant proteolysis may occur without increase in tryrosine or tryptophan (Wallace and Fox, 1998).

2.5.1.6 Gel electrophoresis

This is one of the most widely used analytical techniques for separating and resolving individual component of protein mixtures. Separation is based on size and net charge. The difference in mobility of individual proteins in an electric field, and the sieving effect imparted by the gel, make protein separation possible (Harris and Angal, 1989). Soluble proteins may be analysed under native conditions, which has the advantage of retaining the biological properties. However, the less soluble proteins are subjected to vigorous denaturing conditions usually by sodium dodecyl sulphate (SDS) resulting in the loss of biological and

biochemical activity. Polyacrylamide, a sieving gel and agarose, a non-sieving gel are the most commonly used gel media for electrophoresis (Janson and Ryden, 1989).

Casein degradation bands induced by plasmin can be detected by gel electrophoresis methods such as PAGE, SDS-PAGE, urea-PAGE (Driessen and van der Waals, 1978; Lu and Nielsen, 1993; Kelly and Foley, 1997). This technique allows to differentiate between products of plasmin degradation (γ-casein) from those of bacterial proteases (para-κ-casein) (Lopez-Fandino *et al.*, 1993 b). However, SDS-PAGE is not very effective for resolving the 4 caseins because of their similar molecular mass (α_{s1}casein 23,000 Da; α_{s2} casein 25,000 Da; β-casein 24,000 Da and κ-casein 19,000 Da). β-Casein has a high surface hydrophobicity and therefore will tend to bind more SDS compared to the rest of the caseins and hence a higher electrophoretic mobility than α_{s1}casein (Tremblay *et al.*, 2003).

The main limitation of this technique is that not only is it slow and cumbersome but also can not resolve low molecular mass peptides because they are not fixed in gels and hence are lost during staining and destaining (Recio *et al.*, 1997). This limitation however may be overcome by using the capillary electrophoresis which is faster.

2.5.1.7 High Pressure Liquid Chromatography (HPLC)

This technique is useful to distinguish between peptides produced by plasmin from those produced by bacterial proteases (Datta and Deeth, 2003). Separation is based on the hydrophobicity/hydrophilicy balance of peptides. Due to strong interactions between the solutes and stationary phase, the hydrophobic peptides from plasmin elute slower than the hydrophilic peptides from bacterial proteases (Datta and Deeth, 2003)

This method is considered very sensitive in the measurement of plasmin activity. However, lack of suitable standards limits its use for quantitative measurements (Chen *et al.*, 2003).

Moreover, the time required for analysis may be 30 to 60 min per sample which is time consuming. The use of auto-injectors allows the samples to be run 24 h a day.

2.5.1.8 Other methods

Other procedures such as Kjeldahl, Lowry, Hull and dye binding have been used for protein or peptide quantification. The Kjeldahl procedure, though highly repeatable, is tedious and time consuming and the exposure to dangerous chemicals is inevitable. The Lowry and Hull procedures depend on the reaction of Folin-Ciocalteau reagent with tryptophan or tyrosine, with the formation of blue colour whose absorbance is read at 750nm. The limitation of the two procedures is that significant proteolysis may occur without the formation of tryptophan or tyrosine (Wallace and Fox, 1998). Dye binding involves the reaction of positively charged amino acid residue in proteins with an acid dye (eg Amido black 10B, Orange G) at ~pH 3 to form an insoluble complex which is removed by centrifugation. The supernatant is determined spectrophotometrically, where the absorbance is inversely proportional to the concentration of proteins/peptides in the solution (Wallace and Fox, 1998).

2.5.2 Activity assay methods

Since about 85-90% of plasmin exists as plasminogen (Bastian and Brown, 1996), it is necessary to convert plasminogen to plasmin before analysis. This is usually accomplished by the use of urokinase (Grufferty and Fox, 1988b).

2.5.2.1 Colorimetric methods

Synthetic substrates which are based on 4-nitroaniline (pNA) chromogen are used to measure plasmin activity. On reacting with plasmin, they release a yellow product which is measured at 405 nm either continually or after stopping the reaction with organic acids. These

commercially available substrates such as Spectrozyme PL-S-2251 also known as H $_{D}$-Val-L-Leu-L-Lys p nitroanilide have been used by various researchers (Rollema *et al.*, 1983; Bastian *et al.*, 1991c); V7127 also known as H $_{D}$- valyl-L-leucyl-L-lysyl-4-nitroanilide (Politis *et al.*, 1992c) and S-2403 also known as pyro Glu-Phe-Lys-pNA-HCl (Korycka-Dahl *et al.*, 1983).

These methods are very simple and sensitive. The disadvantages of these methods are high cost and the presence of plasmin inhibitors. Casein and whey proteins (β-lactoglobulin and α-lactalbumin) interfere with plasmin determination by interacting with the chromogenic substances of S2251 (Rippel *et al.*, 2003). Casein is a natural substrate of plasmin and competes with the chromogenic substrate of both S2251 and V 1727 for the active site of plasmin, (Bastian *et al.*, 1991c; Politis *et al.*, 1993). As a result, the synthetic substrate becomes unavailable for reaction with plasmin. Since the substrate V 7127 follows competitive inhibition patterns, Bastian *et al.* (1991c) suggested that inhibition of plasmin by casein could be eliminated by increasing the concentration of chromogenic substrate or by decreasing the casein concentration. These problems may also be avoided by pre-treatment such as centrifugation of milk although the treatments may affect the methods for analysis (Bastian *et al.*, 1991c; Politis *et al.*, 1993). In addition, the chromogenic substances can be hydrolysed by bacterial proteinases and peptidases and hence are not specific for plasmin. Other techniques such as electrophoretic or chromatographic should be used for confirmation.

2.5.2.2 Fluorimetric methods

These methods use various coumarin derivatised peptides for the assay of plasmin activity. They include 7-amino-4-methylcoumarin (AMC), 7-amino-4-trifluoromethylcoumarin, N-methyl N-tosyl-L-lysine naphthol ester and gluconoylpeptidyl-3-amido-9-ethylcarbazole.

A non-fluorescent substrate (N-succinyl-L-alanyl-phenyl-L-lysyl-7-amino-4-methyl coumarin) is used to release a fluorescent product 7-amino-4-methyl-coumarin upon hydrolysis by plasmin (Richardson and Pearce, 1981). The rate of increase of fluorescence intensity is proportional to the quantity of plasmin present.

This method is more sensitive than the colorimetric methods using the pNA substrates. However, the method is also susceptible to interference by whey proteins and caseins as well as plasmin inhibitors (Saint-Denis *et al.*, 2001).

2.5.3 Enzyme quantification method

2.5.3.1 ELISA

The ELISA technique is based on the interaction of a specific antibody with plasmin, as an antigen. This method quantifies the amount of enzyme of plasmin and plasminogen (vs activity). The ELISA test was found to be more sensitive than the fluorimetric method of Richardson and Pearce (1981) although the technique was not able to distinguish between plasmin and plasminogen. The assay is also interfered with casein and can not distinguish between active and inactive enzyme hence it needs to be used with other methods for direct activity determinations.

2.5.3.2 Fourier transform infrared spectroscopy (FT-IR) methods

This method is able to quantify low concentrations of plasmin and plasminogen in the presence of sodium caseinate and whey protein concentrates. Although the method is inferior to the ELISA technique, it gives information about structural changes of proteins and can differentiate between denatured and non denatured proteins (Hayes *et al.*, 2003).

CHAPTER 3 MATERIALS AND METHODS

Unless otherwise stated, all materials were from Fisher (Fisher Scientific UK Ltd, Leicestershire, UK).

3.1 Introduction

This chapter covers the following methods: trinitrobenzene sulphonic acid (TNBS), fluorescamine, gel electrophoresis, reverse phase high performance liquid chromatography (RP- HPLC), and the amino-methyl-coumarin (AMC) protocols.

3.2 Milk sample preparation

Unless otherwise stated, bulk and individual raw milks were obtained from the Centre of Dairy Research (CEDAR), University of Reading. Commercial pasteurised and UHT skim milk were supplied by Dairy Crest, Shropshire, UK. Pasteurisation (APV TRADEMARK, The APV Company LTD, UK) and high temperature heat treatments on an APV Junior Indirect heat exchanger (APV, Crawley, UK) were also carried out at the pilot plant in the Department of Food Biosciences. Raw milk samples were also incubated in a laboratory water bath (Grant Instrument Cambridge Ltd, Cambridge, UK) usually at 37^0C, but occasionally other temperatures were used and will be discussed either below or in the respective chapters. Sodium azide 0.05% (w/v) (Sigma-Aldrich Gillingham, UK) was added to milk samples before incubation to inhibit bacterial activity. A pH meter was used to record the pH of milk samples before and after incubation. Unless otherwise stated, all experiments were carried out at the natural pH of the milk.

3.3 Addition of trypsin and plasmin

Raw and pasteurised milk were incubated at 37^{0}C for 2, 4, 6 and 16 h followed by clarification to obtain pH 4.6 and 6% TCA soluble extracts as described in section 3.6. The aim of this experiment was to establish the appropriate time of incubation to study the effect of proteolysis on incubation time.

Trypsin (10,100 units/ mg protein) supplied by Sigma-Aldrich (St Louis, USA) was added to commercial pasteurised and UHT skim milk to study its effect on proteolysis. One BAEE (N-α- benzoyl-L-arginine ethyl ester) unit will produce a ΔA253 of 0.001 per min at pH 7.6 at 25^{0}C using BAEE as substrate. Samples were incubated for 3 days as described in section 4.2. This enzyme was used because it has similar activity to plasmin and costs significantly less. It is a serine enzyme which also cleaves the same peptide bond Lys-Arg through catalytic triad mechanism as indicated in Figure 2-2.

Clarification procedures to obtain 6% TCA and pH 4.6 soluble extracts were carried out after incubation. The TNBS, fluorescamine, gel electrophoresis and RP-HPLC methods were used to monitor proteolysis. Gelation was checked daily by observing the first appearance of a clot in the samples. The pH of milk was also monitored.

The same procedure was repeated for plasmin (4 units/ mg protein; Sigma-Aldrich, St Louis, USA). One unit will produce one μmole of p-Nitroanilide from D-Val-Leu-Lys-p-Nitroanilide per minute at pH 7.5 at 37^{0}C. The aim of the experiment was to monitor proteolysis during storage by the TNBS, fluorescamine, gel electrophoresis and RP-HPLC. Comparison was made of all the methods used.

3.4 Bacterial sample preparation

3.4.1 Preparation of *Pseudomonas fluorescens* strains and its enzymes

Two strains of *Pseudomonas fluorescens* NCIMB 702085 (416) and NCIMB 701274 (414) were obtained from the departmental stock culture, maintained at -80^{0}C. They were grown overnight in nutrient broth followed by overnight agar slants at 30^{0}C. Streaking at 30^{0}C for 24 h was carried out to check purity of samples followed by Gram staining. Both strains were grown on nutrient agar with CFS (cetrimide fucidin cephaloridine) at 30^{0}C for 24 h to confirm the presence of the fluorescence characteristics of *Pseudomonas fluorescens*. Strains that were positive were incubated in plate count agar (PCA) plates at 30^{0}C for 24-48 h followed by microbial counting. An overnight culture was grown on nutrient broth and inoculated into skimmed UHT and/or pasteurised milk for the detection of proteolysis in milk. Samples were stored at 20^{0}C and 4^{0}C for 7 days. These temperatures were selected because *Pseudomonas fluorescens* grows well at 20^{0}C, and being psychrotrophic, it grows at refrigeration temperatures as well. Following proteolysis, breakdown products were analysed by fluorescamine, TNBS and RP-HPLC after clarification by either trichloroacetic acid 12% (w/v) or isoelectric precipitation to pH 4.6.

In a separate experiment, enzymes from both strains of *Pseudomonas fluorescens* NCIMB 702085 (416) and NCIMB 701274 (414) were extracted. These strains were cultured into UHT skim milk followed by incubation at 37^{0}C for 3 days. Cells were removed by centrifugation (24,000 g for 10 min at 5^{0}C), resulting in a clear supernatant which contained the active enzyme. The crude enzyme extract was stored at -20^{0}C until further required.

To further purify the enzyme, the supernatant was dialysed against distilled de-ionised water (with 0.05% sodium azide). Ten mL of crude enzyme extract from each of the two strains of *Pseudomonas fluorescens* NCIMB 702085 (416) and NCIMB 701274 (414) were dialysed in 14 kDa molecular weight cut-off tubular porous membrane (Medicell International Ltd., London, UK) of. The tubes were clipped securely and placed in 1500 mL of distilled water. Dialysis was performed at 20^0C for 24 h, with one change of water. The semi-purified enzyme was cultured into UHT skim milk and incubated for 24 h. Control samples were samples without added enzymes. Samples were drawn after 2, 6 and 24 h to be analysed by TNBS, fluorescamine, RP-HPLC and gel electrophoresis.

To determine the optimum enzyme concentration to inoculate into UHT skim milk, various concentrations of the enzyme extracts [1%, 5%, 10% and 20% (v/v)] were inoculated into UHT skim milk. Incubation at 37^0 C for 2 and 24 h was followed by analysis of breakdown products by TNBS, fluorescamine, HPLC and gel electrophoresis. After these preliminary experiments, 10% of the enzyme extract was inoculated into UHT skim milk because 1% and 5% (v/v) produced too low concentration of peptides on RP-HPLC as well as lower absorbance and fluorescence readings (results not shown) by TNBS and fluorescamine as compared to 10% and 20%. The detection limit was reached earlier at 20% (v/v) enzyme concentration by the fluorescamine protocol, and therefore 10% was considered appropriate for further study.

Non-dialysed enzyme extracts were also analysed for breakdown products by the same procedures as mentioned above to compare the efficiency of dialysis technique in the partial purification of the *Pseudomonas fluorescens* enzymes.

In another experiment, the effect of storage temperature on proteolysis by *Pseudomonas fluorescens* NCIMB 702085 (416) and *Pseudomonas fluorescens* NCIMB 701274 (414) was investigated. These bacteria were inoculated in UHT and pasteurised skim milk followed by incubation at 20^0C and 37^0C for 7 days. Control samples were samples without added bacteria. All experiments were carried out at the natural pH of the milk. The pH optimum of most heat stable proteases is between 6.5 and 8 (Stepaniak and Fox, 1983). Samples were analysed on days 0, 3 and 7.

3.4.2 Preteolysis by *Bacillus licheniformis* enzymes

Proteolysis by commercial enzymes from the strain of *Bacillus licheniformis* (Sigma-Aldrich Gillingham, England) was also studied. The enzyme (12.9 units/ mg protein; 94 mg protein/ mL; 53.19 mL) was incubated in UHT milk to check the effect of concentration on enzyme activity and proteolysis. One unit will hydrolyse casein to produce colour (Folin-ciocalteu reagent) equivalent to 1μmole of tyrosine per minute at pH 7.5 at 37^0C. The experiment also aimed at assessing the suitability of the selected methods in monitoring proteolysis. A stock solution of *Bacillus licheniformis* enzyme containing 12.1 x 10^{-4} units/ mL was prepared by adding 10μL of *Bacillus licheniformis* enzyme into 100 mL of milk. This was a stock solution, where aliquots containing 24.3 x 10^{-4}, 48.5 x 10^{-4} , 72.8 x 10^{-4}, 97 x 10^{-4} and 121 x 10^{-4} units were each added to 100 mL of UHT skim milk with 0.05% sodium azide to prevent bacterial growth.

Proteolysis was monitored by incubating the samples at 37^0C for 0, 2, 6, 12 and 24 h followed by clarification as described in section 3.6. Samples were then analysed by TNBS, fluorescamine, electrophoresis and RP-HPLC. The experiment was replicated three times. Lower concentrations of enzymes were later used (6.06 x 10^{-4} units/ mL) to study the

breakdown profiles by gel electrophoresis since the levels used previously (12.1 x 10^{-4}, 18.2 x 10^{-4} and 24.3 x 10^{-4} unit/ mL) resulted in complete breakdown of all the caseins.

3.5 Heat treatment experiments

Raw milks were processed on an APV junior UHT plate heat exchanger (APV, Crawley, UK). The details of the procedure are given in section 6.1 and the batches were as follows:

Batch 1: Unheated raw milk (Control)

Batch 2: Raw milk was heated at 85 ^{0}C for 15 s (T_1)

Batch 3: Raw milk was heated at 110 ^{0}C for 2 s (T_2)

Batch 4: Raw milk was heated at 120 ^{0}C for 2 s (T_3)

Batch 5: Raw milk was heated at 130 ^{0}C for 2 s (T_4)

Batch 6: Raw milk was heated at 142 ^{0}C for 2 s (T_5)

After incubation at 37^{0}C for 28 days, clarification to obtain pH 4.6 and 6% TCA soluble extracts was carried out as given in section 3.6. The soluble extracts were analysed by TNBS, fluorescamine, gel electrophoresis and RP- HPLC.

3.6 Clarification of milk

3.6.1 Clarification by TCA

3.6.1.1 Procedure

The concentration of TCA used in this study (12%), was selected after preliminary experiments (results not shown) using 4%, 7.5%, 12% and 24% (w/v). In the experiment, 5 mL TCA (at the 4 levels previously mentioned) were mixed with equal volumes of UHT/ pasteurised skim milk to extract TCA soluble peptides after proteolysis. The peptides from 4% and 7.5% TCA produced too high absorbance readings (results not shown), but too low

readings were produced at 24% TCA, whereas 12% TCA produced appropriate on scale readings.

Prior to clarification, all milk samples were heated at 100^{0}C for 10 min to denature the whey proteins.

Five mL of 12% (w/v) trichloroacetic acid (TCA) was added to an equal volume of milk (raw, pasteurised/ UHT). 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.

3.6.2 Clarification by isoelectric precipitation (pH 4.6)

3.6.2.1 Procedure

Five mL of milk was dispensed into a beaker. Fifty mL of warm water (40^{0}C) was added, followed by 0.5 mL 10% (w/v) acetic acid. The mixture was left at room temperature for 10 min, after which 0.5 mL of 1M sodium acetate was added. The resulting solution was mixed and placed under cold water for 10 min to cool. This was followed by filtration on Whatman No 41 filter paper. The beaker was washed three times and the filter paper twice. The clear filtrate obtained was diluted with water to 100 mL. The extract obtained was further filtered by 0.2 μm Millipore filter.

3.7 Electrophoresis

The protocol was based on Laemli (1970).

3.7.1 SDS-PAGE

This technique was used to observe proteolysis induced by trypsin, *Pseudomonas fluorescens* and *Bacillus licheniformis* enzymes.

3.7.1.1 Reagents and solutions

Acrylamide-bisacrylamide stock solution (30 %T; 29:1) (Sigma-Aldrich Gillingham, UK): The solution was used directly from the bottle.

Separating gel buffer (1.5 M Tris-HCl, pH 8.8): Tris (18.171 g) was dissolved in 100 mL distilled water and pH adjusted to 8.8 with 5 M HCl (Sigma-Aldrich Gillingham, England). The solution was stored at 4^0C.

Stacking gel buffer (0.5 M Tris-HCl, pH 6.8): Tris (6.507 g) was dissolved in 100mL of distilled water. pH was adjusted to 6.8 by 5 M HCl and stored at 4^0C.

Electrode buffer: Tris (15.143 g), glycine (72.067 g) and SDS (5 g) were dissolved in 100 mL of distilled water. No pH adjustment was made (The pH was 8.3). The solution was stored at 4^0C but diluted 5 times before use.

Reducing sample buffer: 0.5 M Tris-HCl (12.5 mL), SDS (2 g) and bromophenol blue (10 mg), were dissolved in 60 mL of distilled water and thoroughly mixed. Glycerol (25 mL) and 2-β-mercaptoethanol (5 mL) were added and the volume made up to 100 mL. All reagents for the reducing sample buffer were from Fisher Scientific UK Ltd, Leicestershire, UK. The solution was stored at 4^0C.

All the solutions below were stored at 25^0C.

Ammonium persulphate (10% APS): This solution was freshly prepared by dissolving 0.1 g of APS in 1 mL of distilled water.

Sodium Dodecyl Sulphate (10% SDS): SDS (10 g) was dissolved in 100 mL distilled water.

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

Staining solution: Coomasie blue R250 (1 g) was dissolved in 400 mL methanol and 100 mL acetic acid. The volume made up to 1000 mL with distilled water.

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.

3.7.1.2 Preparation of the gel

Reagents	*15% Separating Gel*	*4% Stacking Gel*
Separating Gel buffer (mL)	1.50	-
Stacking Gel buffer (mL)	-	0.50
Water (mL)	1.41	1.20
30% acrylamide solution (mL)	3.00	0.27
10% SDS (µL)	60	20
TEMED (µL)	3	2
10% APS (µL)	30	10
Total volume (mL)	**6.003**	**2.002**

3.7.1.3 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^{0}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^{0}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)

3.7.2 Urea-PAGE

3.7.2.1 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^{0}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^{0}C.

Electrode buffer: Tris base (1.5 g) and glycine (7.3 g) were dissolved in 1000mL of distilled water. No pH adjustment was made (the pH was 8.3). The solution was freshly prepared and cooled to 4^{0}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^{0}C.

Ammonium persulphate (10% APS): As in 3.7.1.1

Sodium Dodecyl Sulphate (10% SDS): SDS (10 g) was dissolved in 100 mL distilled water and stored at 25^{0}C.

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^{0}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

3.7.2.2 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**

3.8 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 x 4 mm reverse phase column (SGE 150 GL4-C-P-8/5, Australia) at 40^{0}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.

3.9 Rapid methods for the detection of proteolysis in milk

3.9.1 Trinitrobenzene Sulphonic Acid (TNBS)

This method was a slight modification from the original method by (Fields, 1971) as modified by (McKellar, 1981).

3.9.1.1 Reagents and solutions

All reagents and solutions in this protocol were obtained from Sigma-Aldrich (Gillingham, UK) except potassium hydroxide which was from Fisher Scientific UK Ltd (Leicestershire, UK).

- 5% (v/v) trinitrobenzene sulphonic acid
- 2 M monobasic sodium phosphate
- 18 mM sodium sulphite
- 0.05% (w/v) sodium azide
- potassium borate buffer (pH 9.2) was made from

  1M potassium hydroxide and 1M boric acid

3.9.2 The TNBS protocol

Duplicate 0.2 mL samples of the supernatants (from 3.2.1 and 3.2.2) were mixed with 2 mL of 1 M potassium borate buffer (pH 9.2) and 0.8 mL of 5 mM TNBS. After 30 min

incubation in the dark at 25^{0}C, 0.8 mL of freshly prepared 2 M monobasic Na_2PO_4 [containing 18 mM Na_2SO_3] and 5 mL of distilled water were added. Absorbance was read at 420 nm by spectrophotometer (Cecil CE 1021 1000 series Cambridge, England).

3.9.3 Fluorescamine method

The micro method (Castel *et al.*, 1979) was adopted in this research, as small quantities of reagents are required. This method was initially developed (Weigele, 1972) and was later modified by Beeby (1980). It is based on the reaction of primary amino groups with fluorescamine.

3.9.3.1 Reagents

- 0.03% fluorescamine (Sigma-Aldrich Gillingham, UK): 0.03 g was dissolved in 100 mL acetone.
- Potassium borate buffer pH 8.5*:* This was prepared as described by (Gomori, 1952).

3.9.3.2 The procedure

Duplicates of clarified samples (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 of fluorescamine (0.03% in acetone). The reaction was instantaneous and after 1 min, 3.5 mL of 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. Prior to analysis, the spectrofluorimeter was zeroed with blank comprising distilled water.

3.10 AMC method for the determination of plasmin activity

This was based on the method of Richardson and Pearce (1981), using the fluorescent substrate N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin as a substrate. Plasmin reacts with the non-fluorescent substrate and hydrolyses it to give 7-amino-4-methyl coumarin which is fluorescent and can be measured by a spectrofluorimeter. Activities were expressed in AMC units/ mL, where 1 AMC unit of plasmin releases 1 nmol of 7-amido-4-methyl coumarin/min under the condition of the assay.

3.10.1 Reagents and solutions

N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin dipeptide (substrate), 7-amino 4-methyl coumarin (AMC) as a standard, 0.05 M Tris buffer, dimethyl suphoxide were from Sigma-Aldrich Gillingham, England. Trisodium citrate was obtained from Fisher Scientific UK Ltd, Leicestershire, UK.

3.10.2 Procedure

Milk samples were diluted in 0.4 M trisodium citrate in a ratio of 3:1 followed by centrifugation at 27,000 g for 10 min (Richardson and Pearce, 1981). The supernatant was used to determine the plasmin activity.

Approximately 7.5 mg of coumarin peptide was dissolved in 2.0 mL dimethyl sulphoxide and 8 mL of 0.05 M Tris buffer, pH 7.5 was added to give a final concentration of 1mM - coumarin peptide (Richardson and Pearce, 1981). This solution was kept at -20^{0}C and protected from light. Standard curves were prepared over the range of 0-4 x 10^{-7} M.

Citrate treated milk (100 µL) was added to a cuvette and the volume made to 1.280mL with 0.05M Tris buffer, pH 7.5. After equilibration for 5 min at 25^{0}C, 320 µL of 1mM-coumarin peptide solution was added. Rate assays were conducted by measuring fluorescence for 30

min (recorded after every 5 minutes) at 380 nm excitation and 460 nm emission, while excitation and emission bandpass widths were 6 and 10 nm respectively.

One AMC unit of plasmin activity releases 1 nanomol AMC per min under the standard assay conditions- total volume 1.6 mL; 0.2 mM coumarin peptide; Tris buffer pH 7.5; 25^{0}C (Richardson and Pearce, 1981)

3.11 Ca manipulation

3.11.1 Sample preparation

Ionic calcium measurement is important as it is the main stabilising factor among all minerals and their fractions in milk. Sequestering agents trisodium citrate (TSC) and sodium hexametaphosphate (SHMP) were used to manipulate the levels of ionic calcium in milk and study the effect of trypsin, *Pseudomonas fluorescens* and rennet on gelation as caused by ionic calcium manipulation.

To ensure sterile environment, sample preparation was done under flow hood cabinet. All glassware were sterilised before use. Sequestering agents were filtered through 0.2 µm membrane filters prior to addition into milk. Both pasteurised and UHT milks were used in this experiment. Raw milk was pasteurised at 72^{0}C for 15 s in the pasteurisation unit at the pilot plant in the department of Food Biosciences. Raw milk was heated by plate heat exchangers by hot water at 77 ^{0}C, followed by the holding tube for 15 s after which the milk was cooled to 4^{0}C. The milk (72^{0}C / 15 s) was used for Ca^{2+} manipulation experiments.

UHT was produced as follows. Raw milks were processed on an APV junior UHT plate heat exchanger with two stages of heating involving hot water (80^{0}C) and steam (112—144^{0}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^{0}C for 2 s.

Pasteurised milk was filtered through 0.20 μm membrane filters to make it sterile. This was however very difficult to achieve, hence used only in the first trial.

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 and pasteurised skim milk. Lower levels of sequestering agents were later used (0.04 - 0.08%) to observe the effect of ionic calcium on gelation. Samples were thoroughly mixed using magnetic stirrers for 30 min.

The mechanism of gelation by trypsin, chymosin (chymax Plus batch 071101, Hansen, Denmark) and *Pseudomonas fluorescens* were studied by adding 8.705 x 10^{-3} Units of trypsin , 0.03% (v/v) rennet and10^6 cfu/mL *Pseudomonas fluorescens* 416 to pasteurised milk. Samples were stored at both 25^{0}C and 4^{0}C to monitor gelation and ionic calcium.

UHT milk samples were stored at 25^{0}C for 2½ weeks whereas pasteurised milk samples were stored at both 25^{0}C and 4^{0}C for 2 - 11 days. Pasteurised milk was stored at 4^{0}C because it is the natural storage environment for pasteurised milk whereas UHT is usually stored at room temperature. Gelation was indicated by the high resistance of milk to flow when poured out from the container. Ionic calcium, gelation and proteolysis were monitored.

To study proteolysis, samples were incubated at 37^{0}C for 2 h. Clarification of the milk samples to obtain pH 4.6 soluble extracts was done as described in section 3.5. Proteolysis was monitored by RP-HPLC as discussed in section 3.8.

3.11.2 Ionic Ca measurements

Ionic calcium levels were measured before inoculation 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 minimum required sample is 35 µL. 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, 2.3 and 3.0 mM. The readings (in mV) were an average of 3 measurements and were plotted against log (Ca) mM. The concentration of calcium was obtained from the equation of the calibration curve. All analysis was carried out at room temperature (20 ± 0.5^0C).

3.11.3 Principle of determination

It is a potentiometric method that determines free calcium ions that interact with the calcium ion electrode. It consists of 3 electrodes; a calcium ion electrode, pH electrode and a reference electrode. Each electrode consists of a neutral carrier based calcium sensor, immobilised in a polyvinyl chloride (PVC) membrane. When the sample comes into contact with the membrane of the calcium ion electrode, calcium ions interact with the membrane and a membrane potential develops. The membrane potential is compared to the constant potential of the external reference sensor. The final measured potential is proportional to the free calcium ion concentration in the sample.

3.12 STATISTICAL ANALYSIS

Statistical analysis was carried out by using Statistical Package for Social Sciences (SPSS 14). 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 ($p<0.05$).

CHAPTER 4 EFFECT OF PROTEOLYSIS CAUSED BY TRYPSIN AND PLASMIN

4.1 Introduction

Native enzymes in milk play a crucial role in the proteolysis of milk. Many enzymes are naturally occurring in milk including plasmin, cathepsins B and D, thrombin and elastase (Fox, 1981). Plasmin occurs in the highest amount and is one of the most heat resistant enzymes in milk (Datta and Deeth, 2003). As described in the introduction, plasmin is part of a complex system consisting of plasminogen (PG), plasminogen activators (PA), plasminogen activator inhibitors (PAI), plasmin (PL) and plasmin inhibitors (PI) (Crudden and Kelly, 2003). It causes breakdown of milk proteins in a variety of dairy products, which result in the change of texture and flavour in these products (Ma and Barbano, 2003). The hydrolysis may have positive or negative effects depending on the product and the level of activity (Nielsen, 2005). It plays a positive role in cheese ripening for many varieties of cheese such as Swiss and Gouda (Borda *et al.*, 2004). As discussed in chapters 1 and 2, defects induced by plasmin include development of age-gelation in UHT milk (Datta and Deeth, 2001), bitterness and other off-flavours in dairy products (Ma and Barbano, 2003) and reduction in coagulation properties of milk and cheese (Mara *et al.*, 1998).

In this chapter, the TNBS, fluorescamine, RP-HPLC and gel electrophoresis methods will be assessed by examining their suitability for the detection of proteolysis by native and added plasmin and trypsin in raw, pasteurised and UHT milk. Comparison will be made of the effect of these enzymes in raw milk and milk pasteurised at different temperature-time combinations.

Due to the high cost of plasmin, trials were initially carried out by using trypsin which is much cheaper. Trypsin is also an endopeptidase serine enzyme with similar activity to plasmin. Like plasmin, it attacks the carboxyl groups of lysine and arginine (Roy, 1981).

4.2 Materials and methods

Preliminary experiments were carried out in raw and laboratory pasteurised milk (72^0C/ 15s) without trypsin or plasmin to monitor proteolysis during incubation at 37^0C for 6 and 16 h then clarified by 7.5 and 12% TCA. Pasteurised milk was made from the same batch of raw milk. Sodium azide (0.05%) was used in all experiments to prevent bacterial contamination. The incubation times (6 and 16 h) were selected after preliminary experiments (results not shown) showed that incubation up to 4 h resulted in very low absorbance readings. The soluble extracts obtained were analysed by the TNBS method. In order to study proteolysis and gelation as well as assessing the suitability of the TNBS method in monitoring proteolysis, trypsin was added to commercial pasteurised milk at 745.35 and 1484.7 BAEE units which corresponded to 0.3 and 3 mg/L, the levels normally found in milk. Samples were stored at 20^0C for 7 days. Sampling was done on days 0, 3 and 7.

Following the experiment of commercial pasteurised milk, another set of experiments was set up to observe proteolysis in milks pasteurised at different temperatures. The aim of the experiment was based on the fact that pasteurisation inactivates the plasminogen activator inhibitors, which would result in high plasmin activity due to plasminogen activation. Thus, raw milk was pasteurised in the water bath in the laboratory at 75^0C, 85^0C and 95^0C for 15 s followed by immediate cooling to 25^0C. The effect of inactivation at these temperatures was assessed. Trypsin was also added to the pasteurised milk at 247 and 2475 BAEE units

followed by incubation at 37^{0}C for 2 h to monitor proteolysis by the TNBS method. Sodium azide (0.05%) was added to prevent microbial growth. Raw milk was a control.

After several trials (results not shown), trypsin was added at 248, 742 and 1485 BAEE units which corresponds to 0.1, 0.3 and 3.0 mg/L. Plasmin was added to UHT skim milk at 8.71 x10^{-3}, 26.1 x10^{-3} and 261 x10^{-3} units. The levels were selected because plasmin in milk is usually between 0.1-0.3 mg/ L. The highest level (3 mg/ L) was selected to predict the behaviour of plasmin in milk at 10 times the levels normally found in milk. Higher levels of trypsin and plasmin were chosen in this study so as to accelerate the proteolytic effects. The milk was then incubated at 37^{0}C for 7 days. Sodium azide (0.05%) was added to prevent microbial growth. UHT milk was used because it is considered to be sterile. Clarification of the samples in all cases was obtained by adding 12% TCA or acetic acid and sodium acetate as described in section 3.6. Analysis of breakdown products was monitored by TNBS, fluorescamine, gel electrophoresis and RP-HPLC. In addition, samples of milk with added plasmin were also analysed by the AMC method as described in section 3.10. Control samples were samples without added trypsin or plasmin.

4.3 Results and discussion

4.3.1 Proteolysis caused by trypsin

4.3.1.1 Analysis by TNBS

4.3.1.1.1 Proteolysis in raw and pasteurised milk

This experiment was aimed at investigating the suitability of the TNBS method in monitoring proteolysis in raw and pasteurised milk from the same source. Comparison was made of proteolysis in raw and pasteurised milk.

Results from preliminary experiments to determine proteolysis of raw and pasteurised milk are presented in Table 4-1. It is clear from the table that longer incubation times of 16 h have

resulted in higher absorbance readings by the TNBS method. However, since absorbance after 16 h gave lower values in pasteurised milk; it was decided to study the system for a longer time period. Thus, the incubation time was increased to 7 days, to give sufficient time for proteolysis to take place and to study the effect of proteolysis by trypsin during storage. The higher absorbance readings observed for 7.5% TCA than 12% TCA soluble extracts were due to decreased solubility with increasing concentration of TCA as more peptides were precipitated and consequently less peptides were present in the soluble extract (Yvon *et al.*, 1989).

Table 4-1 Absorbance of raw and pasteurised milk samples clarified with 7.5 and 12 % TCA after incubation at 37^{0}C for 6 and 16h as analysed by the TNBS method

Samples	Absorbance at 420 nm after incubation at 37^{0}C for 6 /16 h	
	6h	16h
Raw milk (clarified with 7.5 % TCA)	0.429±0.017	0.735±0.012
Raw milk (clarified with 12 % TCA)	0.079±0.045	0.591±0.011
Laboratory pasteurised milk (clarified with 7.5% TCA)	0.221±0.008	0.253±0.003
Laboratory pasteurised milk (clarified with 12 % TCA)	0.072±0.0017	0.185±0.001

The same table also shows that raw milk had higher proteolytic activity than pasteurised milk at both levels of TCA and incubation times. This was unexpected and contradicts previous findings (Richardson, 1983b). It is documented that during pasteurisation, plasminogen activator inhibitors, being heat labile, are destroyed causing more plasminogen to be converted to plasmin resulting in increased proteolysis (Grufferty and Fox, 1988b; Richardson, 1983b). Our results suggest that this is not the case. One possible explanation for the high proteolytic activity in raw milk could be the presence of extracellular bacterial enzymes produced by psychrotrophic bacteria. These heat stable enzymes attack all caseins, with preferential hydrolysis of κ-casein, then β-casein and finally α-casein (Fairbairn and Law, 1986). Hydrolysis of κ-casein causes destabilisation of casein micelles and coagulation

with the formation of para- κ-casein, followed by extensive non-specific hydrolysis (Law *et al.*, 1977a). Although no coagulation was evident in the raw milk sample, the high absorbance observed in raw milk by the TNBS protocol could be a result of bacterial activity. However, as the milk was fresh, this seems unlikely. It is possible that the native enzyme (plasmin) was already present in high amount in the raw milk. This will need to be further investigated in the future.

Addition of 248 and 1485 BAEE units of trypsin to commercial pasteurised milk resulted in increased proteolytic activity in the samples stored at 20^{0}C for 7 days. A consistent increase in activity with time of incubation and trypsin concentration was clearly observed (Figures 4-1 and 4-2), with trypsin added sample (1485 BAEE units) almost reaching a reading of 1 absorbance unit on the seventh day (Figure 4-2). Gelation was observed on day 2 for the same sample but on day 3 for 742 BAEE units added trypsin sample. Control samples were stable throughout the incubation period and did not gel.

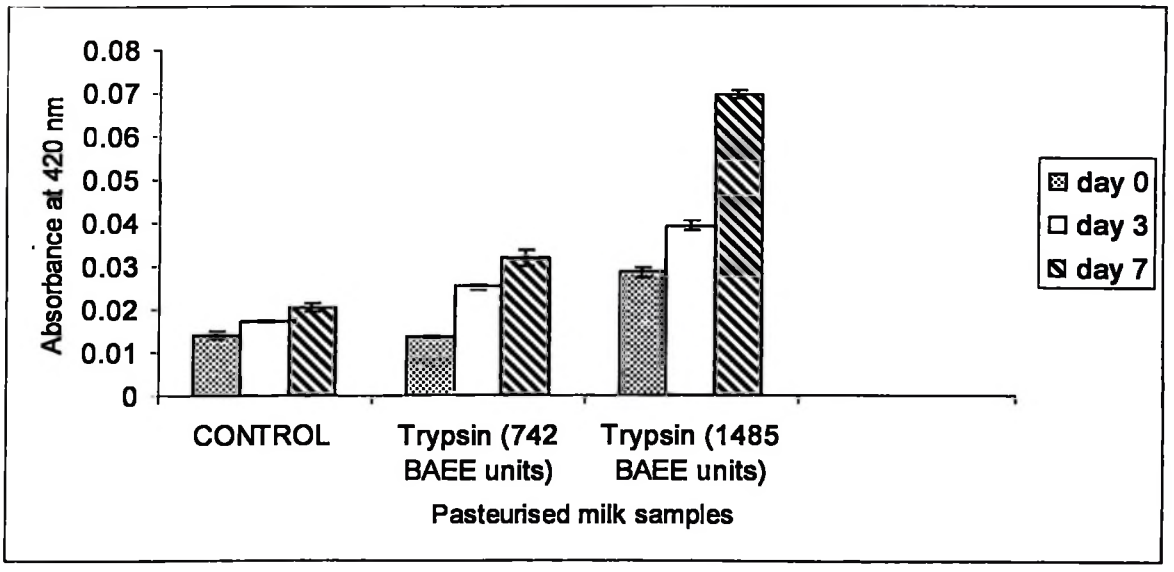


Figure 4-1 Absorbance of pH 4.6 soluble extracts of commercial pasteurised milk samples with added trypsin incubated for 7 days at 20^{0}C as analysed by the TNBS method; Error bars represent standard deviation (N=6); pH 4.6 soluble extracts were diluted (x20).

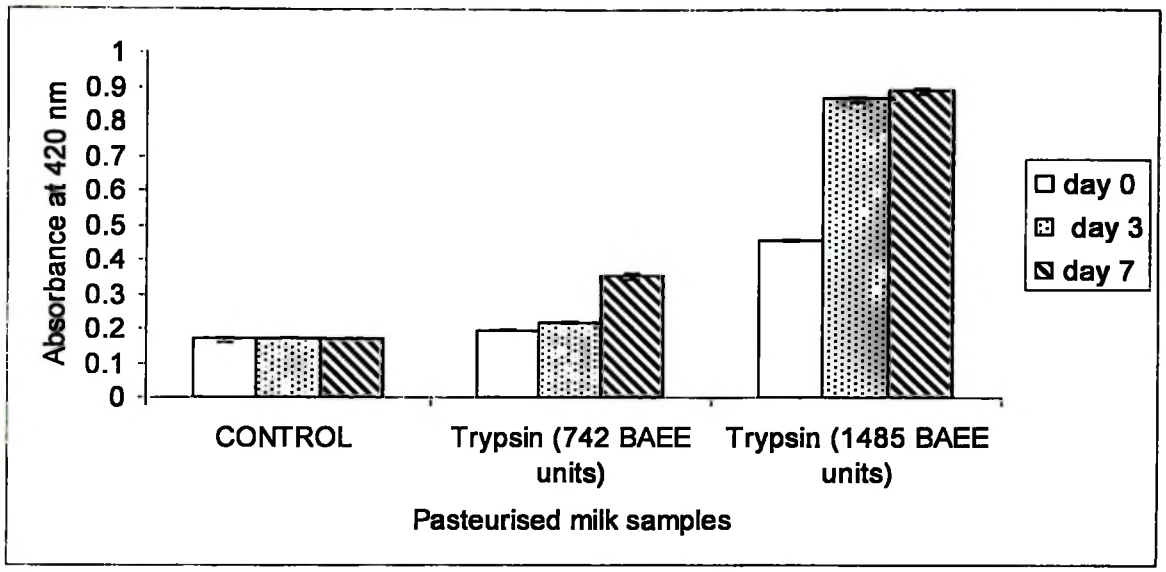


Figure 4-2 Absorbance of 6% TCA soluble extracts of commercial pasteurised milk samples with added trypsin and incubated for 7 days at 20^{0}C as analysed by the TNBS method; Error bars represent standard deviation (N=6); 6% TCA soluble extracts were diluted (x2).

Although pasteurised milk is not usually stored at 20^{0}C, this experiment was aimed at assessing the suitability of the TNBS method in monitoring proteolysis in pasteurised milk. Since trypsin was used in the experiment to represent plasmin, conclusions will be made by referring to plasmin. In the current study it was revealed that residual levels of trypsin in pasteurised milk may cause gelation if the milk is left at 20^{0}C for 3 days. However, pasteurised milk would undergo spoilage when left at 20^{0}C for 24 h. Moreover, in reality such high levels of enzyme are unlikely to be encountered in pasteurised milk as the maximum storage life of pasteurised milk is about 2-3 weeks under refrigeration, which is not sufficient to increase proteolysis to levels causing gelation. However, in the case of inactivation of plasminogen activator inhibitor during pasteurisation, plasmin activity could increase due to increased plasminogen activation (Grufferty and Fox, 1988b). It is obvious that several factors have to be considered when drawing conclusions from these studies as the plasmin system is quite complex. The TNBS method was useful in monitoring proteolysis at

the levels studied, however lower levels of trypsin (247.45 BAEE) units were used in the next experiment to confirm its suitability.

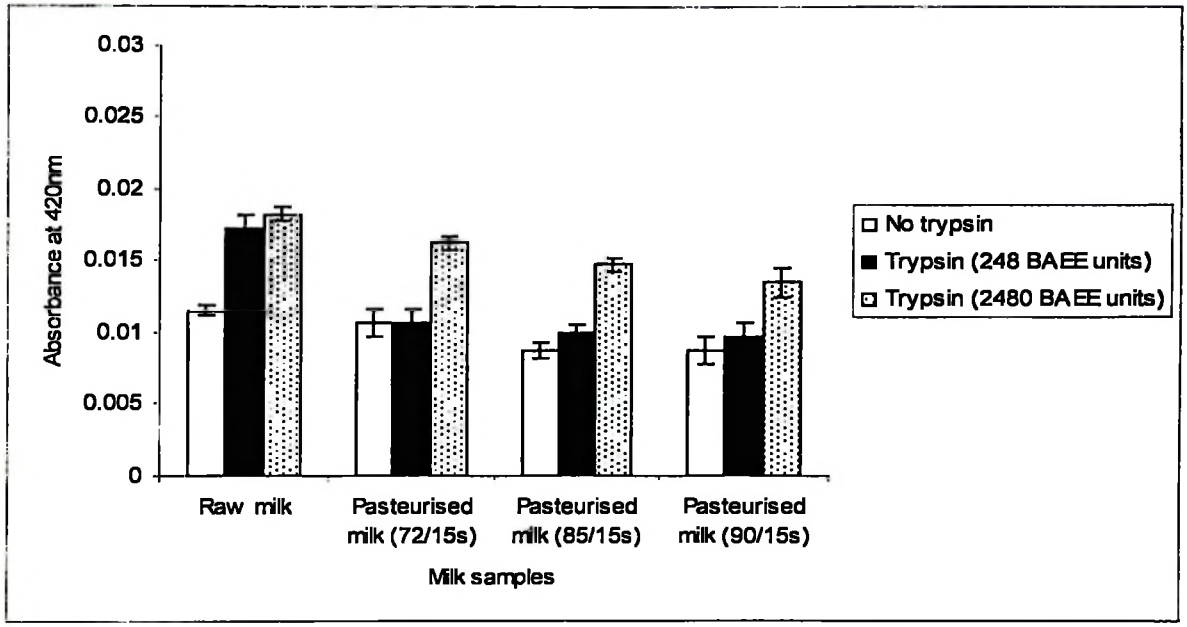


Figure 4-3 Absorbance of pH 4.6 soluble extracts of pasteurised milk incubated at 37^{0}C for 2 h as analysed by the TNBS method; Error bars represent standard deviation (N=6); the experiment was repeated 3 times

The effect of pasteurisation at different temperatures with or without the addition of trypsin (247.45 and 2474.5 BAEE units) is illustrated in Figure 4-3. Trypsin was added after pasteurisation. From this figure, it is evident that raw milk had highest absorbance at all levels of trypsin added (247.45 and 2474.5 BAEE units) compared to pasteurised milk, confirming the results found earlier. However, these differences were small as shown in the figure. All control samples (without trypsin) had residual proteolytic activity probably from native enzymes, which slightly decreased with heating temperature. Milk pasteurised at 90^{0}C had lower absorbance than at 85^{0}C which had lower absorbance than at 72^{0}C at all levels of added trypsin. This meant that more breakdown products were present at the lower rather than higher pasteurisation temperatures. However, since the same raw milk was used for pasteurisation, it is possible that some proteolytic activities were reduced after pasteurisation

because the control pasteurised samples (without added trypsin) had slightly lower absorbance than control raw milk samples. More experiments on the controls to study the effect of plasmin addition will be done later on in the chapter, whereas the effects of heat treatment on proteolysis will be discussed in chapter 6. Based on the current experiment, it may be concluded that pasteurisation slightly affected enzyme activity which meant that pasteurising at 72, 85 and 90 ^{0}C for 15 s did not cause sufficient inactivation of the inhibitors to cause increase in absorbance at higher pasteurisation temperatures.

Several studies regarding the inactivation of plasmin have been documented. A study was reported where pasteurisation at 72^{0}C for 15 s and 63^{0}C for 30 min increased proteolytic activity by 30-40 % and 8-24% respectively, after incubating at 37^{0}C for 3-6 days (Noomen, 1975). Increased proteolysis after pasteurisation was also reported in another study by Andrews (1983c). As discussed earlier, several authors have documented that pasteurisation destroys the plasminogen activator inhibitors and thereby more plasminogen is converted to plasmin by the plasminogen activators (Fox, 1981; Grufferty and Fox; 1988; Nielsen, 2000; Datta and Deeth, 2001; Richardson, 1983b).

Other researchers however, suggested that at higher pasteurisation temperatures, the effect of heat stability was more closely associated with heating time than the heating temperature. The D values (decimal reduction time or the heating time required to reduce the initial activity to 10% of its initial value) of plasmin as reported by Driessen and Van der Waals (1978) was 35.7 min at 72.5^{0}C and 6.4 min at 85^{0}C whereas St–Dennis *et al.* (2001) reported a D value of 2.9 min at 95^{0}C. From these observations it may be argued that since the D-value at 72^{0}C is higher than at 85^{0}C and at 95^{0}C, then more proteolytic activity would be expected at 72^{0}C than at 85^{0}C and at 90^{0}C. The holding time in the current experiment was constant (15 s) which is the standard pasteurisation time at 72^{0}C. Although the time was

shorter than the D-values reported above, proteolytic activity was lower with increasing pasteurisation temperature. This is consistent with results observed in this study. A study by Igarashi (1990) revealed that the degree of proteolysis decreased on heating at temperatures higher than 70^0C. The same author also observed complete loss of plasmin activity at 90^0C for 10 min. The similarity in observations between the current study and that by Igarashi (1990) is probably due to the shorter time of incubation (2 and 20 h respectively) compared to 3-6 days by Noomen (1975).

However, it was pointed out that kinetic data on heat inactivation of plasmin were not very consistent (Farke and Imafidon, 1995). Due to the complex nature of the plasmin system, differences in experimental conditions would result in different conclusions (Metwalli *et al.*, 1998). Heat inactivation of plasmin strongly depended on conditions applied as well as on the behaviour of plasminogen activators, plasminogen activators inhibitors and plasmin inhibitors all of which greatly affect plasmin activity.

4.3.1.1.2 Proteolysis in UHT skim milk

In another experiment, trypsin (247, 742 and 1484 BAEE units) was inoculated into commercial UHT skim milk and proteolysis monitored for 7 days at 37^0C. Sodium azide (0.05%) was added to prevent bacterial contamination.

To examine the effect of storage time at the optimum temperature of activity for trypsin, two soluble extracts of UHT milk (pH 4.6 and 6% TCA) were studied by the methods previously described. Table 4-2 indicates significant differences in proteolysis ($p<0.05$) between the days of incubation as well as between samples with added trypsin levels. By the third day of incubation, samples with 742 and 1484 BAEE units of added trypsin were clarified with a

clear separation between casein and serum. This suggested that extensive proteolysis had taken place by the third day.

Table 4-2 Absorbance of pH 4.6 and 6% TCA soluble extracts of commercial UHT skim milk samples incubated with and without trypsin at 37^{0}C for 7 days to examine the effect of proteolysis on storage time by the TNBS method at 420 nm

Day of incubation	Treatment	Absorbance of pH 4.6 soluble extracts at 420 nm	Absorbance of 6% TCA soluble extracts at 420 nm
Day 0	Control (No trypsin)	0.001 ± 0.0000 (a) J	0.103 ± 0.0012 (a) I
	Trypsin (248 BAEE units)	0.003 ± 0.0001 (b) A	0.111 ± 0.0036 (a) A
	Trypsin (742 BAEE units)	0.003 ± 0.0002 (b) D	0.115 ± 0.0019 (b) C
	Trypsin (1485 BAEE units)	0.003 ± 0.0004 (c) G	0.120 ± 0.0027 (c) F
Day 3	Control (No trypsin)	0.002 ± 0.0016 (d) K	0.117 ± 0.0024 (d) J
	Trypsin (248 BAEE units)	0.042 ± 0.0020 (e) B	0.123 ± 0.0023 (d) A
	Trypsin (742 BAEE units)	0.060 ± 0.0080 (e) E	0.391 ± 0.1802 (e) D
	Trypsin (1485 BAEE units)	0.240 ± 0.7000 (f) H	1.241 ± 0.4039 (f) G
Day 7	Control (No trypsin)	0.002 ± 0.0019 (g) L	0.118 ± 0.0023 (g) J
	Trypsin (248 BAEE units)	0.054 ± 0.0024 (h) C	0.220 ± 0.0727 (h) B
	Trypsin (742 BAEE units)	0.160 ± 0.0406 (i) F	1.089 ± 0.0733 (i) E
	Trypsin (1485 BAEE units)	0.312 ± 0.0552 (j) I	1.764 ± 0.0196 (j) H

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment: The experiment was repeated 3 times; N=9; TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20)

Although the absorbance of pH 4.6 soluble extracts appeared low with similar values on the day of incubation, it was chiefly due to high sample dilution (x 20). However, absorbance increased linearly with time. The control sample had similar activity on days 3 and 7 as compared to the day of incubation (d 0), indicating constant activity during incubation. At 247 BAEE units of added trypsin, the increase was 14 and 18 times on days 3 and 7 respectively. The increase in activity was 20 times for 742 BAEE units of added trypsin on

day 3 and 53 times on day 7. At 1485 BAEE units of added trypsin, the increase was 80 times on day 3 and 104 times on day 7. Based on these observations, it could be suggested that the rapid increase in proteolytic activities was due to both added trypsin and probably other residual enzymes from milk which would have survived heat treatment, presumably plasmin. As sodium azide was used to prevent microbial activity, the small increase in proteolytic activity in the control sample in pH 4.6 soluble extract was probably derived from plasmin. As the increase in proteolytic activity was negligible in 6% TCA soluble extracts of control samples (no significant differences between day 3 and day 7 at $p > 0.05$), it is more likely the peptides produced were large, presumably originating from native enzymes, possibly plasmin and so were precipitated by TCA. Unlike TCA soluble extracts in which TCA precipitates large peptides (Datta and Deeth, 2003), pH 4.6 soluble extracts solubilise these large peptides, hence their presence in the extracts.

The 6% TCA soluble extracts show significant differences in proteolysis ($p < 0.05$) at the various levels of added trypsin. As expected, absorbance values increased proportionally with both trypsin concentration and the time of incubation. As explained above, no significant differences were found between days 3 and 7 for control samples ($p>0.05$). Likewise, added trypsin at 248 BAEE units did not show significant differences ($p>0.05$) between day 0 and day 3, but increased almost two fold on day 7 (~1.78 times). The increase in absorbance between day 0 and day 3 at 742 BAEE units of added trypsin was 3 times whereas it was 10 times on day 7. For 1485 BAEE units of added trypsin the increase was 10 times on day 3 but almost 15 times on day 7 respectively. This trend indicates that at lower added trypsin levels, the large peptides are precipitated by TCA and hence this results in lower absorbance readings. It is speculated that with increasing trypsin levels and the availability of the

substrate, more peptides are continuously being broken down and hence increasing absorbance readings.

The results are similar to those observed by Datta and Deeth (2003) when plasmin was added at higher levels. Although the levels of plasmin added by the latter authors were much higher (>1000 mg/ L), the incubation time was only for 2 hours at 40^0C. Thus, given that in the current experiment the incubation was for 7 days, it is most likely that extensive proteolysis resulted in higher absorbance values.

Figure 4-4 shows a strong positive correlation (R^2= 0.974) between pH 4.6 and 6% TCA soluble extracts of UHT skim milk. In addition, the TNBS protocol was able to detect low levels of added trypsin (248 BAEE units). This suggests that the TNBS protocol is a useful technique to study proteolysis and may be used for the purpose of routine monitoring of proteolysis in the laboratory. This method was also recommended as an indicator of shelf life studies since it could detect proteolysis prior to development of off-flavours which is crucial for quality assessment (McKellar, 1981). However, comparison with other methods will be necessary to evaluate the best method for monitoring proteolysis.

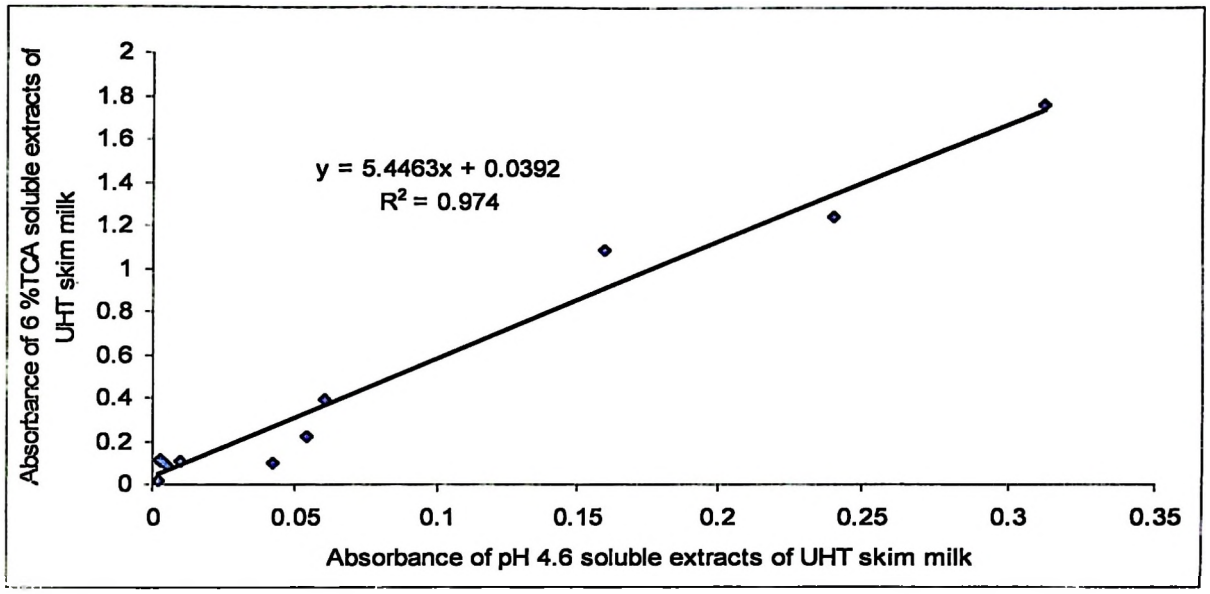


Figure 4-4 Correlation between absorbance values of pH 4.6 and 6% TCA extracts of UHT skim milk incubated with trypsin at 37^{0}C for 7 days as analysed by the TNBS

4.3.1.2 Analysis by Fluorescamine

The suitability of the fluorescamine method in monitoring proteolysis was studied in UHT skim milk samples treated with 248, 742 and 1485 BAEE units of added trypsin during storage at 37^{0}C for 7 days. The pH 4.6 and 6% TCA soluble extracts of UHT skim milk from these samples were monitored with time.

Table 4-3 Relative fluorescence of pH 4.6 and 6% TCA soluble extracts of UHT skim milk samples incubated with and without trypsin at 37^{0}C for 7 days to examine the effect of proteolysis on storage by the fluorescamine method

Day of incubation	Treatments	Relative fluorescence of pH 4.6 soluble extracts of UHT skim milk at 475 nm		Relative fluorescence of 6% TCA soluble extracts of UHT skim milk at 475 nm	
Day 0	Control (No trypsin)	134 ± 2.0	(a) A	91 ± 3.4	(a) A
	Trypsin (248 BAEE units)	158 ± 2.4	(c) D	98 ± 7.0	(b) D
	Trypsin (742 BAEE units)	154 ± 2.1	(b) G	100 ± 3.2	(b) G
	Trypsin (1485 BAEE units)	277 ± 3.4	(d) J	234 ± 4.0	(c) H
Day 3	Control (No trypsin)	145 ± 5.2	(e) B	112 ± 3.0	(d) B
	Trypsin (248 BAEE units)	382 ± 8.3	(f) E	364 ± 7.4	(e) E
	Trypsin (742 BAEE units)	860 ± 8.1	(g) H	1100	(f) *
	Trypsin (1485 BAEE units)	1100	(h) *	1100	(f) *
Day 7	Control (No trypsin)	163 ± 3.3	(i) C	134± 4.2	(g) C
	Trypsin (248 BAEE units)	446 ± 3.4	(j) F	580 ± 5.2	(h) F
	Trypsin (742 BAEE units)	931 ± 34.9	(k) I	1100	(i) *
	Trypsin (1485 BAEE units)	1100	(l) *	1100	(i) *

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment; The experiment was replicated 3 times; * upper detection limit
The TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20)

Table 4-3 clearly indicates increasing fluorescence units with both trypsin levels and incubation time. Although the upper detection limit of the equipment was reached on day 3, significant differences in proteolysis were observed among same samples on all days of incubation ($p<0.05$). In addition, proteolysis was significantly different ($p<0.05$) in all samples on each day of incubation for both extracts except at 248 and 742 BAEE units of added trypsin in 6% TCA soluble extract.

The increase in pH 4.6 soluble extracts between days 0 and 7 was 3, 6 and more than 4 times respectively for 248, 742 and 1485 BAEE units of added trypsin but only once for control. Likewise, for 6% TCA soluble extracts the increase was 1 time for control sample but 6, 11 and more than 5 times for added trypsin at 248, 742 and 1485 BAEE units. It is evident that the increase in proteolytic activities on day 7 was much higher in 6% TCA than the pH 4.6 soluble extract. However accurate results on the rate of increase of proteolytic activity can not be predicted because the detection limit (1100) was reached on days 3 and 7 for both pH 4.6 and 6% TCA soluble extracts. It is more likely to have higher rate of proteolysis at the highest level of added trypsin than lower.

Due to the low upper detection limit of the equipment (1100 fluorescence units), the rate of increase in proteolytic activity was approximately calculated. Thus, the major limitation with the method is the low upper detection limit particularly with the equipment used in the current experiment. It has been mentioned that some spectrofluorimeters can record readings up to 10,000 fluorescence units and these though more expensive, would be more useful.

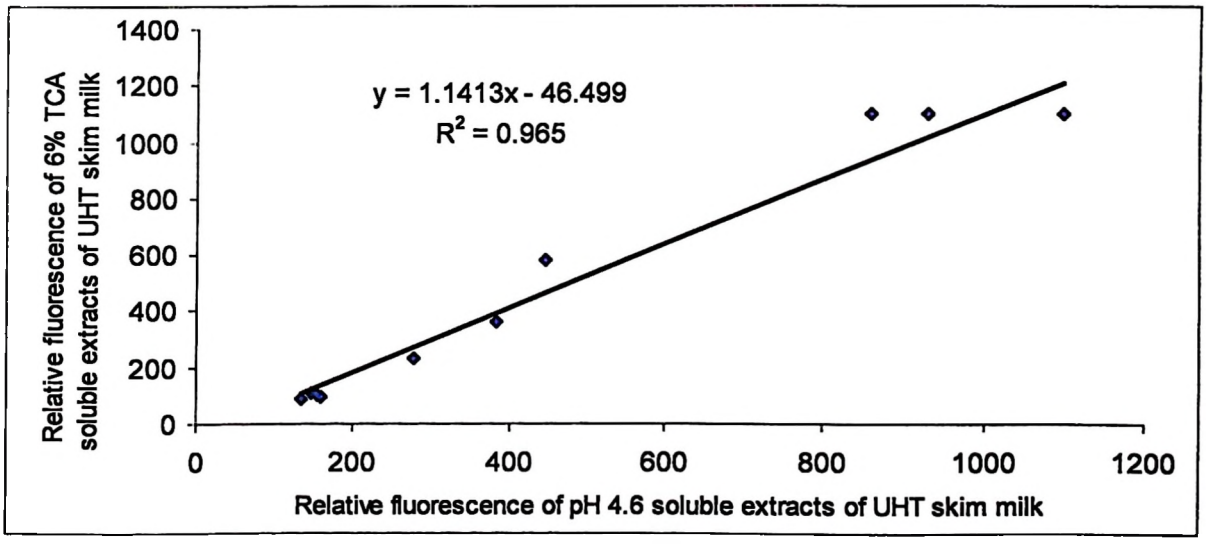


Figure 4-5 Relative fluorescence of pH 4.6 and 6% TCA soluble extracts of UHT skim milk with added trypsin incubated at 37^{0}C for 7 days as determined by fluorescamine method.

Although there were problems with the upper detection limit, the pH 4.6 and 6% TCA extracts were strongly correlated (R^2= 0.965) as shown in Figure 4-5, indicating the suitability of the fluorescamine method in monitoring proteolysis in the two extracts. The method was highly recommended by Beeby (1980). It was also shown to be superior to TNBS (Schwabe, 1973). Other researchers recommended it as a sensitive method for measuring protease activity that is specific for primary amino groups and free of interference (Chism *et al.*, 1979). The method was regarded to be most reliable followed by TNBS, absorbance at 280 nm and finally the Lowry method (Kwan *et al.*, 1983).

4.3.1.3 Analysis by RP-HPLC

The RP-HPLC protocol was also used to analyse the pH 4.6 and 6% TCA soluble extracts as described in section 3.9 following proteolysis by 248, 742 and 1485 BAEE units of added trypsin at 37^{0}C for 7 days. Comparison was made of the proteolysis between the two soluble extracts. The suitability of the RP-HPLC method in assessing proteolysis was compared against other methods, in section 4.4.

Table 4-4 Peak areas of pH 4.6 and 6% TCA soluble extracts of UHT skim milk samples incubated with and without added trypsin at 37^0C for 7 days to examine the effect of proteolysis on storage by the RP-HPLC

Days of incubation	Treatments	Total peak areas of pH 4.6 soluble extracts of UHT skim milk		Total peak areas of 6% TCA soluble extracts of UHT skim milk	
Day 0	Control (No trypsin)	20.9±1.81	(a) A	53.0 ±1.75	(a) A
	Trypsin (248 BAEE units)	20.2±0.82	(a) D	60.8 ±1.26	(b) D
	Trypsin (742 BAEE units)	31.4±1.26	(b) G	61.0 ±1.73	(b) G
	Trypsin (1485 BAEE units)	38.5±1.45	(c) J	63.3 ±1.83	(c) J
Day 3	Control (No trypsin)	29.0±1.79	(d) B	62.4 ±2.22	(d) B
	Trypsin (248 BAEE units)	54.1± 3.91	(d) E	146 ±3.44	(e) E
	Trypsin (742 BAEE units)	246± 69.8	(e) H	499 ±6.79	(f) K
	Trypsin (1485 BAEE units)	342 ±1.89	(f) K	1404 ±15.3	(g) H
Day 7	Control (No trypsin)	33.9±1.86	(g) C	66.3 ±4.14	(h) C
	Trypsin (248 BAEE units)	290 ±3.97	(h) F	228 ±8.83	(i) F
	Trypsin (742 BAEE units)	383±36.5	(i) I	1340 ±22.9	(j) I
	Trypsin (1485 BAEE units)	679±15.2	(j) L	2110 ±37.5	(k) L

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment; The experiment was replicated 3 times; TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20)

Table 4-4 shows significant differences in proteolysis ($p<0.05$) between the same samples on different days of incubation in both soluble extracts. The increase in proteolytic activity from day 0 to 7 was highest at 1485 BAEE units of added trypsin for both pH 4.6 and 6% TCA soluble extracts (18 and 33 times) respectively. The increase in 742 BAEE units of added trypsin was 12 and 22 times respectively for each soluble extract but 14 and 4 times at 248 BAEE units. The control sample was almost constant (the increase was less than double) respectively in pH 4.6 and 6% TCA soluble extracts.

As previously explained (section 4.3.1.1.2), the low but constant activity in control samples was probably due to naturally occurring enzymes in milk.

As expected, the increase in peak area was highest at 1485 BAEE units of added trypsin, with 6% TCA soluble extracts having higher peak areas than in pH 4.6 soluble extract. As discussed earlier, TCA precipitates large peptides, such as those formed by trypsin and therefore those peptides should essentially be absent in TCA soluble extracts. Previous researchers have reported that an increase in proteolytic activity in 6% TCA soluble extracts was due to bacterial enzymes, whereas an increase in pH 4.6 was due to both bacterial and native enzymes and hence higher values are usually found in the latter extract (Datta and Deeth, 2003). The increased proteolytic activity in 6% TCA extract was most likely to be due to further degradation of large peptides by trypsin at such high levels, since sodium azide was used to prevent bacterial contamination and no bacterial proteases were added. In addition, control samples had constant activity implying low or negligible contribution from naturally occurring enzymes. As more trypsin was available, peptides that had been cleaved were exposed to reveal new sites which were further broken down into much smaller peptides and amino acids. Some researchers suggested that the extent of proteolysis of milk proteins by pancreatic enzymes depended on the number of target sites available for each enzyme and on the accessibility of these target peptide bonds by proteases (Salami *et al.*, 2008). This observation is also supported by the fact that, the trend of increased proteolytic activity in 6% TCA than pH 4.6 was especially observed at 742 and 1487 BAEE units of added trypsin but not at 248 BAEE units of added trypsin. It is possible that at this low level, although the substrate was available, the enzyme level was too low to cause significant proteolysis, and hence fewer peptides were detected. Moreover, the pH 4.6 soluble extract was more dilute (x10) than 6% TCA soluble extract, thus lower readings in pH 4.6 soluble extracts.

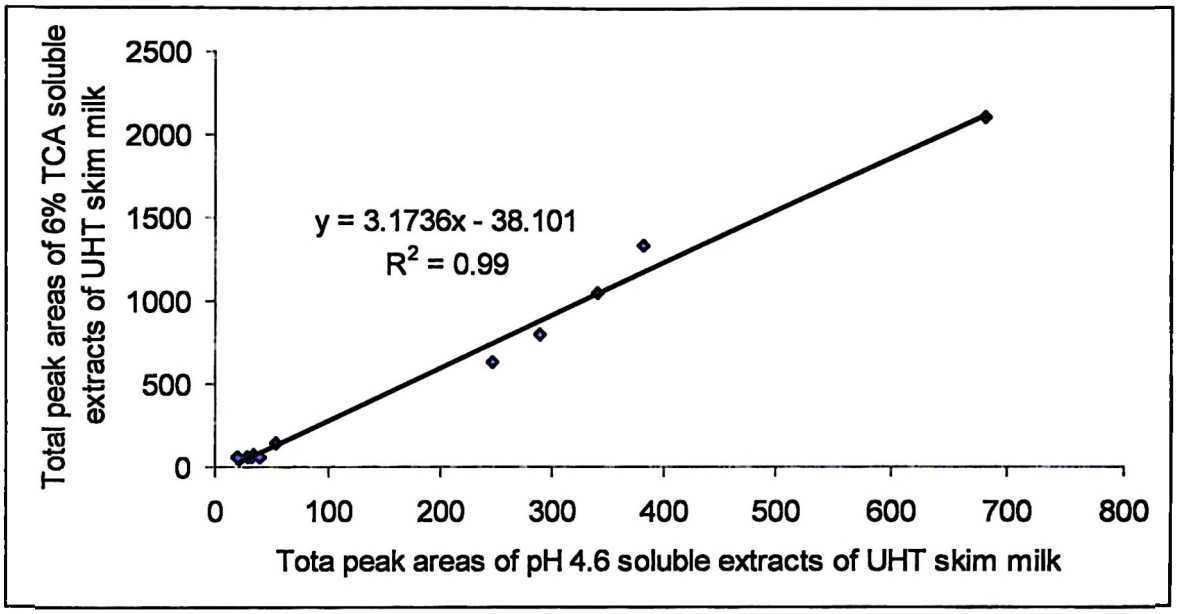


Figure 4-6 Correlation between total peak areas of pH 4.6 and 6% TCA soluble extracts of UHT skim milk incubated at 37^{0}C for 7 days as determined by the RP-HPLC

A strong correlation (R^2 = 0.99) was observed between pH 4.6 and 6% TCA soluble extracts as shown in Figure 4-6. This suggests that RP-HPLC method is an accurate and efficient method for detecting proteolysis in milk. It was found to be 600 times more sensitive than the fluorescamine (Le *et al.*, 2006).

4.3.1.4 Analysis by gel electrophoresis

Urea-PAGE electrophoresis is a useful procedure for monitoring proteolysis of caseins by enzymes because unlike SDS-PAGE, the caseins are well separated because separation is based on charge and not molecular weight. In the current experiment UHT skim milk with and without added trypsin were analysed by this method during incubation at 37^{0}C for 7 days and the results compared against those of TNBS, fluorescamine and RP-HPLC.

Figure 4-7 shows protein breakdown of samples incubated with added trypsin at 37^{0}C for 0, 3 and 7 days. There was no difference in protein breakdown between control (lane 4) and samples with 248 and 742 BAEE units of added trypsin (lanes 1 and 2) on day 0 indicating

low trypsin activity. On lane 3 however, there was disappearance of β-casein with concomitant appearance of γ-casein, which confirmed extensive proteolysis by trypsin at such high concentration.

On day 3, there was a progressive disappearance of β-caseins from lane 5 to 7 where γ-caseins (products of β-caseins degradation by trypsin) were clearly visible in samples with 248 BAEE units of added trypsin but faintly at 742 BAEE units of added trypsin and none at 1485 units of added trypsin (lane 7). β- casein was completely degraded on lane 6 and neither α- nor β-caseins were undegraded on lane 7. On the same lanes (6 and 7), further hydrolysis of the α-caseins resulted in decreased intensity of the bands, indicating increased activity on day 3. The single band on lane 7 is due to α-casein degradation products, formed after cleavage of α-casein. On day 7, β-caseins were degraded further on lane 9 (248 BAEE units of added trypsin) and produced more γ-caseins. On lane 10 however, the γ-caseins previously formed on day 3 were absent. It is most likely that many small peptides (which would not be fixed in the gel and hence would be lost during staining and destaining) may have been formed as a result of extensive proteolysis at such high levels of added plasmin.

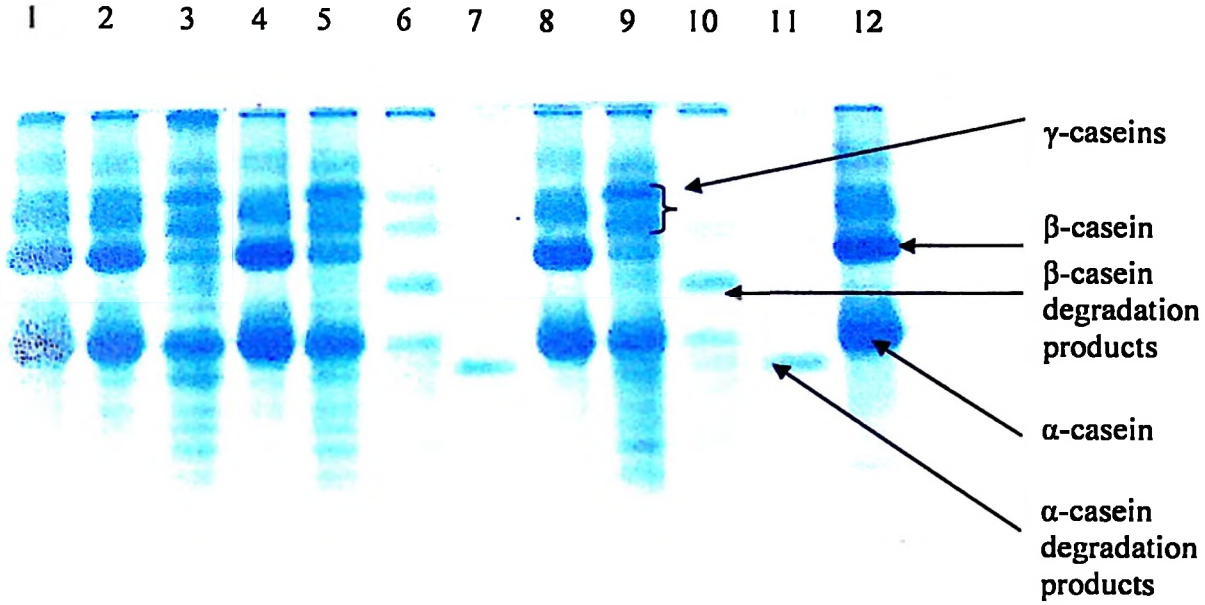


Figure 4-7 Urea-PAGE electrophoretogram (T=12%, C=4%, pH 8.9) of trypsin inoculated in UHT milk at various levels and incubated for 7 days at 37^{0}C.**Lanes 1-3**: trypsin-treated UHT milk (248, 742 and 1485 BAEE units) on day 0. **Lane 4:** Control UHT milk on day 0 **Lanes 5-7** trypsin-treated UHT milk (248, 742 and 1485 BAEE units) on day 3 **Lane 8:** Control UHT milk on day 3. **Lanes 9-11:** trypsin-treated UHT milk (248, 742 and 1485 units) on day 7 **Lane 12:** Control UHT milk on day 7.

Gradual to complete disappearance of γ-caseins was evident at 742 BAEE units of added trypsin (from lanes 2 to 6 to 10) and 1485 BAEE units of added trypsin from (lanes 3 to 7 to 11) from day 0 to 7, which suggests that extensive proteolysis by trypsin further degraded the γ-caseins formed. This finding has never been reported before. A study by Igarashi (1989) revealed that incubating at 37^{0}C for 20 h initially increased the γ-caseins but a decline in the rate of increase during the later half of incubation was observed. Other researchers have observed increase in γ-caseins with incubation time. However these studies were performed for shorter time than in the current experiment such as 6 and 24 h (Crudden *et al.*, 2005c; Rham and Andrews, 1982 a).

Since both on days 3 and 7, β-casein degradation products were formed at 742 BAEE units of added trypsin but α-casein degradation products were evident on the same days at 1485 BAEE units of added trypsin, it implies preferential cleavage of β-casein over α-casein, a finding that has been documented for plasmin (Datta and Deeth, 2003). Both the α- and β-caseins in the control sample remained intact and did not show any proteolytic activity, from day 0 to 7. This is contradictory to the TNBS and fluorescamine results where significant differences in proteolysis were observed in the control samples between the various days of incubation. However, since electrophoresis was used as a qualitative tool to monitor proteolysis, direct comparison to the quantitative methods such as the TNBS method should be indicative only, otherwise it may be misleading as quantitative methods can measure and detect very low quantities.

In order to compare breakdown products of raw and pasteurised milk with those from UHT milk, raw and pasteurised milk were injected with trypsin at 248 BAEE units of added trypsin followed by incubation at 37^0C for 3 days to monitor proteolysis. Sodium azide (0.05%) was added to all samples. From Figure 4-8, no differences were observed between raw and pasteurised milk on day 0. However, on day 3, both control and trypsin added samples showed increased proteolytic activity, with concomitant formation of γ- caseins (lanes 7, 9, 10 and 12). As the amount of added trypsin was low (248 BAEE units of added trypsin), neither β-casein nor α-casein degradation products were observed on day 3 as in contrast to Figure 4-6. The bands were slightly fainter in pasteurised than raw milk (comparing lanes 7 with 10 or lane 9 with 12), indicating slightly higher activity from the former than the latter. The same observation was found in other studies (Somers *et al.*, 2003; Andrews and Alichanidis, 1983 b). The presence of γ-caseins in the control samples was probably due to proteolysis by native enzyme, possibly plasmin. Generally, caseins are more

rapidly hydrolysed because of their greater flexibility and open structures (Salami *et al.*, 2008). As control and trypsin added (248 BAEE units of added trypsin) samples did not show much difference in the casein breakdown profiles, it may be concluded that 248 BAEE units of added trypsin was too low to cause significant proteolysis to be detected by the electrophoresis method.

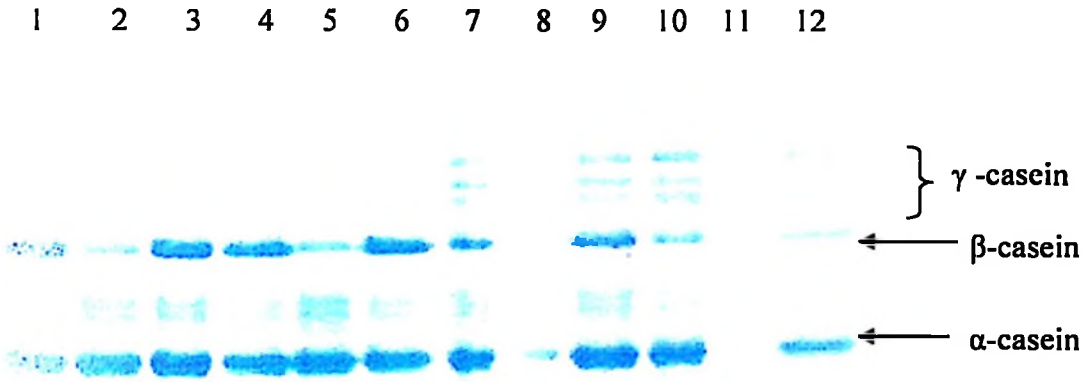


Figure 4-8 Electrophoretogram of UREA PAGE (T= 12%, C= 4%, pH 8.9) of trypsin and *Bacillus* inoculated in raw and pasteurised milk and incubated for 3 days at 37^{0}C. **Lanes 1-3:** Raw milk incubated with trypsin (248 BAEE units), *Bacillus* (6.06 x 10^{-4} units), control on day 0. **Lanes 4-6:** Pasteurised milk incubated with trypsin (248 BAEE units), *Bacillus* (6.06 x 10^{-3} units) , control on day 0 **Lanes 7-9:** Raw milk incubated with trypsin (247.5 BAEE units), *Bacillus* (6.063 x 10^{-4} units), control on day 3 **Lanes 10-12:** Pasteurised milk incubated with trypsin (248 BAEE units), *Bacillus* (6.06 x 10^{-4} units), control on day 3; **NOTE:** The same gel was run for *Bacillus licheniformis* and trypsin samples; the *Bacillus* sample however, will be discussed in chapter 5 section 5.3.3.3.

Following proteolysis of pasteurised milk by trypsin at 37^{0}C for 14 days, electrophoresis by SDS-PAGE was carried out to analyse the breakdown products. Figure 4-9 clearly shows that the rate of casein breakdown increased with time. As observed earlier (Figure 4-8) both

control and sample with 248 BAEE units of added trypsin showed similar breakdown pattern at all incubation times. Proteolysis from control sample is probably due to residual plasmin that was present. There were no major changes on day 3, but on day 7 however, both α and β-caseins were degraded from both trypsin added (248 BAEE units) and control samples indicating some degree of hydrolysis had occurred. Bands below 14.2 kDa appeared concentrated on days 3 and 7 but disappeared on day 14 signifying extensive proteolysis. The new bands on lanes 5 and 7 (just above 14.2 kDa) were casein degradation products which were further degraded until their disappearance on day 14 (lanes 8 and 10). Gradual to complete disappearance of β-casein band was observed from day 7 to day 14 (lanes 4 and 7; 8 and 10) also indicating high rate of casein hydrolysis.

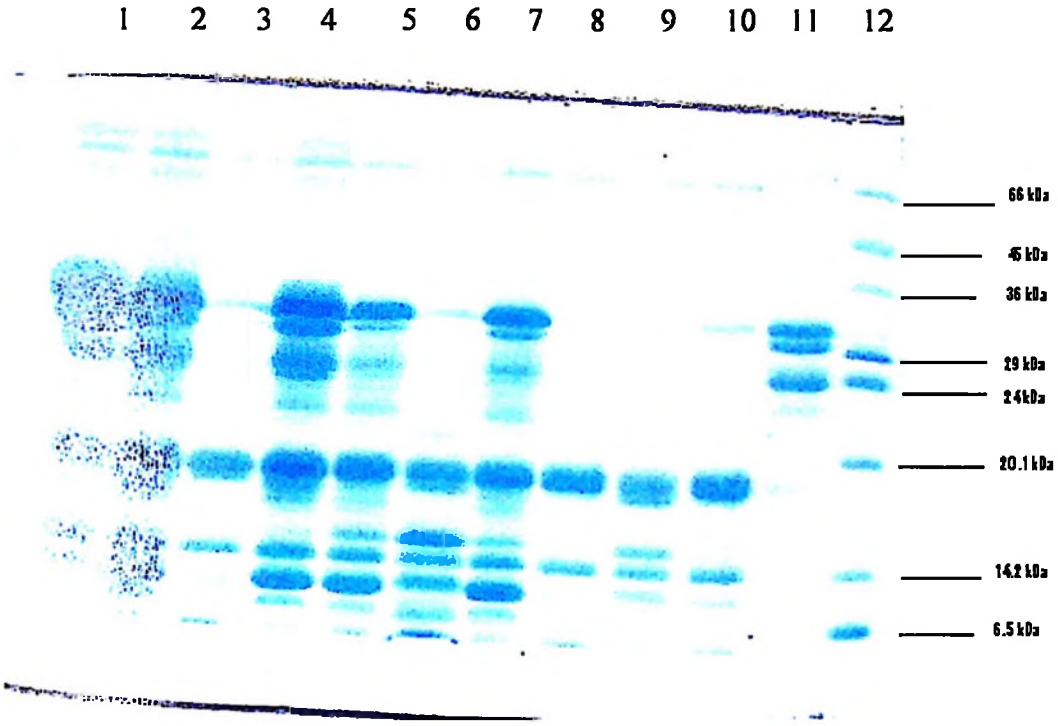


Figure 4-9 SDS-PAGE (T=15%, C=4%, pH 8.9) of pasteurised milk samples incubated with trypsin (248 BAEE units) and *Bacillus* (12.1 x 10^{-4} units) at 37^{0}C for 3, 7 and 14 days.**Lane 1:** pasteurised milk control on d0 **Lane 2-4:**pasteurised milk on d3 incubated with trypsin, *Bacillus*, control respectively **Lane 5-7:**pasteurised milk incubated on d7 with trypsin, *Bacillus*, control **Lane 8-10:** pasteurised milk incubated on d14 with trypsin *Bacillus*, control **Lane 11:** caseins (α β κ) **Lane 12:** Low molecular weight markers (6.5 – 66 kDa) **NOTE:** The same gel was run for *Bacillus licheniformis* and trypsin samples; the *Bacillus* samples however, are not discussed in this chapter

4.3.2 Proteolysis caused by plasmin

4.3.2.1 Analysis by TNBS

Following proteolysis by trypsin, a similar procedure was repeated to analyse proteolysis by added plasmin using the TNBS, fluorescamine, RP-HPLC and gel electrophoresis methods to monitor proteolysis during storage. Plasmin (8.71 x 10^{-3}, 26.1 x 10^{-3} and 261 x 10^{-3} units) was injected into commercial UHT skim milk and proteolysis monitored for 7 days at 37^{0}C. Clarification to obtain pH 4.6 and 6% TCA soluble extracts was done as described in section 3.6. As trypsin was documented to have similar proteolytic activity to plasmin, comparison was also made of the breakdown products from these enzymes at the concentrations used.

Although no significant differences in proteolysis were observed on day 0 for pH 4.6 and 6% TCA soluble extracts, analysing the same samples at each day of incubation showed significant differences ($p<0.05$) as shown on (Table 4-5). This observation implies that significant proteolysis occurred in these samples during incubation on each day.

Table 4-5 Absorbance of pH 4.6 and 6% TCA soluble extracts of UHT skim milk incubated with or without added plasmin at 37°C for 7 days as analysed by the TNBS method at 420 nm

Incubation time (days)	Treatments	Absorbance of pH 4.6 soluble extracts at 420 nm	Absorbance of 6 % TCA soluble extracts at 420 nm
d0	Control (no plasmin)	0.011±0.0011 (a) A	0.117±0.0044 (a) A
	Plasmin (8.71×10^{-3} units)	0.012±0.0016 (a) D	0.115±0.0028 (a) D, E
	Plasmin (26.1×10^{-3} units)	0.011±0.0009 (a) G	0.112±0.0024 (a) G
	Plasmin (261×10^{-3} units)	0.012±0.0013 (a) J	0.118±0.0026 (a) J
d3	Control (no plasmin)	0.035±0.0017 (b) B	0.078±0.0276 (c) B
	Plasmin (8.71×10^{-3} units)	0.041±0.0018 (b) E	0.092±0.3520 (c) D
	Plasmin (26.1×10^{-3} units)	0.049±0.0033 (b) H	0.139±0.0286 (d) H
	Plasmin (261×10^{-3} units)	0.194±0.0660 (c) K	0.691±0.0230 (e) K
d7	Control (no plasmin)	0.051±0.0041 (d) C	0.101±0.0489 (f) C
	Plasmin (8.71×10^{-3} units)	0.054±0.0049 (d) F	0.135±0.0039 (f,g) E
	Plasmin (26.1×10^{-3} units)	0.069±0.0063 (e) I	0.177±0.0051 (g) I
	Plasmin (261×10^{-3} units)	0.263±0.0055 (f) L	0.835±0.0644 (h) L

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day of analysis; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment; The experiment was repeated 2 times; N=6;
The TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20)

The increase in absorbance from day 0 to 3 was 3, 3.4, 4 and 16 times respectively for control, 8.71×10^{-3}, 26.1×10^{-3} and 261×10^{-3} units of added plasmin. It is clear from the trend that rate of activity increased proportionally with the concentration of added enzyme.

On analysing same samples of 6% TCA soluble extracts on the different days of incubation, Table 4-5 indicates that with the exception of 8.71×10^{-3} units of added plasmin all other samples were significantly different ($p < 0.05$) in proteolysis.

Like pH 4.6 soluble extracts on day 0, no significant differences in proteolysis were found in the 6% TCA soluble extracts because it was the day of incubation so low or absence of

significant proteolysis was not unexpected. The increases in absorbance on day 3 were less than twice for 8.71 x 10^{-3}, 26.1 x 10^{-3} units of added plasmin respectively and almost 6 times for 261 x 10^{-3} units of added plasmin. On day 7, the increase was less than twice for both 8.71 x 10^{-3}, 26.1 x 10^{-3} units of added plasmin but 7 times for 261 x 10^{-3} units of added plasmin. This trend confirms earlier observations that with increasing enzyme concentration and incubation time, higher activities were evident. Proteolytic activities for control and 8.71 x 10^{-3} units of added plasmin on day 3 were low and significantly different from other samples (26.1 x 10^{-3} and 261 x 10^{-3} units of added plasmin). On day 7 however, 261 x 10^{-3} units of added plasmin had highest activity and was significantly different from all other samples.

The lower absorbance readings in pH 4.6 soluble extracts compared to 6% TCA soluble extracts were as discussed in section 4.3.1.3 and also mainly due to sample dilution.

The two soluble extracts are strongly correlated (R^2 = 0.947) and thus this method proved to be useful for the determination of proteolysis in milk (Figure 4-10).

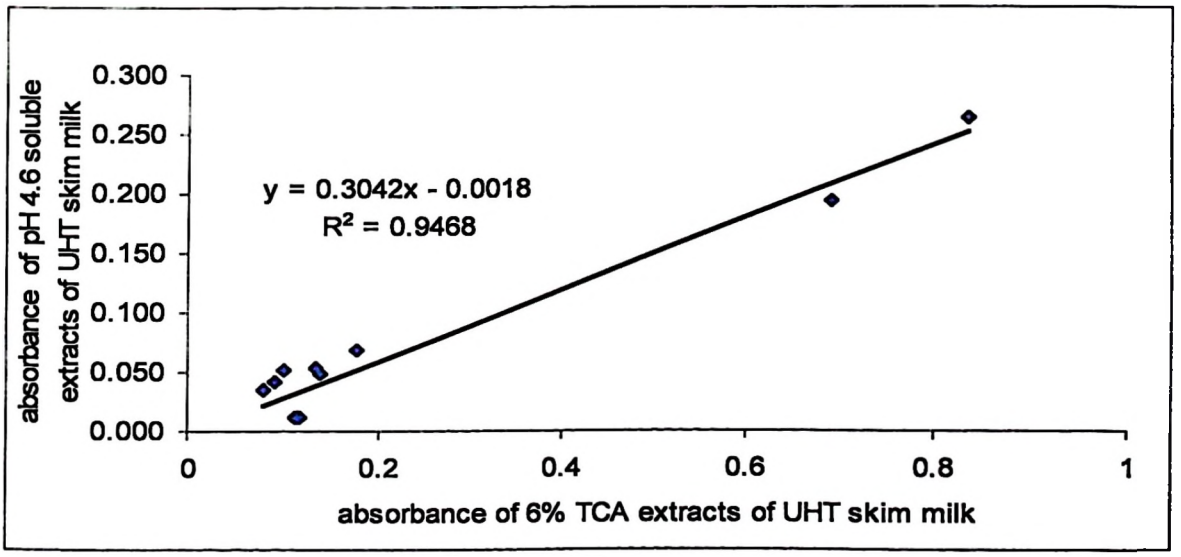


Figure 4-10 Correlation between absorbance values of pH 4.6 and 6% TCA soluble extracts of UHT skim milk with or without added plasmin incubated at 37^{0}C for 7 days as determined by the TNBS method

4.3.2.2 Analysis by Fluorescamine

The fluorescamine method was used to assess its suitability to monitor proteolysis in milk caused by added plasmin. As previously employed in monitoring trypsin activity in pH 4.6 and 6% TCA soluble extracts, this section investigates plasmin activity in the same extracts.

Table 4-6 shows no significant differences in proteolysis ($p > 0.05$) between samples on day 0 for both pH 4.6 and 6% TCA extracts which was similar to observation by the TNBS method (Table 4-5) which meant that the samples had low or inactive native enzymes and hence limited proteolysis. Analysing the same samples on each day of incubation revealed that, only 261.12×10^{-3} units of added plasmin was statistically different in proteolysis ($p < 0.05$) on each day of incubation, whereas other samples did not differ on days 3 and 7 in pH 4.6 soluble extracts.

For 6% TCA soluble extracts however, no significant differences were observed for control sample on each day of incubation. This suggests that these samples had low activity. On day 3, sample with 8.71×10^{-3} units of added plasmin was not statistically different from either day 0 or day 7. Plasmin added samples (261×10^{-3} units) on day 0, were statistically different in proteolysis to samples from days 3 and 7.

Table 4-6 Relative fluorescence of pH 4.6 and 6% TCA soluble extracts of UHT skim milk incubated at 37^{0}C for 7 days as analysed by the fluorescamine method at 475nm

Day of incubation	Treatments	Relative fluorescence of pH 4.6 soluble extracts of UHT skim milk at 475 nm	Relative fluorescence of 6% TCA soluble extracts of UHT skim milk at 475 nm
Day 0	Control (no plasmin)	98±15.6 (a) A	110±11.5 (a) A
	Plasmin (8.71 x 10^{-3} units)	97±19.3 (a) C	117±18.3 (a) B
	Plasmin (26.1 x 10^{-3} units)	98±16.9 (a) E	120±22.5 (a) D
	Plasmin (261 x 10^{-3} units)	98±16.5 (a) G	134±39.3 (a) F
Day 3	Control (no plasmin)	148±5.1 (b) B	114±16.5 (b) A
	Plasmin (8.71 x 10^{-3} units)	199±11.9 (c) D	151±38.0 (b) B, C
	Plasmin (26.1 x 10^{-3} units)	292±26.7 (d) F	261±61.9 (c) E
	Plasmin (261 x 10^{-3} units)	806±60.4 (e) H	1048±57.0 (c) G
Day 7	Control (no plasmin)	153±1.6 (f) B	114±17.2 (d) A
	Plasmin (8.71 x 10^{-3} units)	198±18.0 (f) D	171±45.6 (d) C
	Plasmin (26.1 x 10^{-3} units)	299±63.5 (g) F	313±104.4 (e) E
	Plasmin (261 x 10^{-3} units)	974±13.0 (h) I	*1100 (f) H

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment; The experiment was repeated 3 times; * upper detection limit of the instrument
The TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20)

At the highest level of plasmin, statistical differences in proteolysis were observed ($p < 0.05$) for each day of incubation suggesting higher enzymes activity due to high enzyme concentration. The observed trend suggests that as enzyme concentration increases, high rates of proteolysis take place on each day of incubation, resulting in significance differences as observed for trypsin samples.

Although Table 4-6 shows some differences between the fluorescence values of the two soluble extracts, they were strongly correlated (R^2=0.980). This implies that the

fluorescamine method is suitable for monitoring proteolysis of added plasmin in both extracts. The method was also highly recommended by other authors (Kwan *et al.*, 1983).

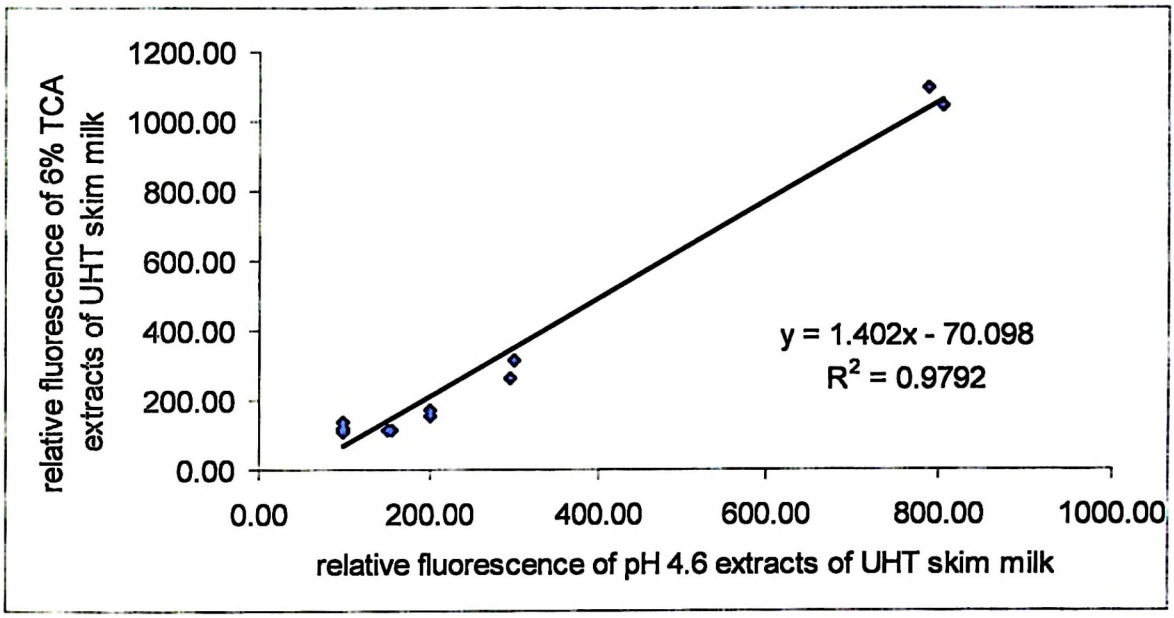


Figure 4-11 Correlation between pH 4.6 and 6% TCA extracts of UHT skim milk with or without added plasmin incubated at 37^{0}C for 7 days as determined by the fluorescamine method

4.3.2.3 Analysis by RP-HPLC

To examine the breakdown products of proteolysis in milk by added plasmin (8.71 x 10^{-3}, 26.1 x 10^{-3} and 261 x 10^{-3} units) during storage, pH 4.6 and 6% TCA soluble extracts were analysed by the RP-HPLC. Comparison was made of the peptide profiles from the two soluble extracts. Results from RP-HPLC method were compared to the TNBS, fluorescamine and gel electrophoresis.

Figures 4-12 and 4-13 show increasing peptide peaks as plasmin levels were increased in both extracts on the seventh day. It should be noted however that peak heights were higher for 6% TCA than pH 4.6 due to lower dilution (x 2) as compared to pH 4.6 (x 20). The peak areas from 8.71 x 10^{-3} and 26.1 x 10^{-3} units of added plasmin samples however, were too low

in both figures compared to 261 x 10^{-3} units of added plasmin samples. This is consistent with results from Table 4-6 where no significant differences in proteolysis ($p > 0.05$) were found between these samples. From the same figures, five similar prominent peaks were observed at 261 x 10^{-3} units of added plasmin (see single sided arrows). All these peptides were obviously soluble in both extracts indicating that they were not precipitated by 12% TCA. Some more small peptides were also evident in samples containing 8.71 x 10^{-3} units and especially 26.1 x 10^{-3} units of added plasmin in 6% TCA soluble extract (Figure 4-12) although at very low quantities. However, the peptide eluted at 35 min in pH 4.6 soluble extract was absent in 6% TCA (double-sided arrow) indicating precipitation by the TCA. This observation was also reported in previous studies (Datta and Deeth, 2003; Lopez-Fandino *et al.*, 1993 b).

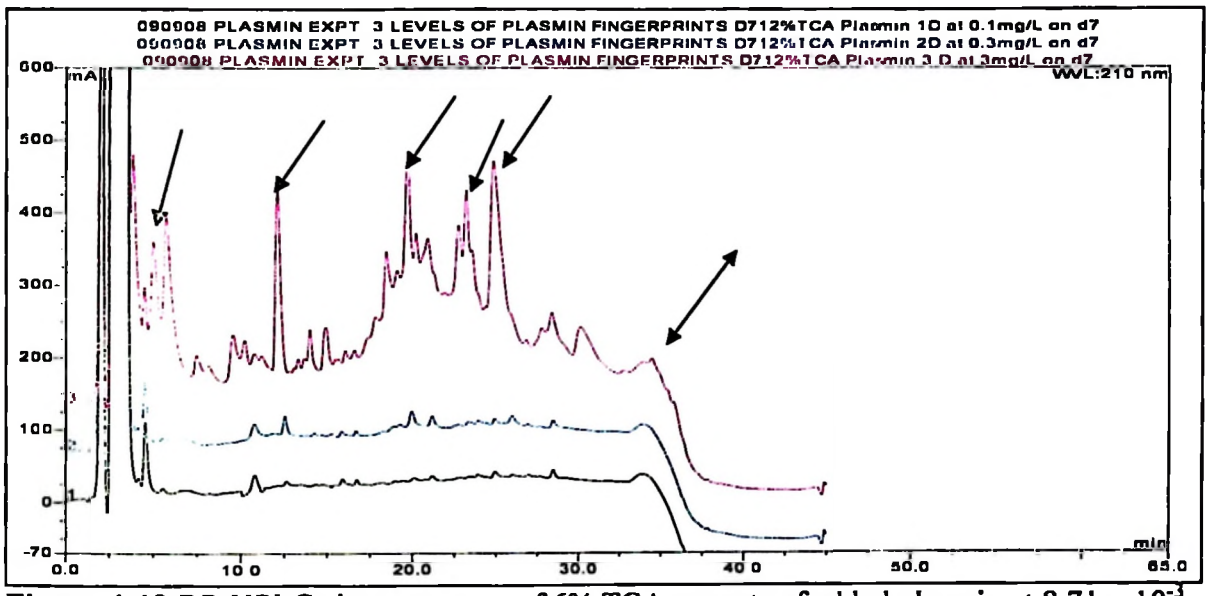


Figure 4-12 RP-HPLC chromatogram of 6% TCA extracts of added plasmin at 8.71×10^{-3}, 26.1×10^{-3} and 261×10^{-3} units after 7 days of incubation at 37^0C

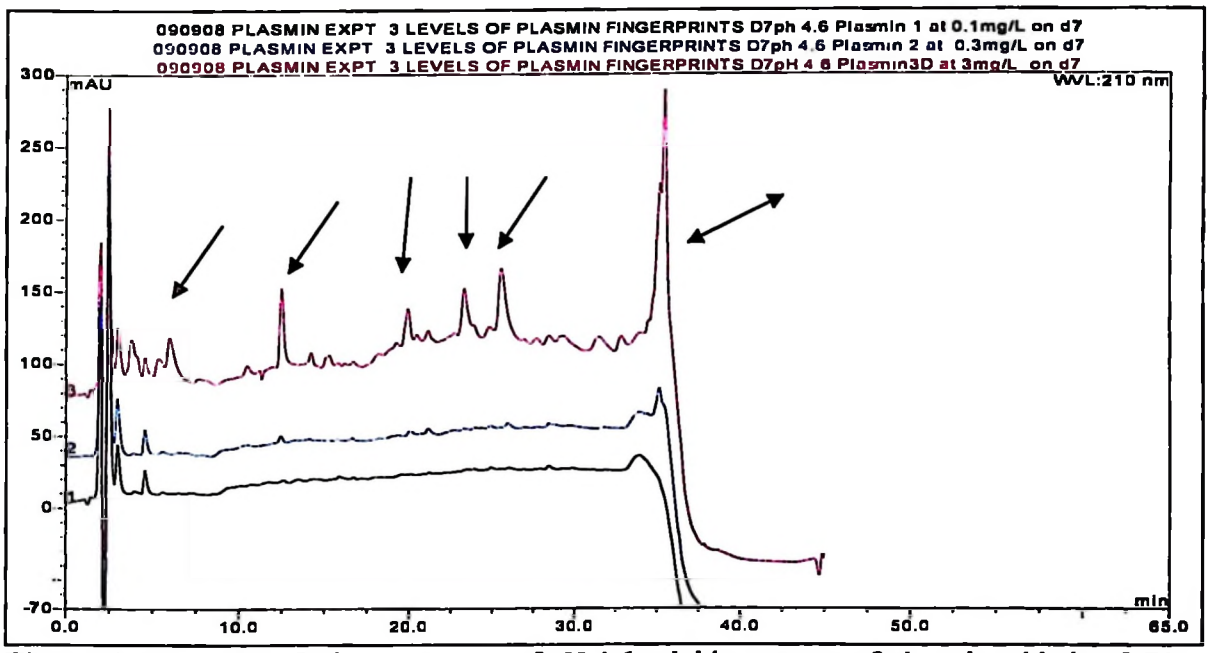


Figure 4-13 RP-HPLC chromatogram of pH 4.6 soluble extracts of plasmin added at 8.71×10^{-3}, 26.1×10^{-3} and 261×10^{-3} units after 7 days of incubation at 37^0C.

Table 4-7 shows significant differences in proteolysis ($p<0.05$) on each day of incubation for both extraction methods in all samples, except the control. The non-significant differences in control samples on days 0 and 3 in pH 4.6 soluble extracts and on days 3 and 7 in 6% TCA extracts were probably due to low proteolytic activities from native enzymes.

Table 4-7 Total peak areas of pH 4.6 and 6%TCA soluble extracts of UHT skim milk with or without added plasmin incubated at 37^{0}C for 7days as analysed by the RP-HPLC method

Day of incubation	Treatments	Total peak areas of pH 4.6 soluble extracts of UHT skim milk		Total peak areas of 6% TCA soluble extracts of UHT skim milk	
day 0	Control (no plasmin)	20.2 ± 1.22	(a) A	31.2 ± 2.66	(a) A
	Plasmin (8.71 x 10^{-3} units)	19.8 ± 2.57	(a) C	34.5 ± 1.66	(a) C
	Plasmin (26.1 x 10^{-3} units)	19.7 ± 1.63	(a) F	34.2 ± 3.44	(a) F
	Plasmin (261 x 10^{-3} units)	21.0 ± 2.13	(a) I	33.6 ± 4.27	(a) I
day 3	Control (no plasmin)	21.5 ± 1.70	(b) A	37.0 ± 2.45	(b) B
	Plasmin (8.71 x 10^{-3} units)	24.3 ±1.33	(b) D	66.7 ± 3.31	(c) D
	Plasmin (26.1 x 10^{-3} units)	47.1 ± 3.65	(c) G	87.5 ± 3.33	(c) G
	Plasmin (261 x 10^{-3} units)	193.5 ± 8.89	(d) J	469.6 ± 44.89	(d) J
day 7	Control (no plasmin)	26.8 ± 3.91	(e) B	39.9 ± 2.39	(e) B
	Plasmin (8.705 x 10^{-3} units)	32.9 ± 1.85	(f) E	76.0 ± 2.79	(f) E
	Plasmin (26.1 x 10^{-3} units)	42.9± 1.85	(f) H	92.9 ± 3.60	(f) H
	Plasmin (261 x 10^{-3} units)	463.7 ±10.51	(g) K	647.2 ± 35.39	(g) K

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment; The experiment was repeated 2 times; N=6;
The TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20)

For pH 4.6 soluble extracts, peptide increase in control and at 8.705 x 10^{-3} units/ mL of added plasmin between days 0 and 3 was constant indicating low proteolytic activity in these samples. The same observation was made on day 7. At 26.1 x 10^{-3} units and 261 x 10^{-3} units of added plasmin, the peptide peak areas increased by two-folds and almost ten folds respectively on day 3 whereas it increased by two fold and more than twenty folds on day 7. As previously observed, highest proteolytic activity was at 261.12 x 10^{-3} units of added plasmin, reaction rate increases with enzyme concentration. For 6% TCA extracts, on days 3 and 7, added plasmin at 8.71 x 10^{-3} units and 26.1 x 10^{-3} units did not show any significant differences in proteolysis ($p<0.05$) unlike the other samples which were significantly different. A strong correlation (R^2 = 0.928) between pH 4.6 and 12%TCA soluble extracts (Figure 4-14) emphasises the significance of this method in monitoring proteolysis. The 6%

TCA extracts were higher than pH 4.6 extracts mainly due to higher sample dilution of the latter.

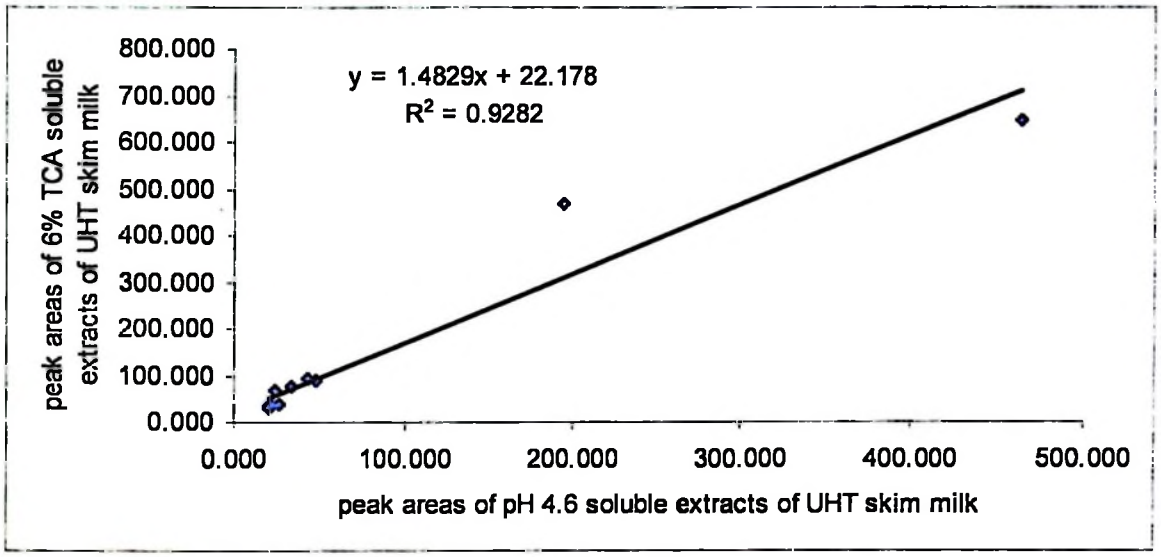


Figure 4-14 Correlation between pH 4.6 and 6% TCA extracts of UHT skim milk with or without added plasmin and incubated at 37^{0}C for 7 days as determined by RP-HPLC.

4.3.2.4 Analysis by gel electrophoresis

This technique was used to identify breakdown products of proteolysis after incubation with added plasmin at 37^{0}C for 7 days. Evaluation of proteolysis by gel electrophoresis by plasmin and trypsin was assessed to compare their activities during storage.

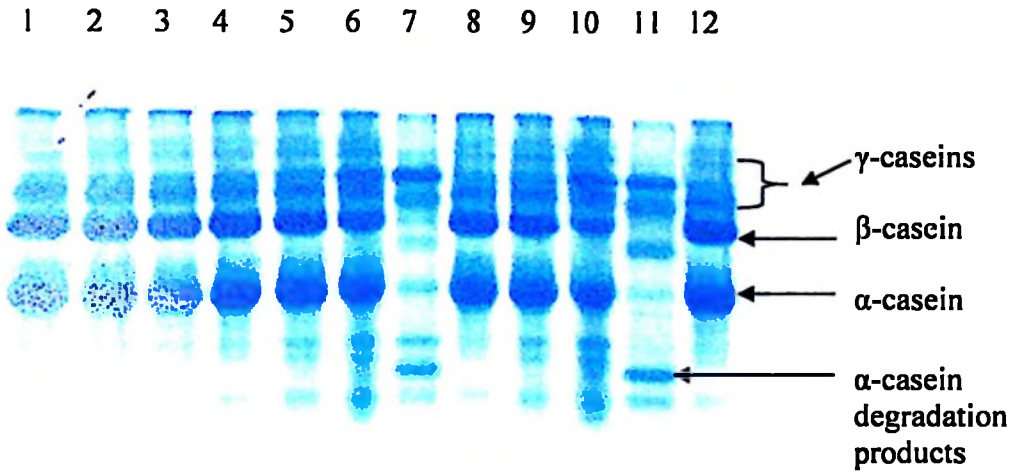


Figure 4-15 Urea PAGE electrophoretogram (T=12%, C=4%, pH 8.9) of UHT skim milk inoculated with plasmin at various levels and incubated for 7 days at 37^{0}C. **Lanes 1-3:** plasmin treated milk (8.71 x 10^{-3}, 26.1 x 10^{-3} and 261 x 10^{-3} units) on day 0. **Lane 4:** Control UHT milk on d 0. **Lanes 5-7:** plasmin treated milk (8.71 x 10^{-3}, 26.1 x 10^{-3} and 261 x 10^{-3} units) on d 3. **Lane 8:** Control UHT milk on d 3. **Lanes: 9-11:** plasmin treated milk (8.71 x 10^{-3}, 26.1 x 10^{-3} and 261 x 10^{-3} units) on d 7. **Lane 12:** Control UHT milk on d 7.

Figure 4-15 illustrates the breakdown profile of UHT skim milk following incubation with plasmin. Control samples on days 0 and 3, showed no casein breakdown during storage (lanes 4 and 8). The bands on lanes 1-3 were all intact and similar to the control (lane 4), demonstrating that no casein breakdown occurred on day 0. On the third day however, gradual breakdown of caseins resulted in the formation of new bands (γ-caseins) on lanes 5 and 6 as right above β-casein. Samples with 261 x 10^{-3} units of added plasmin showed a faster rate of breakdown of both α and β-casein bands (which were almost disappearing) with the concomitant formation of γ-caseins. Plasmin activity on β-caseins results in the formation of γ-caseins (Fox and McSweeney, 1998). It has been documented that α- and κ-caseins are more resistant to proteolysis by plasmin , but susceptible to attack by Cathepsins D and microbial proteases (Pereda *et al.*, 2008).

A faint band detected between α and β-caseins is a β-casein degradation product (lane 11). The three faint bands below the α-casein which also appeared on lanes 5 and 6 are α-caseins degradation products. Although these bands had low intensity on day 0 (lanes 1-4), they became denser with time (lanes 6 and 7; lanes 10 and 12). On day 7, both the α- and β-casein bands completely disappeared on lane 11, indicating extensive proteolysis. On day 7 (lane 11), the β- and α-casein degradation products had higher intensities than on day 3 implying more products had been formed as a result of casein degradation.

On the third day of incubation, samples of milk with 261 x 10^{-3} units of added plasmin appeared slightly yellow indicating a high level of proteolysis.

4.4 Proteolysis assessed by the AMC method

Citrate clarified UHT milk samples that had been incubated at 37^{0}C for 7 days with or without added plasmin were analysed by the AMC method as described in section 3.11.

Preliminary experiments of raw and laboratory pasteurised milk (72^{0}C for 15s) with added trypsin (to represent plasmin) that had been incubated at 37^{0}C for 14 days resulted in high off scale readings (results not shown) which suggested that the method was very sensitive. Control samples (without added plasmin) provided on scale readings as shown in appendix 3. The same figure shows that raw milk had highest activity in all the days of incubation, compared to pasteurised milk. The same observation was made for samples analysed by the TNBS method where it was concluded that the pasteurisation was probably insufficient to inactivate the plasmin inhibitors to cause increased proteolysis in pasteurised milk.

Figure 4-16 shows plasmin activity of UHT skim milk samples incubated at 37^{0}C for 7 days. It is apparent that proteolytic activity increased with plasmin concentration and with time of incubation as previously observed by the TNBS and fluorescamine methods. Plasmin activity

of control samples (without added plasmin) in the current experiment was found to be between 0.00618 and 0.01031 AMC units/ mL. Richardson and Pearce (1981) found plasmin activity in pasteurised milk samples as analysed by the AMC method ranged between 0.037 – 0.195 AMC units/ mL (Richardson and Pearce, 1981). Although UHT skim milk was used in the current experiment, the values obtained by the previous researchers seem to be in agreement with these results. In another study where UHT milk was stored at 20^{0}C for 180 days, plasmin activity was found to be 0.012 AMC units/ mL on day 0 but 0.015 AMC Units per mL after 180 days (Kelly and Foley, 1997). Plasmin activity in a study by Enright *et al.* (1999) was found to be 0.031 AMC units/ mL on day 1 but 0.038 AMC units/ mL on day 63. However, plasmin activity will vary depending on the experimental conditions. Several researchers have confirmed the suitability of this method for the detection of proteolytic activity in milk (Richardson and Pearce, 1981)

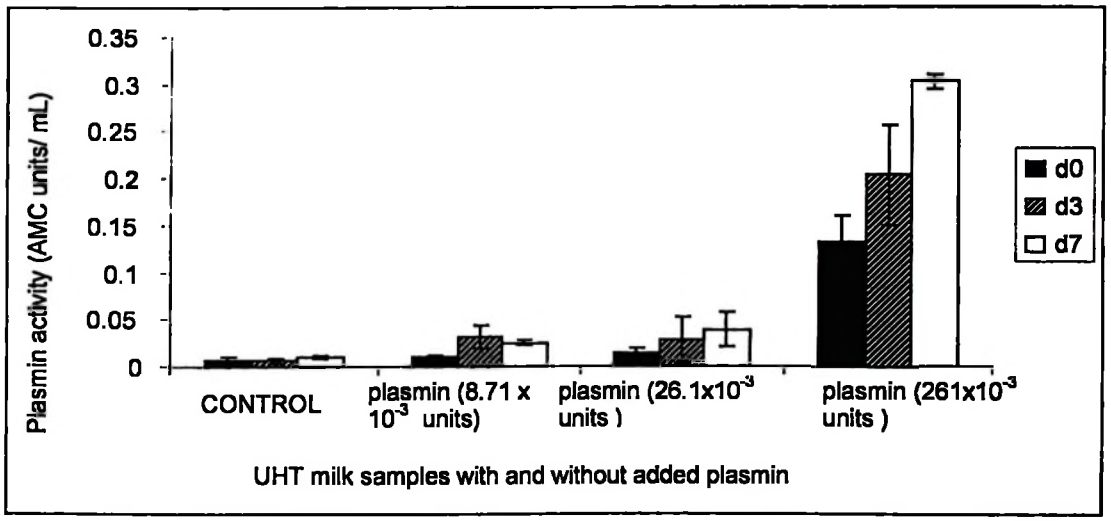


Figure 4-16 Plasmin activity of UHT skim milk with and without added plasmin after incubation at 37^{0}C for 7 days as analysed by the AMC method

4.5 Comparison between the TNBS, Fluorescamine and RP- HPLC methods for monitoring proteolysis by trypsin and plasmin

4.5.1 Measurement of samples with added trypsin

A brief summary of the correlations between the quantitative methods used in the current research is presented in Appendix 2. From the pH 4.6 soluble extracts, the strongest correlation, was between TNBS and RP-HPLC (R^2= 0.867), followed by TNBS and fluorescamine and finally between fluorescamine and RP-HPLC.

Likewise for 6% TCA, the strongest correlation was observed between TNBS and RP-HPLC (R^2= 0.900), followed by TNBS and fluorescamine and then fluorescamine and RP-HPLC. The trend of correlations in both pH 4.6 and 6% TCA extracts was unexpected. This is because the principles for the determination of TNBS and fluorescamine methods are both based on the measurement of primary amines with their respective reagents (TNBS and fluorescamine respectively). The higher the proteolytic activity, the more free amino groups in the peptides released, the greater the absorbance and fluorescence intensities respectively. The RP-HPLC however, measures absorbance of separated peptides and amino acids with response being based approximately on mass (Le *et al.*, 2006). In contrast with the finding of the current study, it was expected for TNBS to be more strongly correlated to fluorescamine than to RP-HPLC.

4.5.2 Measurement of samples with added plasmin

Correlation in pH 4.6 soluble extracts between TNBS and fluorescamine was strongest (R^2= 0.981), followed by TNBS and RP-HPLC and finally fluorescamine and RP-HPLC (Appendix 2). The 6% TCA extracts revealed highest correlation between TNBS and fluorescamine (R^2= 0.980), then TNBS and RP-HPLC and finally fluorescamine and RP-

HPLC. In this respect, TNBS and fluorescamine methods were well correlated as expected because they both measure free amino groups.

In general, it appears that the RP-HPLC and fluorescamine methods were least correlated ($R^2 \leq 0.8$) compared to the other methods when determined separately in pH 4.6 and 6% TCA soluble extracts from proteolysis by added trypsin. It has been reported that proteinase activity determined by the HPLC were approximately 600 times higher than those determined by the fluorescamine methods (Le *et al.*, 2006).

It was interesting to note that the TNBS method was strongly correlated to the fluorescamine and RP-HPLC in both plasmin and trypsin experiments. The difference in response by the three methods between trypsin and plasmin experiments is unclear, but it could be due to higher rate of proteolysis by trypsin compared to plasmin as observed in the results. It was also noted that correlations from plasmin experiments were much higher than the corresponding trypsin experiments in both extracts and by all the three methods. It has been documented that plasmin is more specific than trypsin (Roy, 1981).

The gel electrophoresis technique proved useful for qualitative analysis in monitoring proteolysis. However, the use of hazardous chemicals, poor resolution of lower molecular weight peptides and lengthy analysis limits its application for routine laboratory analysis.

From a practical point of view, the RP-HPLC method is very accurate, sensitive, reproducible and sophisticated compared to the fluorescamine and TNBS methods. However, the requirement for expensive equipment would limit its use as a method for routine analysis in a quality assurance laboratory.

The advantage of the fluorescamine method is that it is simple, rapid and very sensitive to low levels of proteases. However, the main drawback of this method is its low upper

detection limit, interference with TCA as well as the requirement for expensive equipment (spectrofluorimeter) hence impractical for routine laboratory analysis.

Although the TNBS method was less sensitive than the fluorescamine method, it was still possible to detect lower levels of proteases as used in the current study. In addition to being simple and rapid, it also does not require expensive equipment. This method is therefore recommended as a method of choice for routine laboratory analysis.

The AMC method which is specific for plasmin activity is a sensitive method as proved by the results obtained where the rate of increase in fluorescence intensity was proportional to the quantity of plasmin present. The major limitation with this method is the high cost involved in purchasing the chemicals and also the need for spectrofluorimeter.

4.6 Conclusions:

This chapter provided a detailed investigation of the effect of added trypsin and plasmin enzymes on proteolysis of milk during storage. Milk was pasteurised at various temperatures to study the effect of inactivation of the inhibitors in milk. Low and higher levels of enzymes were added to pasteurised and UHT skim milk so as to assess the suitability of the TNBS, fluorescamine, RP-HPLC and gel electrophoresis methods in detecting proteolysis.

Preliminary results from milk pasteurised at various temperatures confirmed that the higher the pasteurisation temperature, the lower the proteolytic products and vice-versa. The same experiment revealed that the pasteurisation temperatures employed (72, 85 and 90^{0}C for 15 s) were insufficient to inactivate the inhibitors in milk. This study also demonstrated that though unexpected, raw milk was more proteolytic than pasteurised milk. This was attributed to the presence of native enzymes in raw milk. Comparison of the pH 4.6 and 6% TCA soluble extracts of UHT skim milk with added trypsin or plasmin by the three selected methods revealed high correlations ($R^2 > 0.93$) which implied that both extracts followed the same trend of increased proteolysis upon storage.

The correlations between the extracts from plasmin added samples were higher than from trypsin added samples probably due to higher substrate specificity of the former enzyme than the latter.

The TNBS, fluorescamine and gel electrophoresis methods were found to be useful rapid methods for monitoring proteolysis in milk. The correlation coefficients observed for the three selected procedures were quite high and all methods could detect low levels of proteases added. The gel electrophoresis method was a useful tool for qualitative determination of proteolysis during storage. However, the TNBS method was recommended

for routine assessment of proteolysis in the laboratories as it was considered to be simple, rapid and cheaper than the other methods.

The AMC method, though rapid and sensitive, is not recommended for routine laboratory analysis due to the high cost of the reagents and the need for a spectrofluorimeter.

CHAPTER 5 EFFECT OF BACTERIAL PROTEOLYSIS CAUSED BY *Pseudomonas fluorescens (Ps)*, ITS ENZYMES AND *Bacillus licheniformis* ENZYMES

5.1 Introduction

Proteolysis in refrigerated milk is usually caused by Gram negative psychrotrophs. Of these, *Pseudomonas spp.* are the predominant ones. Under sanitary condition, <10% of total microorganisms are psychrotrophs compared to >75% under unsanitary conditions (Suhren, 1989). These bacteria can survive in milk at low refrigeration temperatures and produce extracellular endometalloproteases which are heat resistant to both pasteurised and UHT treatments (Kohlmann *et al.*, 1991a). As a result, spoilage in milk may occur after pasteurisation and more likely after UHT heat treatments. As these enzymes are not commercially available, one aim of this research is to extract them followed by partial purification before inoculation into pasteurised and UHT skim milks.

The Gram positive psychrotrophs responsible for spoilage are sporeforming *Bacillus* species. It is documented that about a third of refrigerated pasteurised milk is spoiled by Gram positive bacteria (Nielsen, 2002). Proteolytic enzymes derived from these enzymes are commercially available.

The present study focuses on the significance of *Bacillus licheniformis* and *Pseudomonas fluorescens* and their enzymes on milk proteolysis. Simple rapid methods such as fluorescamine and TNBS, as well as gel electrophoresis are used to monitor proteolysis during storage. The RP-HPLC method is used in addition to electrophoresis to identify markers of proteolytic activities in pH 4.6 and 6% TCA soluble extracts as detailed below.

5.2 Materials and methods

5.2.1 Proteolysis caused by *Pseudomonas fluorescens*

To examine the effect of proteolysis by *Pseudomonas fluorescens* in milk, two strains of *Pseudomonas fluorescens* NCIMB 702085 (416) and NCIMB 701274 (414) were inoculated into skimmed UHT and pasteurised milk. A preliminary experiment to check the effect of storage temperature on proteolysis was conducted at 20^0C and 4^0C for 7 days. Gelation was monitored daily by checking the first appearance of clots. The pH was also monitored.

The details of these procedures are explained in section 3.4.1.

5.2.2 Extraction of *Pseudomonas fluorescens* enzymes

To prevent the possibility of contamination from strains of bacteria other than *Pseudomonas fluorescens*, enzymes were extracted from *Pseudomonas fluorescens* NCIMB 702085 (416) and NCIMB 701274 (414) as described in section 3.4.1. The crude enzyme obtained was further purified by dialysis against distilled de-ionised water into which 0.05% sodium azide (w/v) was added to prevent microbial contamination. Dialysis was performed to remove peptides and amino acids from the enzyme so as to minimise substances which would interfere with the assaying procedures.

5.2.3 Proteolysis caused by *Pseudomonas fluorescens* enzymes

The enzymes extracted above (5.2.2) were inoculated into skim UHT and pasteurised milk as detailed in section 3.4.1.

5.2.4 Proteolysis caused by commercial bacterial enzymes of *Bacillus licheniformis*

To examine the influence of these enzymes in milk, commercial enzyme from a strain of *Bacillus licheniformis* (Sigma-Aldrich Gillingham, UK) was inoculated into UHT skim milk

at five levels. Details of the procedure are given in section 3.4.2. Fluorescamine, TNBS, RP-HPLC and gel electrophoresis methods were employed to detect the proteolysis. In a separate experiment, *Bacillus licheniformis* was inoculated at three levels into UHT skim milk followed by monitoring proteolysis as described in section 3.4.2.

5.3 Results and discussion

5.3.1 Proteolysis caused by *Pseudomonas fluorescens*

5.3.1.1 Analysis by TNBS

5.3.1.1.1 Effect of storage time

This investigation was aimed at examining the effect of storage time on proteolysis by *Pseudomonas fluorescens* as well as the suitability of the selected methods in monitoring proteolysis. Preliminary experiments indicated that the number of viable cells in the stock (inocula) from the departmental culture of *Pseudomonas fluorescens* was 10^7-10^8 cfu per mL. It was stated that a psychrotrophs population of 10^4 cfu/ mL is sufficient to induce proteolysis in milk (Renner, 1988). About 10^5-10^6 cfu/ mL (1% v/v) was inoculated into pasteurised and UHT skim milk to initiate proteolysis.

Table 5-1 Absorbance by the TNBS method on pH 4.6 and 6%TCA extracts to study the effect of storage time on proteolysis of commercial pasteurised skim milk inoculated with 10^5-10^6 cfu/mL *Pseudomonas fluorescens* 414 and 416 and incubated at 20^0C for up to 7 days

Days of incubation	Treatments	Absorbance of pH 4.6 soluble extracts at 420 nm	Absorbance of 6% TCA soluble extracts at 420 nm
day 0	Control (pasteurised milk)	0.015 ± 0.002 (a) A	0.170 ± 0.003 (a) A
	Pseudomonas fluorescens 414	0.017 ± 0.001 (a) C	0.216 ± 0.017 (b) C
	Pseudomonas fluorescens 416	0.041 ± 0.004 (b) F	0.230 ± 0.011 (c) F
day 3	Control (pasteurised milk)	0.020 ± 0.003 (c) B	0.176 ± 0.003 (d) B
	Pseudomonas fluorescens 414	0.178 ± 0.005 (d) D	1.853 ± 0.027 (e) D
	Pseudomonas fluorescens 416	0.252 ± 0.007 (e) G	2.248 ± 0.009 (f) G
day 7	Control (pasteurised milk)	0.019 ± 0.003 (f) B	0.173± 0.005(g)AB
	Pseudomonas fluorescens 414	0.242 ± 0.006 (g) E	2.335 ± 0.072 (h) E
	Pseudomonas fluorescens 416	0.287 ± 0.005 (h) H	2.504 ± 0.017 (i) H

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment: The experiment was repeated 3 times; N=9; TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20)

Table 5.1 shows significant differences in proteolysis ($p < 0.05$) were observed in control, *Pseudomonas fluorescens* 414 and 416 on days 3 and 7 in both soluble extracts studied. Statistical analysis revealed that proteolysis in the inoculated samples was significantly different ($p<0.05$) for each bacterium for each day in each soluble extracts analysed. These samples were also statistically different from the control samples which had low activity. The low but constant activity in the control sample was probably as a result of activity of native enzymes. The high activity of *Pseudomonas fluorescens* 416 was also confirmed by its high absorbance on day 0 (Table 5-1), which was analysed at about 30 - 40 min after inoculation. Gelation occurred on days 1 and 2 (results not shown) respectively for *Pseudomonas fluorescens* 416 (pH = 6.69) and 414 (pH = 6.73) confirming high activity of the former bacterium than the latter. Figure 5-1 also reveals that the pH was stable for 2 days (>6.6) but after day 3, it fell steeply for all samples. Metabolites formed by *Pseudomonas fluorescens*

caused the pH drop in the inoculated samples. The pH of the control sample dropped to 6.1, and this could be due activity of microbial flora from the milk itself.

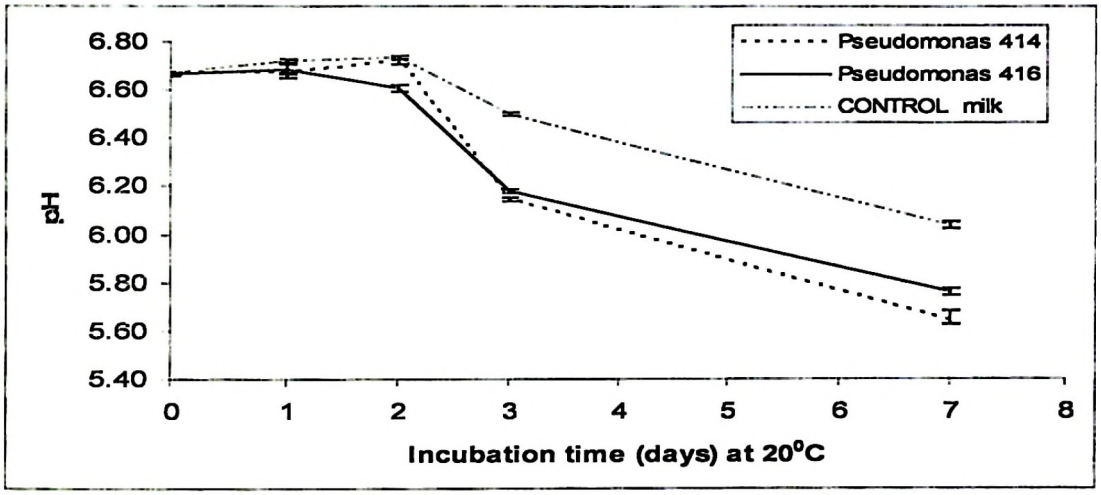


Figure 5-1 Changes in pH of *Pseudomonas fluorescens* 414 and 416 inoculated in pasteurised skim milk (10^5 -10^6 cfu/mL) during incubation at 20^0C for 7 days; Error bars represent standard deviations; the experiment was repeated three times

Although the experiment gave an indication of the effect of storage time on proteolysis, a trial using partially purified *Pseudomonas fluorescens* enzymes was carried out to broaden our understanding of the effect of these enzymes on proteolysis during storage.

Absorbance values for pH 4.6 soluble extracts plotted against 6% TCA soluble extracts in Figure 5-2 indicate that pH 4.6 and 6% TCA soluble extracts from pasteurised milk were well correlated (R^2 =0.989). As proteolysis by bacterial proteases produce smaller peptides and amino acids that are soluble in TCA (Lopez-Fandino *et al.*, 1993 a), higher absorbance values reflect high activities from these bacteria. It has been documented that TCA precipitates large peptides as those formed by plasmin (Datta and Deeth, 2003). However, pH 4.6 soluble extracts contain peptides from both bacterial and native proteases (Datta and Deeth, 2003) and hence their absorbance values also reflect bacterial proteolysis as no native

proteases were added. Moreover, control samples had lower absorbance values, confirming that native proteases were probably too low to be detected.

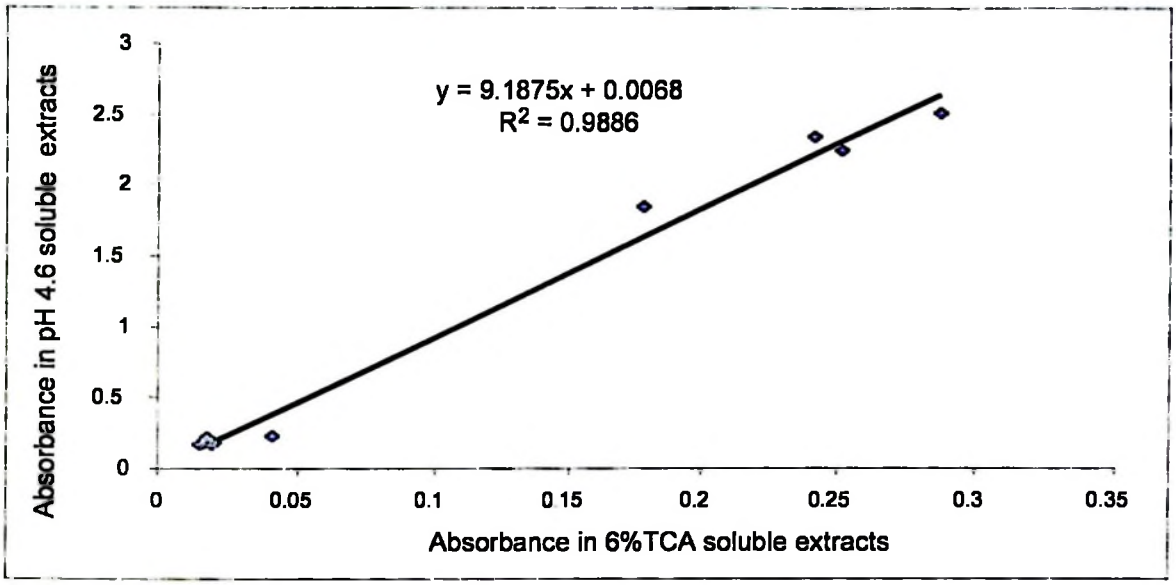


Figure 5-2 Correlation between pH 4.6 and 6% TCA soluble extracts from samples of *Pseudomonas fluorescens* 414 and 416 (10^5 cfu/ mL) incubated in pasteurised skim milk at 20^0C for 7 days as analysed by the TNBS method

Proteolysis was studied by the TNBS method in UHT skim milk inoculated with two strains of *Pseudomonas fluorescens* (414 and 416) following incubation at 20^0C for 7 days. Comparison was made of proteolysis in pH 4.6 and 6% TCA soluble extracts from pasteurised and UHT milks.

Table 5-2 shows significant difference in proteolysis ($p < 0.05$) between all samples for all days of incubation except the inoculated samples from 6% TCA soluble extracts on day 7. Although it appears from the table that these samples are significantly different in proteolysis, it still confirms that *Pseudomonas fluorescens* NCIMB 702085 (416) was the most proteolytic sample which supports the earlier observation. These differences in activity were probably due to the nature of the strains and particularly on the quantity of enzyme produced. Many factors are involved in protease production and proteolysis. Although, there

are contradictory reports regarding the quantity of enzyme produced and the activity of the microorganism, the strain of the bacteria was regarded as crucial factor in determining proteolysis (Haryani *et al.*, 2003).

Table 5-2 Absorbance by the TNBS method on pH 4.6 and 6%TCA extracts to study the effect of storage time on proteolysis of commercial UHT skim milk inoculated with 10^5-10^6 cfu/mL *Pseudomonas fluorescens* 414 and 416 and incubated at 20°C for up to 7 days

Day of incubation	Treatment	Absorbance of pH 4.6 soluble extracts at 420nm	Absorbance of 6% TCA soluble extracts at 420nm
day 0	no microbe (control)	0.017±0.001 (a) A	0.132±0.004 (a) A
	Pseudomonas fluorescens 414	0.094±0.012 (b) D	0.397±0.002 (b) D
	Pseudomonas fluorescens 416	0.130±0.035 (c) G	0.937±0.002 (c) G
day 3	no microbe (control)	0.019±0.002 (d) B	0.158±0.001 (d) B
	Pseudomonas fluorescens 414	0.296±0.002 (e) E	1.98 ±0.012 (e) E
	Pseudomonas fluorescens 416	0.418±0.002 (f) H	2.32 ±0.183 (f) H
day 7	no microbe (control)	0.029±0.002 (g) C	0.17 ±0.002 (g) C
	Pseudomonas fluorescens 414	0.380±0.002 (h) F	2.12 ±0.043 (h) F
	Pseudomonas fluorescens 416	0.458±0.004 (i) I	2.13 ±0.012 (h) I

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment: The experiment was repeated 3 times; N=9; TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20)

In the current study, samples inoculated with *Pseudomonas fluorescens* NCIMB 702085 (416) gelled after overnight incubation at 20°C whereas *Pseudomonas fluorescens* NCIMB 701274 (414) gelled after 3 days. The control samples did not gel. This further suggests that the former strain is more active than the latter. In an attempt to ascertain that *Pseudomonas fluorescens* NCIMB 702085 (416) had higher proteolytic activity than *Pseudomonas fluorescens* NCIMB 701274 (414), an experiment was conducted to extract the enzymes from both strains followed by partial purification by dialysis to monitor proteolysis. The selected methods will be used to assess their suitability to detect proteolysis. This will be discussed on section 5.3.2. A close relationship ($R^2 = 0.957$) between pH 4.6 and 6% TCA soluble extracts in UHT skim milk is shown on Figure 5-3, indicating that the TNBS method is an accurate method for studying proteolysis, using either extracts.

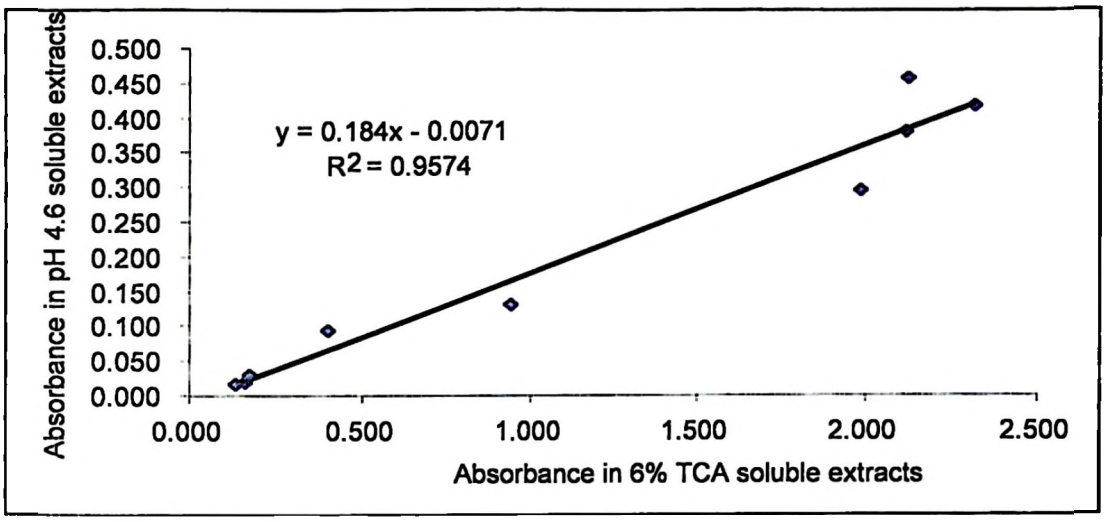


Figure 5-3 Correlation between pH 4.6 and 6% TCA soluble extracts from samples of UHT skim milk inoculated with 10^5 cfu/ mL *Pseudomonas fluorescens* 414 and 416 for 7 days at 20^0C as analysed by the TNBS method.

5.3.1.1.2 Effect of storage temperature

This study was aimed at observing the activity of *Pseudomonas fluorescens* at two temperatures. Being a psychrotrophic, it was expected to grow well under refrigeration and this was compared against room temperature (20^0 C) where UHT milk is usually stored. The TNBS method will be used to detect proteolysis in these samples. Figures 5-4 and 5-5 demonstrate the effect of storage temperature on proteolysis by *Pseudomonas fluorescens* NCIMB 702085 (416) and *Pseudomonas fluorescens* NCIMB 701274 (414) in pH 4.6 soluble extracts of UHT skim milk. From the figures, *Pseudomonas fluorescens* NCIMB 702085 (416) has higher proteolytic activity than *Pseudomonas fluorescens* NCIMB 701274 (414). The same figures also confirm that both strains of *Pseudomonas fluorescens* have higher growth rate at 20^0C than at 4^0C. This implies that these microorganisms would cause more proteolysis at room temperature than under refrigeration.

Other researchers support these findings (Mitchell and Marshall, 1989; Nielsen, 2002; McKellar, 1982; Stepaniak and Fox, 1985). Thus, although these microorganisms grow well

under refrigeration temperatures, they can cause deteriorative changes at 20-30^{0}C which is their optimum temperature of activity (Nielsen, 2002). Thus, the TNBS method has proved useful in detecting proteolysis in these samples.

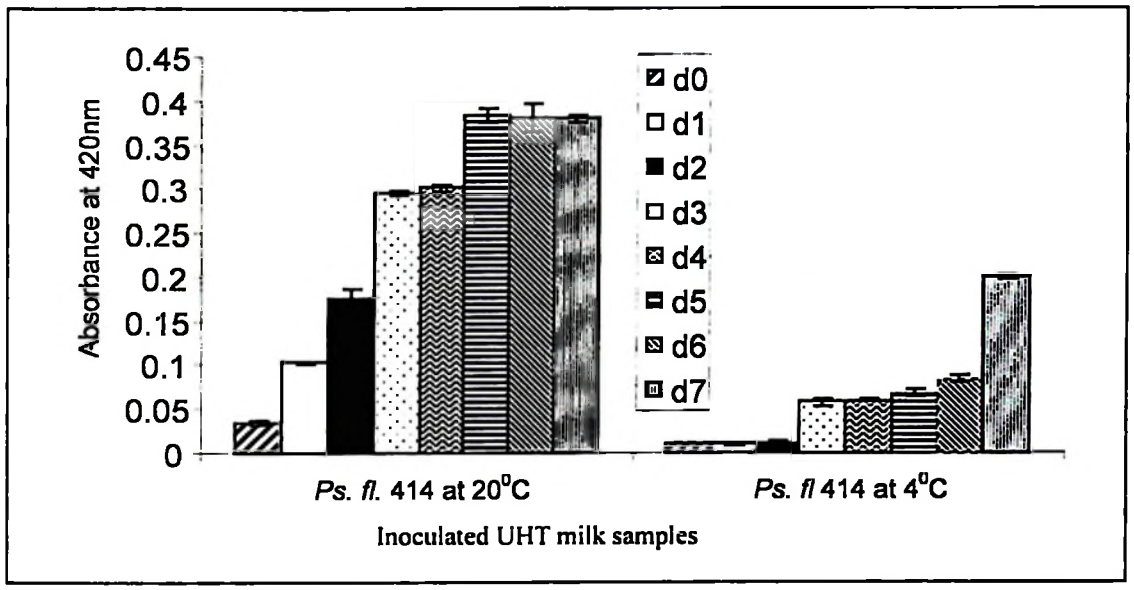


Figure 5-4 The effect of storage temperature on proteolysis by *Pseudomonas fluorescens (Ps. fl.)* 414 in pH 4.6 soluble extracts of UHT skim milk by the TNBS method. Error bars represent standard deviation; N=6; The experiment was repeated 2 times

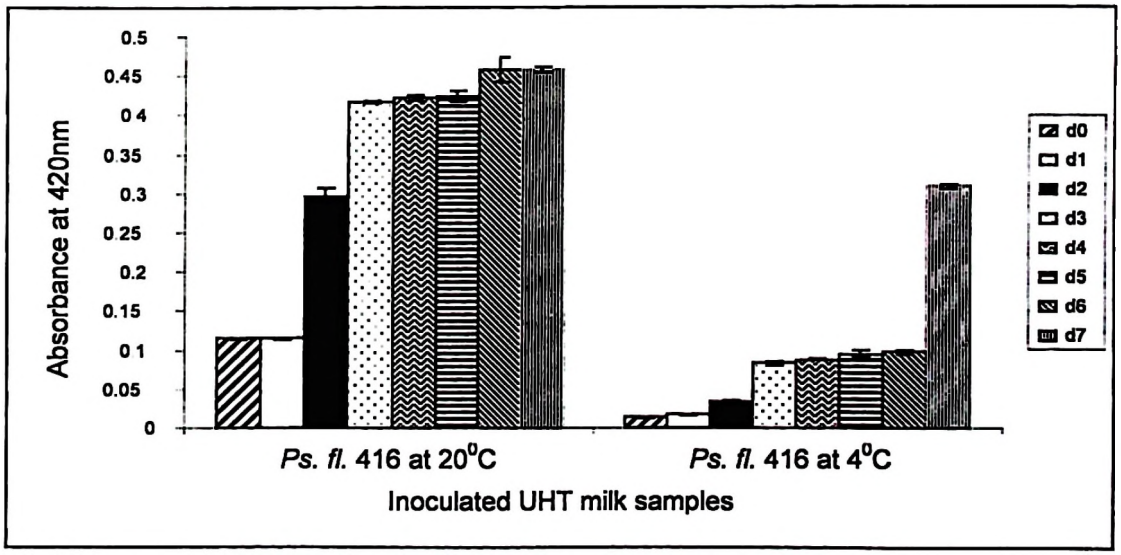


Figure 5-5 The effect of storage temperature on the proteolysis by *Pseudomonas fluorescens (Ps. fl.)* 416 in pH 4.6 soluble extracts of UHT skim milk by the TNBS method. Error bars represent standard deviations. N=6; The experiment was repeated 2 times

5.3.1.2 Analysis by Fluorescamine

The aim of using this method was to validate its suitability in monitoring proteolysis. It has been recommended as a suitable method for measuring microbial enzymes in solutions (Beeby, 1980). The effect of storage time and temperature on proteolysis was investigated. The basis for this method is detailed in section 2.5.1.2.

5.3.1.2.1 Effect of storage time

In support of earlier observations, Table 5-3 indicates that there were statistically significant differences in proteolysis ($p < 0.05$) in all samples on each day of observation. However, on days 3 and 7 for *Pseudomonas fluorescens* 416 it was not possible to draw any conclusions because the assay reached its upper detection limit. The high activities in both inoculated samples on day 0 (about 40 min after incubation), indicate that these bacteria produce significant breakdown products and hence it would be more useful to purify the enzymes by dialysis for detailed study of proteolysis. Again, it was also evident that *Pseudomonas fluorescens* 416 was more proteolytic than *Pseudomonas fluorescens* 414. By the third day, *Pseudomonas fluorescens* 416 strain had almost doubled in fluorescence units and reached the upper detection limit. *Pseudomonas fluorescens* 414 however, reached this limit after a week implying that although the method is useful during short storage and probably for samples with low activities, it may be limiting for longer proteolysis studies. Dialysis provides an opportunity to reduce the breakdown products to detectable levels, and would be a useful technique to use in conjunction with this method to study proteolysis.

5.3.1.2.2 Effect of storage temperature

Figures 5-6 and 5-7 show that storage temperature affects proteolysis. Although activity was lower at 4^0C than at 20^0C, storage at refrigeration temperatures is selective for psychotropic

bacteria which can multiply below their optimal growth temperatures (Law, 1979). This implies that with longer storage time, samples stored at 4^0C could also result in significant proteolysis. The activity was almost constant for the first three days but increased (almost doubled) on the fourth day where it remained constant until day 6. The same figures show that the upper detection limit was reached on days 4 and 3 respectively for *Pseudomonas fluorescens* 414 and 416 at 20^0C, again suggesting higher activity from the latter than the former bacterium. From the current study, it may be concluded that although the method suffers the disadvantage of reaching the upper detection limit faster, it still is a useful method to assess bacterial proteolysis.

Table 5-3 Relative fluorescence by the fluorescamine method on pH 4.6 soluble extracts to examine the effect of storage time on proteolysis of UHT skim milk inoculated with 10^5 cfu/mL *Pseudomonas fluorescens* 414 and 416 and incubated for 7 days at 20^0C

Day of incubation	Sample	Relative fluorescence of pH 4.6 soluble extracts at 475 nm		
day 0	No microbe (Control)	109± 0.8	(a)	A
	Pseudomonas fluorescens 414	532 ± 0.9	(b)	D
	Pseudomonas fluorescens 416	721 ± 2.7	(c)	G
day 3	No microbe (Control)	131± 1.6	(d)	B
	Pseudomonas fluorescens 414	1082± 2.5	(e)	E
	Pseudomonas fluorescens 416	*1100± 0	(f)	H
day 7	No microbe (Control)	136± 2.3	(g)	C
	Pseudomonas fluorescens 414	*1100±0	(h)	F
	Pseudomonas fluorescens 416	*1100±0	(h)	H

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment: The experiment was repeated 3 times (N=9); pH 4.6 soluble extracts were diluted (x20); * upper detection limit of the equipment

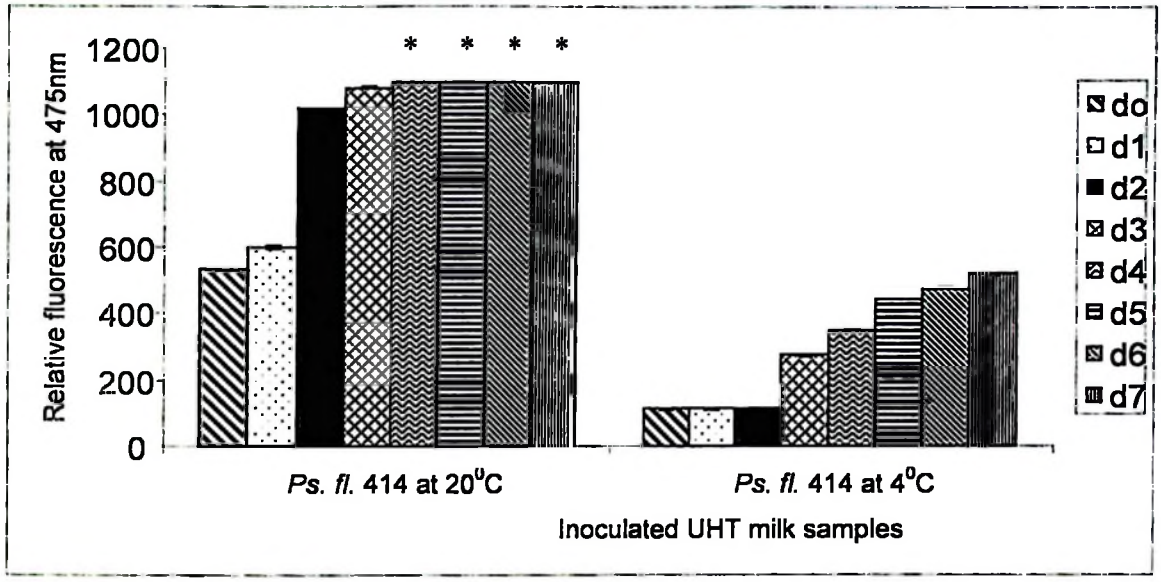


Figure 5-6 Relative fluorescence by the fluorescamine method on pH 4.6 soluble extracts to examine the effect of storage time on proteolysis of UHT skim milk inoculated with 10^5 cfu/mL *Pseudomonas fluorescens* 414 for 7 days at 20°C. N=6; Error bars represent standard deviation; The experiment was repeated 2 times; * upper detection limit of the equipment

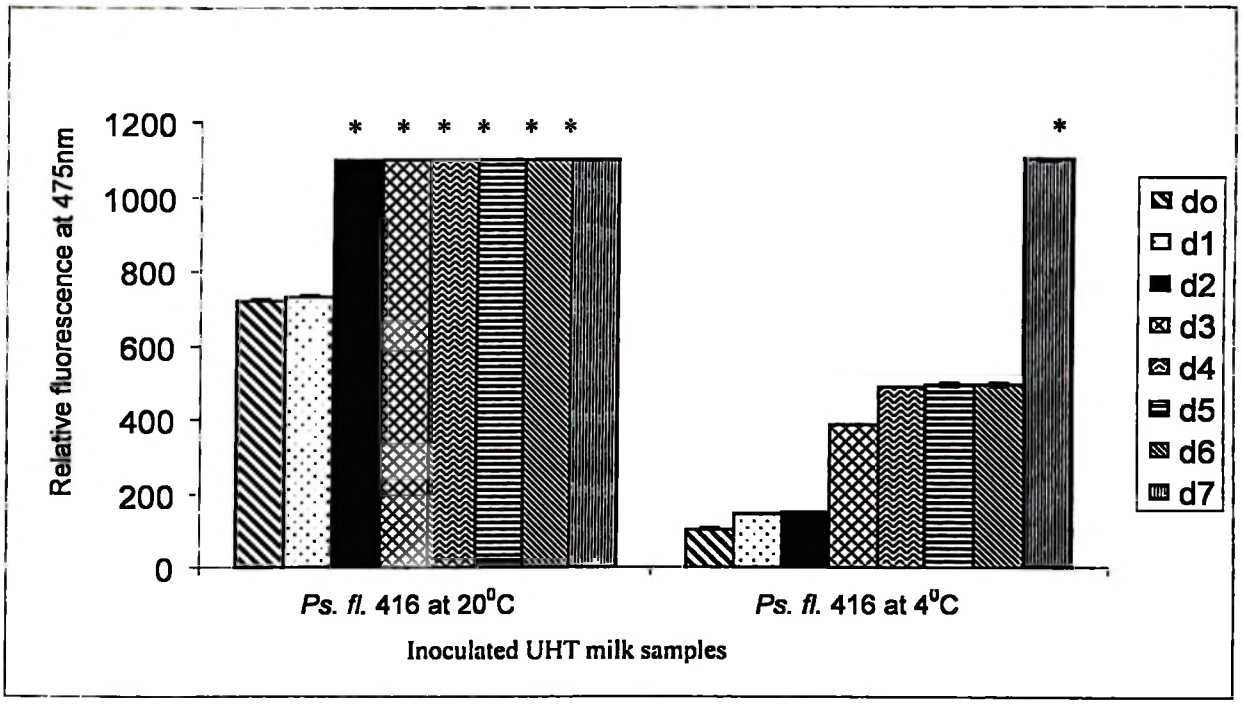


Figure 5-7 Relative fluorescence by the fluorescamine method on pH 4.6 soluble extracts to examine the effect of storage time on proteolysis of UHT skim milk inoculated with 10^5 cfu/mL *Pseudomonas fluorescens* 416 for 7 days at 20°C. The experiment was repeated 2 times. N=6; Error bars represent standard deviation (some error bars are too small to be seen); * upper detection limit of the equipment

5.3.1.3 Analysis by RP-HPLC

5.3.1.3.1 Markers of activity

This study aimed at identifying markers of *Pseudomonas fluorescens* activity from the RP-HPLC profiles. It was reported that RP-HPLC is a useful technique for identifying markers of activity since it separates the hydrophilic from hydrophobic peptides (Datta and Deeth, 2003). Clarified samples of pasteurised milk inoculated with *Pseudomonas fluorescens* and incubated at 20^{0}C for 7 days were analysed by this method. Figures 5-8 and 5-9 show chromatograms of *Pseudomonas fluorescens* 414 in pH 4.6 and 6% TCA soluble extracts respectively. Several small peaks are observed from injection to ~ 28 min in pH 4.6 soluble extracts. The large peak observed at 35 min (see arrow) in Figure 5-8 is most probably hydrophobic peptide resulting from proteolysis by native proteases. These peaks (35 min) are absent in 6% TCA soluble extracts (see the arrow on Fig 5-9). The trend is similar in Figures 5-10 and 5-11 for *Pseudomonas fluorescens* 416. As observed earlier (5.3.1) *Pseudomonas fluorescens* 416 is more proteolytic than *Pseudomonas fluorescens* 414, producing several quite sizeable peptide peaks from the beginning of injection. Peptide peaks by bacterial proteases were observed up to 24 min in pH 4.6 soluble extracts in the work of Datta and Deeth (2003). The same researchers concluded that bacteria produce small peptides which are hydrophilic and hence eluted earlier in the column; whereas plasmin produces large hydrophobic peptides are eluted later in the column.

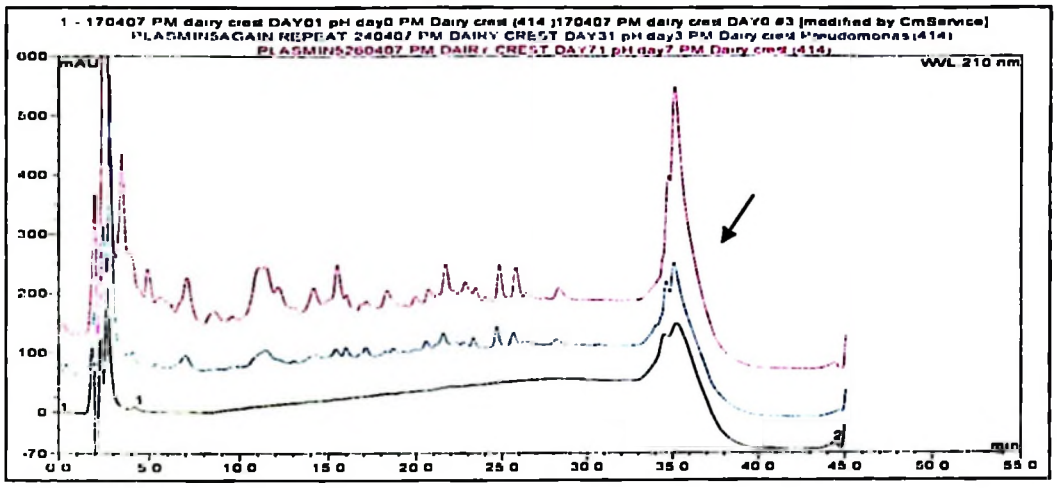


Figure 5-8 Chromatogram (by RP- HPLC) of pH 4.6 soluble extracts of *Pseudomonas fluorescens* 414 incubated in pasteurised milk for 7 days at 20^{0}C (Numbers on the left hand side of each chromatogram refer to **1** : proteolysis on day 0; **2**- proteolysis on day 3 and **3**: proteolysis on day 7)

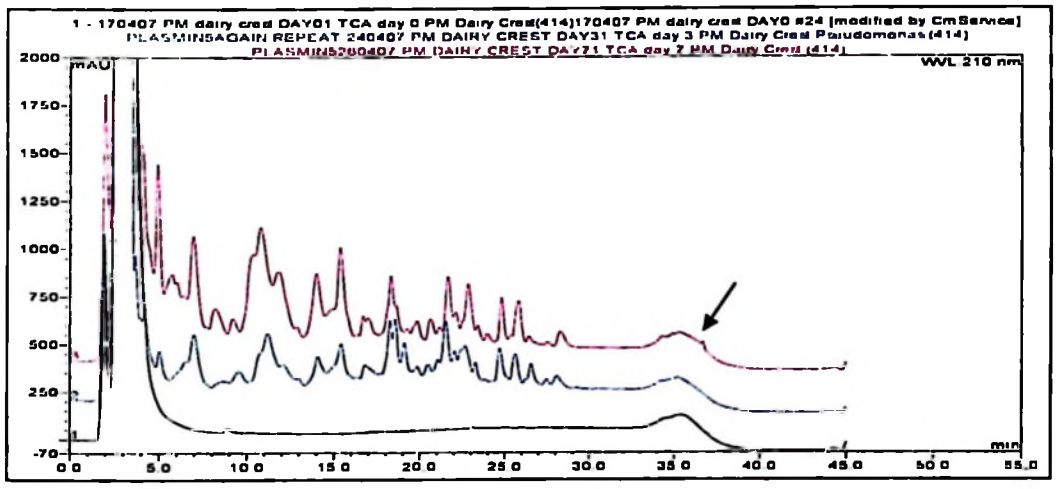


Figure 5-9 Chromatogram (by RP- HPLC) of 6% TCA soluble extracts of *Pseudomonas fluorescens* 414 incubated in pasteurised milk for 7 days at 20^{0}C (Numbers on the left hand side of each chromatogram refer to **1**: proteolysis on day 0; **2**- proteolysis on day 3 and **3**: proteolysis on day 7)

In the current study, peaks were observed up to 28 min, hence peak areas were calculated up to 28 min (see Figure 5-9). Thus, from the Figures (5-8 to 5-11), markers of activity may be identified from the two extraction procedures. The 6% TCA soluble extracts lack the high

peak at 35 min, whereas the pH 4.6 soluble extracts have a sharp peak at 35 min. This has been previously reported (Datta and Deeth, 2003; Lopez-Fandino *et al.*, 1993 b).

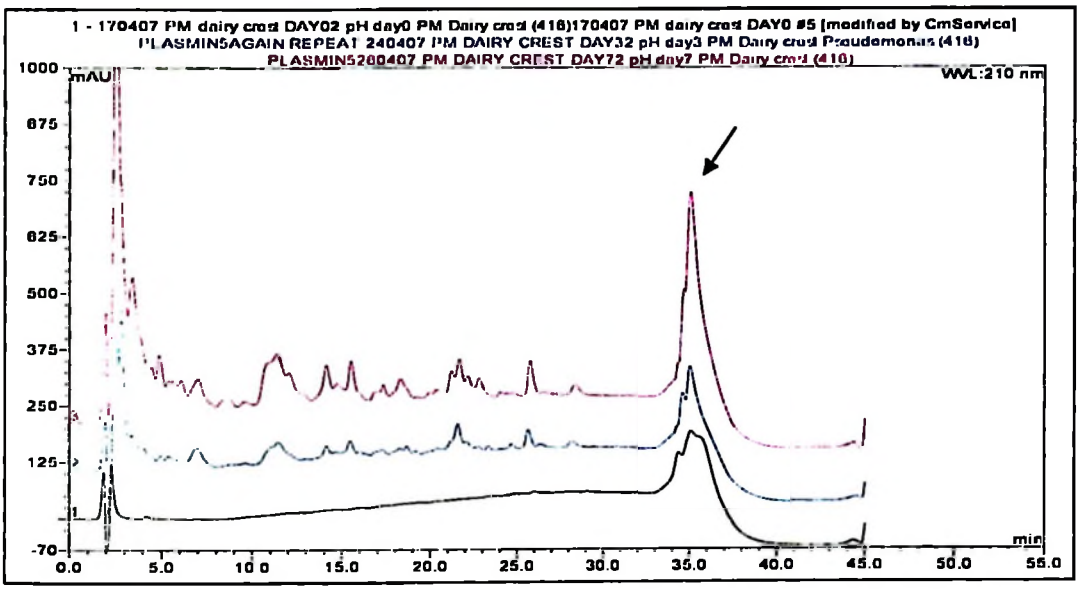


Figure 5-10 Chromatogram (by RP- HPLC) of pH 4.6 soluble extracts of *Pseudomonas fluorescens* 416 incubated in pasteurised milk for 7 days at 20^{0}C (Numbers on the left hand side of each chromatogram refer to **1**: proteolysis on day 0; **2**- proteolysis on day 3 and **3**: proteolysis on day 7)

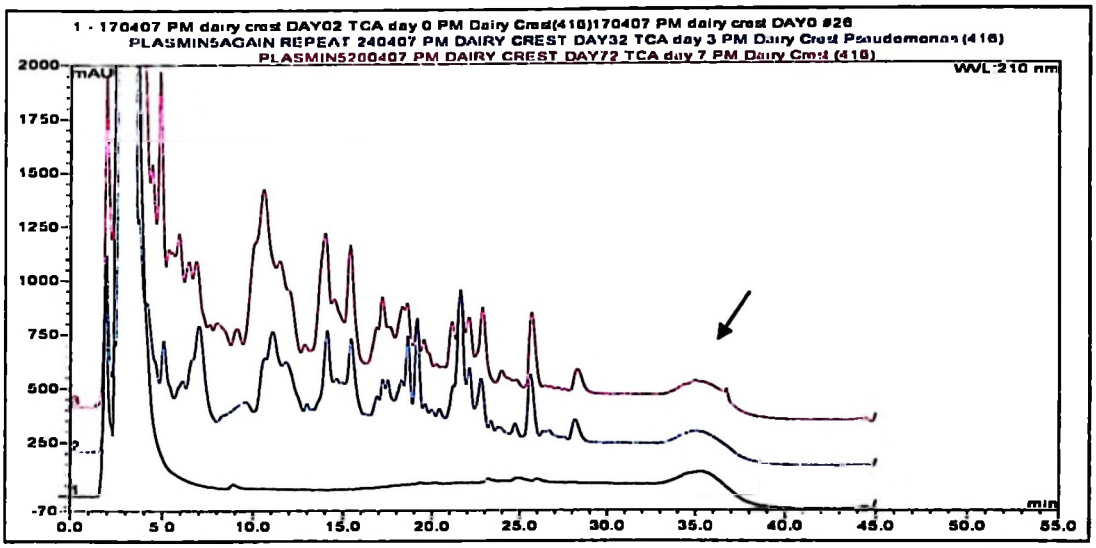


Figure 5-11 Chromatogram (by RP- HPLC) of 6% TCA soluble extracts of *Pseudomonas fluorescens* 416 incubated in pasteurised milk for 7 days at 20^{0}C (Numbers on the left hand side of each chromatogram refer to **1**: proteolysis on day 0; **2**- proteolysis on day 3 and **3**: proteolysis on day 7)

5.3.1.3.2 Peak areas

Figure 5-12 shows significant increase in proteolysis between the various days of incubation. The increase in absorbance between days 0 and 3 is between (100-200 mAU*min) for all samples whereas on day 7, the increase doubled for each sample. Overall, the change in absorbance is linear with the time of incubation. This observation is similar to TNBS results (Table 5-1). As observed earlier, *Pseudomonas fluorescens* 414 was less proteolytic than *Pseudomonas fluorescens* 416 (Figure 5-12). It may be concluded that the RP-HPLC method is suitable to detect lower levels of proteolysis as shown in Figure 5-12.

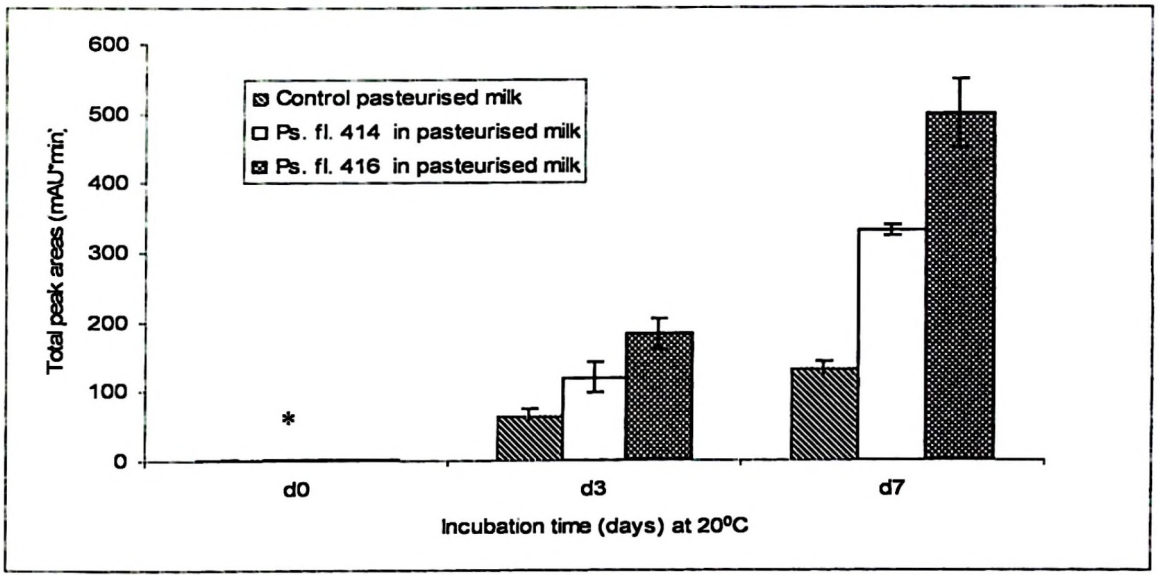


Figure 5-12 Total peak areas of peptides eluted from the pH 4.6 extracts of pasteurised milk incubated at 20^{0}C for 7 days (analysed by RP-HPLC); *Values too low to be seen (<2 mAU*min)

5.3.1.4 Analysis by urea-PAGE electrophoresis

This experiment was set up to monitor casein degradation following inoculation of *Pseudomonas fluorescens* 414 and 416 into skim UHT milk and incubated for 24 h but sampled at 2 and 24 h. Milk proteins may be identified by PAGE since the individual proteins can be separated according to size, or charge to mass ratio. SDS-PAGE does not resolve the 4 caseins properly, because their molecular weights are very similar where α_{s1}-

5.3.1.3.2 Peak areas

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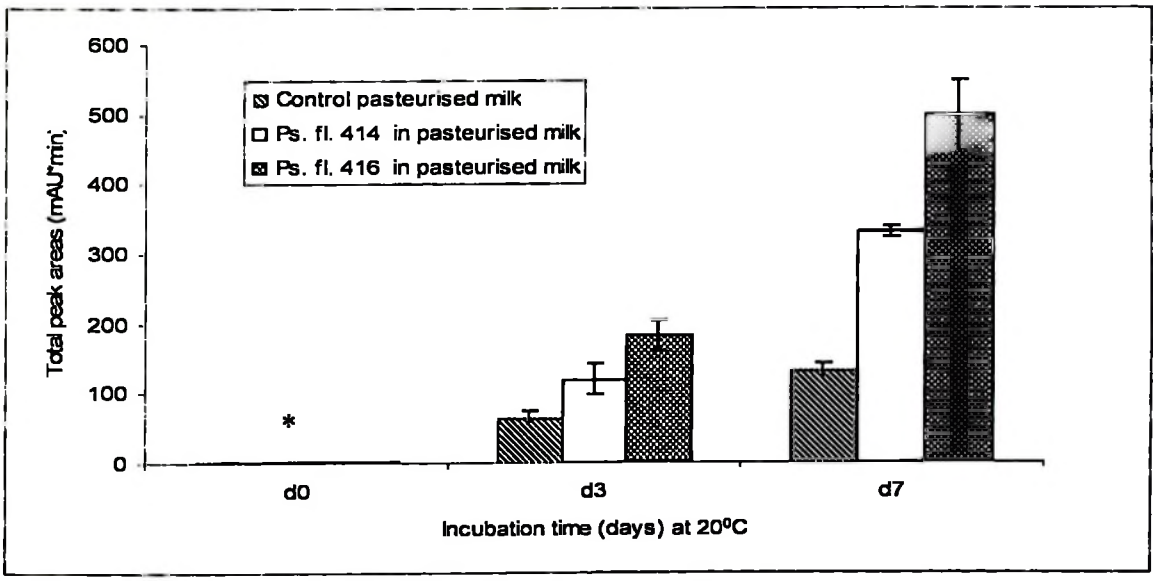

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5.3.1.4 Analysis by urea-PAGE electrophoresis

This experiment was set up to monitor casein degradation following inoculation of *Pseudomonas fluorescens* 414 and 416 into skim UHT milk and incubated for 24 h but sampled at 2 and 24 h. Milk proteins may be identified by PAGE since the individual proteins can be separated according to size, or charge to mass ratio. SDS-PAGE does not resolve the 4 caseins properly, because their molecular weights are very similar where α_{s1}-

caseins- 23 kDa; α_{s2}-caseins - 25 kDa; β- casein – 24 kDa and κ-caseins – 19 kDa (Fox, 2003). Separation by urea-PAGE is based on charge to mass ratio.

Caseins differ in their amino acid sequences and the degree of phosphorylation i.e. the phosphate groups whereby κ –casein has 1 group; β-casein has 5; α_{s1}-casein has 8-9 and α_{s2}-casein consists of 10-13 phosphate groups (Hekken and Thompson, 1992). These properties are exploited in urea-PAGE electrophoresis. Thus, urea-PAGE gives a better separation of the caseins than the SDS-PAGE.

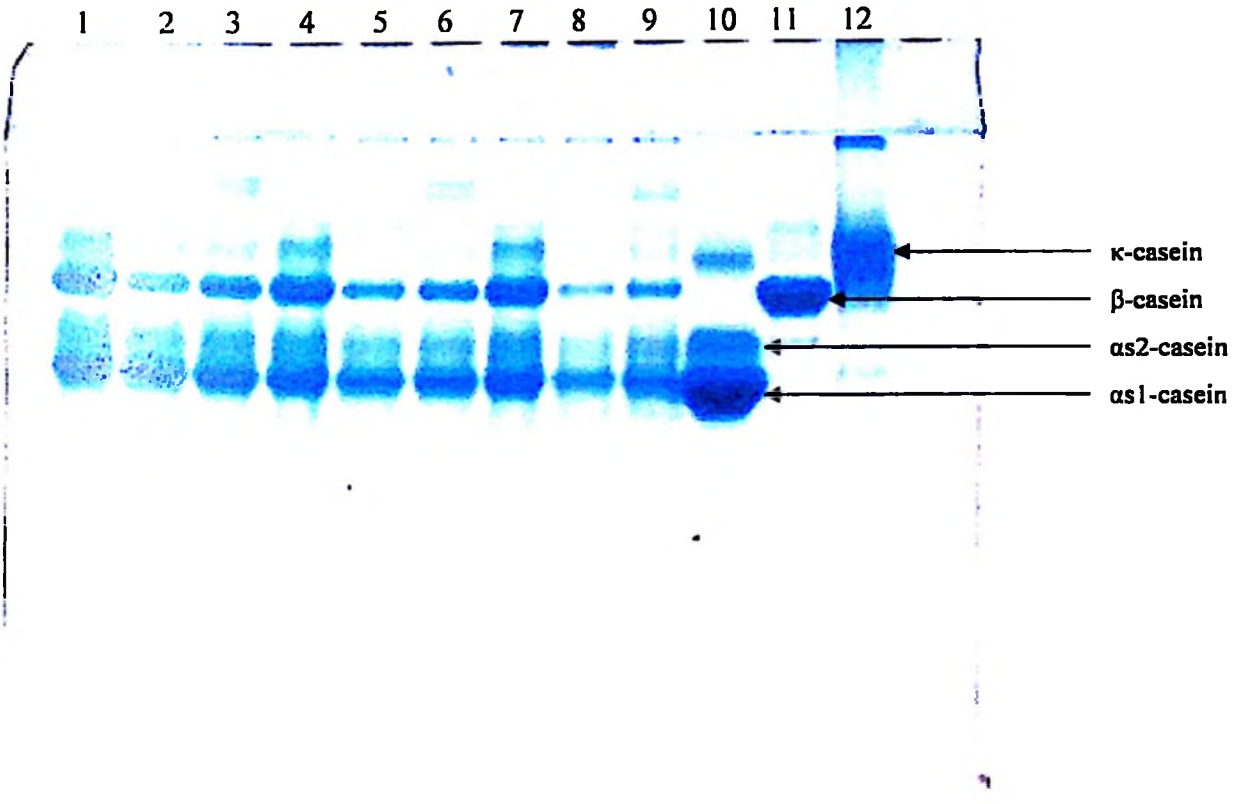

Figure 5-13 Urea PAGE electrophoretogram (T=12%, C=4%) of *Pseudomonas fluorescens (Ps. fl.)* incubated in UHT milk at 37^{0}C for 0, 2 and 24 h. **Lanes 1-3:** Control UHT milk; *Ps. fl.* enzyme 416 and *Ps. fl.* enzyme 414 incubated in UHT milk at 37^{0}C for 0h **Lanes 4-6:** Control UHT milk; *Ps. fl.* enzyme 416 and *Ps. fl* enzyme 414 incubated in UHT milk at 37^{0}C for 2 h. **Lanes 7-9:** Control UHT milk; *Ps. fl.* enzyme 416 and *Ps. fl.* enzyme 414 incubated in UHT milk at 37^{0}C for 24 h **Lane 10:** α-casein **Lane 11:** β-casein **Lane 12:** κ-casein

Incorporation of urea into the samples permits the separation of most caseins and reveal the removal of phosphate groups from individual caseins (Bingham *et al.*, 1976), and thus can monitor proteolysis of caseins during the ageing of cheeses. Hekken and Thompson (1992) observed that 40% dephosphorylation of whole casein slowed down the migration rate compared to the native β-casein. The authors explained that the removal of negatively charged phosphate groups slowed down the migration rate of the protein on the gels. The same researchers also found that κ–casein was poorly resolved in the gel, an observation which was also made in the current study.

Figure 5-13 indicates that the caseins in control samples were not degraded (lanes 1, 4 and 7). This finding concurs with the observation from RP-HPLC peak areas as well as the TNBS where the control samples had too low values and did not gel. Comparison between inoculated samples and the controls confirmed preference for *Pseudomonas fluorescens* 416 (lanes 5 and 8) to degrade β-casein. There was also gradual disappearance of αs_1 and αs_2-caseins which was most evident after 24 h on lane 8. Although κ -casein has shown some degradation, its poor resolution in the gel makes it difficult to conclude on the extent of hydrolysis. It has been documented that κ-casein is the ideal substrate for bacterial proteolysis (Fairbairn and Law, 1986; Datta and Deeth, 2003). However, in one study it was revealed that different strains of *Pseudomonas sp.* showed different hydrolysis rates on caseins whereby some degraded whole casein more than α , β or κ -casein while others degraded β –casein more than α and κ–casein (Mitchell and Marshall, 1989). A study of 6 proteases revealed that 3 were more active against α -casein than whole casein, γ, β or κ-casein; one hydrolysed α -casein and whole casein to the same extent (Patel *et al.*, 1986). It was stipulated that whole casein could have a configuration that made it more susceptible to enzyme action (Mitchell and Marshall, 1989). In addition, a great deal of variability was

observed in the ability of the proteases to hydrolyse κ- casein. The conformational differences in proteins can affect substrate specificity.

5.3.2 Proteolysis caused by *Pseudomonas fluorescens* enzymes

5.3.2.1 Analysis by TNBS

Pseudomonas fluorescens enzymes were dialysed against water, so as to remove peptides and amino acids which interfered with analysis. Inoculating the milk with live *Pseudomonas fl.* presented problems because other microorganisms could grow in milk as no sodium azide was added. Addition of partially purified enzyme into milk is important because sodium azide, which controls growth of microorganisms, can be used. Hence, by adding NaN_3, no additional bacterial proteases could be produced on storage and thus only proteases that survived treatments could act on caseins.

Figures 5-17, 5-18, 5-19 and 5-20 of UHT skim milk inoculated with dialysed *Pseudomonas fluorescens* 414 and 416 enzymes (10%), indicate that the dialysis procedure further purified the samples since the breakdown products in dialysed samples were lower after 24 h than the non-dialysed samples.

Table 5-4 reveals that for pH 4.6 soluble extracts, absorbance increased almost 5 times for *Pseudomonas fluorescens* 416 and 4 times for *Pseudomonas fluorescens* 414 between 0-2 h incubation. The activity almost doubled between 6 and 24 h for both strains. It may thus be concluded that significant changes in absorbance at pH 4.6 soluble extracts occurs in the first 2 h of incubation. The control sample was more or less constant throughout the incubation time. The pH of the samples was constant (6.60± 0.020).

Table 5-4 Absorbance of the pH 4.6 soluble extracts of UHT skim milk inoculated with dialysed *Pseudomonas fluorescens* 414 and 416 enzymes at 37^{0}C for 24 h as analysed by the TNBS method .

	pH 4.6 soluble extracts			6% TCA soluble extracts		
Time (h)	Control	*Ps fl.* 414	*Ps fl.* 416	Control	*Ps fl.* 414	*Ps fl.* 416
0	0.024±0.001	0.026±0.002	0.038±0.002	0.107±0.008	0.184±0.003	0.201±0.011
2	0.022±0.003	0.106±0.007	0.187±0.005	0.140±0.014	0.504±0.005	0.836±0.018
6	0.035±0.003	0.121±0.004	0.191±0.004	0.131±0.011	0.580±0.019	0.942±0.026
24	0.038±0.005	0.231±0.015	0.350±0.007	0.141±0.021	1.212±0.040	1.539±0.078

* pH 4.6 soluble extracts were diluted (x 20) whereas 6%TCA soluble extracts were diluted (x 2)

As with the previous observation, the 6% TCA soluble extracts show that *Pseudomonas fluorescens* 414 increased by almost 3 times whereas *Pseudomonas fluorescens* 416 increased by 4 times between 0 and 2 h of incubation. Almost constant absorbance was observed between 2 and 6 h incubation for both strains of *Pseudomonas fluorescens.* This observation appears to suggest that between 2 to 6 h, proteolysis is constant however, after 6h there is an increase in activity as observed by increased absorbance readings.

Dialysed samples were also analysed by the TNBS protocol to examine the effectiveness of the technique. The dialysis technique is useful in removing interfering substances from the enzyme, hence purifying the enzyme. Figure 5-14 clearly indicates the effect of dialysis on purification of *Pseudomonas* spp, where absorbance readings are lowered by 60 and 72% *Pseudomonas fluorescens* 414 and 416 respectively from pH 4.6 soluble extracts. The decrease of absorbance as a result of dialysis in the same figure is 73% and 80% respectively for *Pseudomonas fluorescens* 414 and 416 in 6% TCA soluble extracts, indicating the usefulness of this technique in partial purification of the enzymes. It is documented that enzyme purification by dialysis removes the peptides and amino acids that are associated with the enzyme preparation (Schokker and VanBoekel, 1997). From the above discussion, it may be concluded that dialysis was useful in removing the interfering substances.

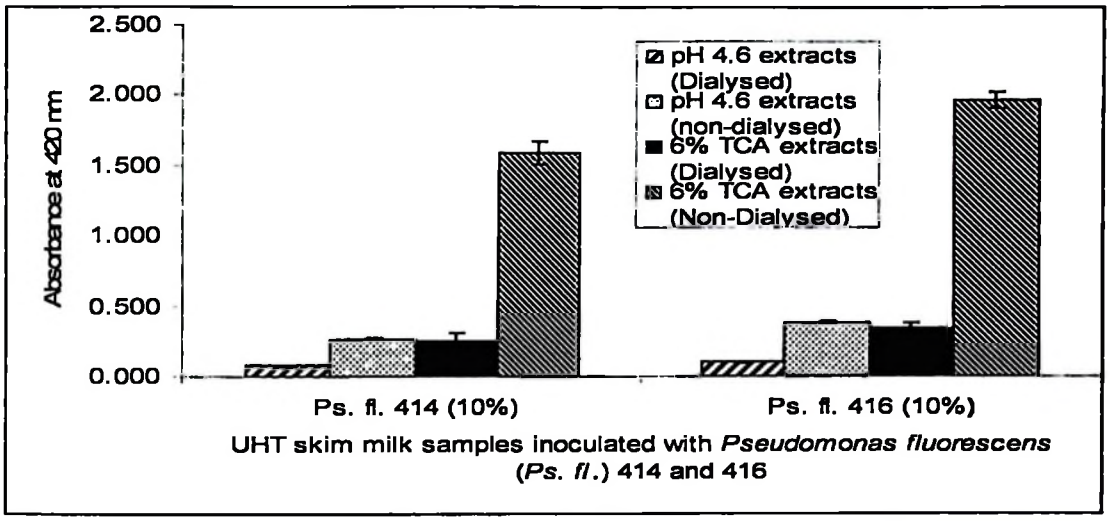

Figure 5-14 The effect of dialysis on purification of *Pseudomonas fluorescens* enzymes in pH 4.6 and 6% TCA soluble extracts after 24 h at 37^{0}C in UHT milk as analysed by the TNBS protocol (N=4)

5.3.2.2 Analysis by Fluorescamine

The experiment was conducted to assess the suitability of this method in analysing proteolysis in dialysed and non-dialysed samples of the enzyme extract. Comparison was made of pH 4.6 and 6% TCA soluble extracts following proteolysis by the two strains of *Pseudomonas fluorescens* (414 and 416) that were inoculated into UHT milk in dialysed and non-dialysed samples. Figure 5-15 shows the effect of dialysis on the relative fluorescence of the breakdown products of proteolysis in pH 4.6 and 6% TCA soluble extracts. Non-dialysed samples reached the upper detection limit just after incubation (results not shown). After dialysis however, the relative fluorescence was detectable up to the end of the experiment which shows that the dialysis procedure reduced the peptides and amino acids to levels which could be detected. This is consistent with the results by TNBS method (5.3.2.1).

Further purification of the *Pseudomonas fluorescens* 414 and 416 enzyme by ammonium sulphate precipitation was carried out by some researchers and this was found to increase the specific activity by 13 fold (Schokker and VanBoekel, 1997). In the current experiment,

however, no further purification was done because the aim was to reduce fluorescence to detectable levels which was achieved by dialysis alone.

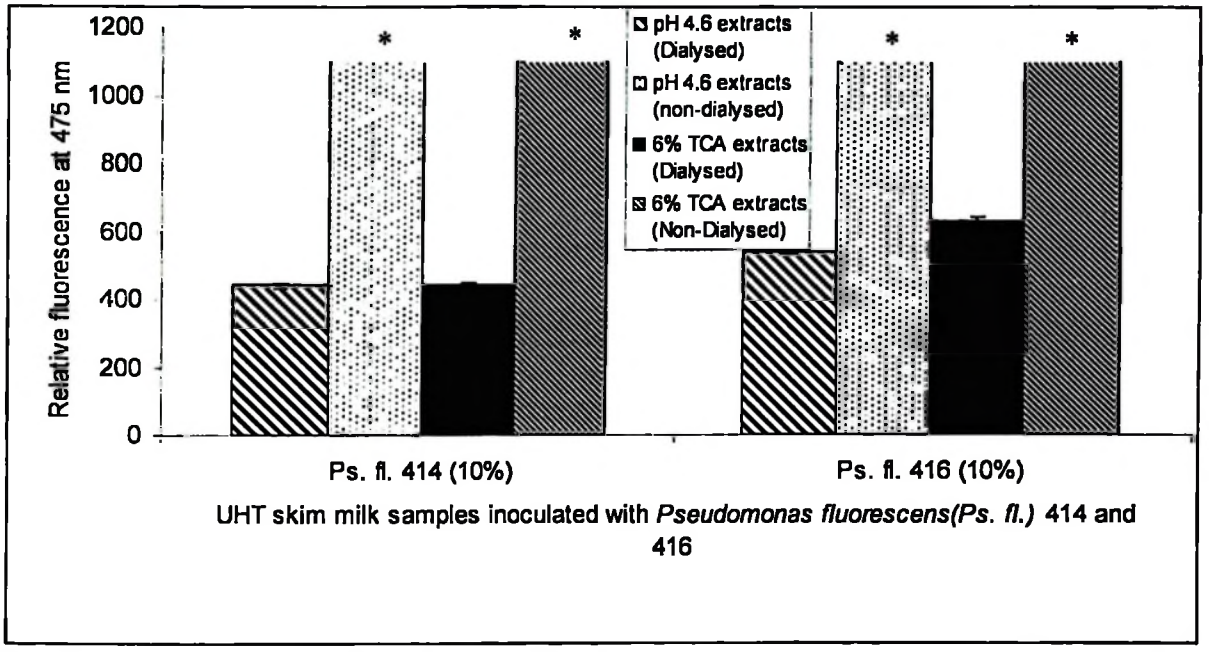

Figure 5-15 The effect of dialysis on purification of *Pseudomonas fluorescens* enzymes in pH 4.6 soluble extracts after 24h at 37^{0}C by the fluorescamine protocol (N=4); *upper detection limit of the instrument

5.3.2.3 Analysis by RP-HPLC

Several peaks were produced after 24 h incubation of dialysed *Pseudomonas* 414 and 416 at 1%, 5%, 10%, 15% and 20% (v/v) as observed in Figures 5-16 and 5-17. In Figure 5-16, there were significant peaks between 20 - 30 min (see the arrow), which increased rapidly with concentration of the enzyme. The same peaks appear in Figure 5-17 but were much smaller probably because as observed earlier *Pseudomonas fluorescens* 416 had higher activity than *Pseudomonas fluorescens* 414. The peak areas increased with the concentration of *Pseudomonas fluorescens* (v/v) inoculated into the UHT milk.

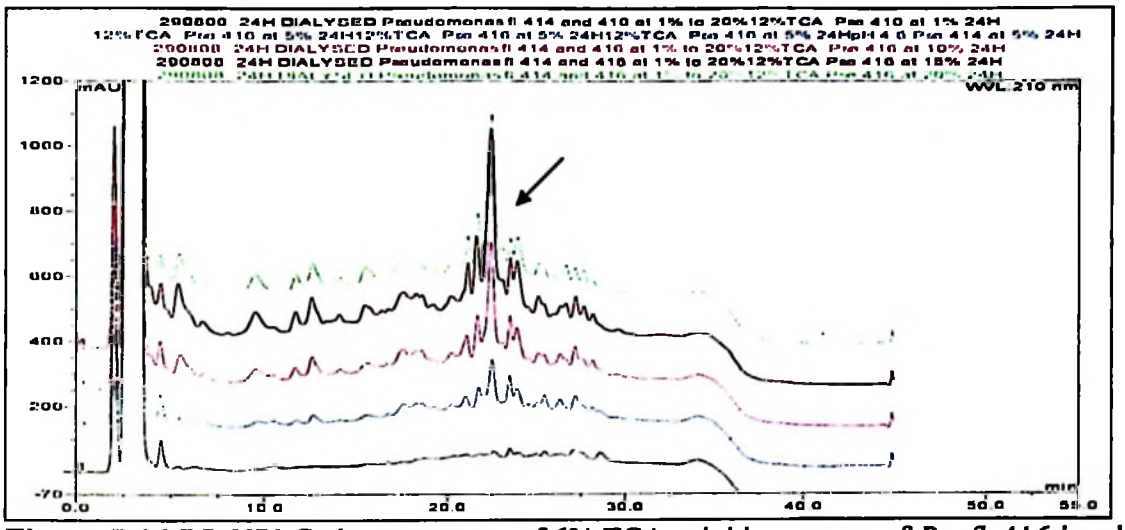

Figure 5-16 RP-HPLC chromatogram of 6% TCA soluble extracts of *Ps. fl.* 416 incubated in UHT milk for 24 h at 37^{0}C [numbers on the left hand side refer to **1**:1%, **2**:5%, **3**:10%, **4**:15% and **5**:20% (v/v)]

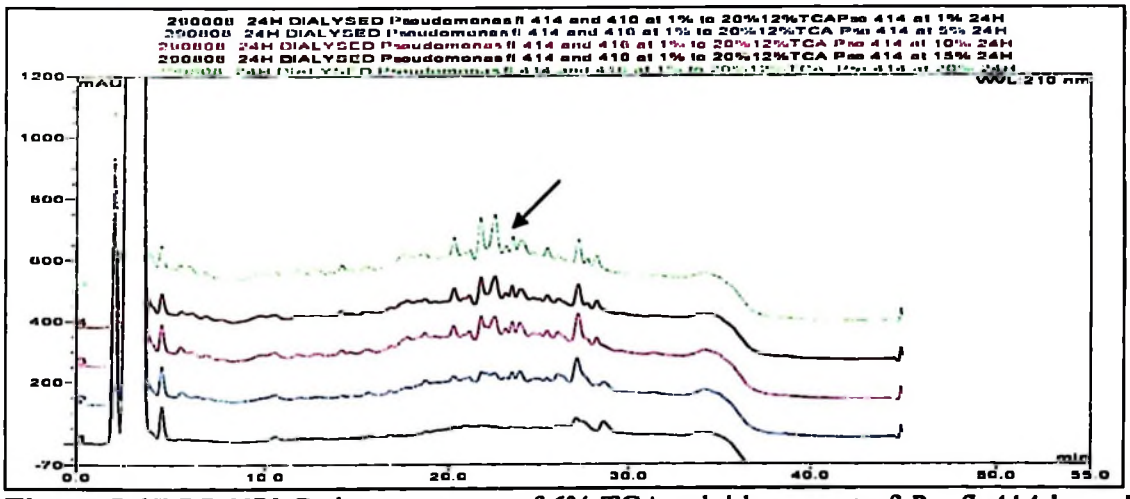

Figure 5-17 RP-HPLC chromatogram of 6% TCA soluble extract of *Ps. fl.* 414 inoculated in UHT milk for 24 h at 37^{0}C [numbers on the left hand side refer to **1**:1%, **2**:5%, **3**:10%, **4**:15% and **5**:20% (v/v)]

5.3.2.4 Analysis by urea-PAGE

Figure 5-18 shows the effect of incubating the various concentrations of *Pseudomonas fluorescens* 414 at 5%, 10% and 20% for 2 and 24 h at 37^{0}C on proteolysis. The bands for *Pseudomonas fluorescens* 414 were denser at 2 h at all concentrations compared to 24 h as expected. The α_{s1}-casein bands were denser than the β-caseins and α_{s2}-casein bands, indicating preference of *Pseudomonas fluorescens* to hydrolyse β-caseins and α_{s2}-casein.

Proteolysis was most evident at 20% enzyme concentration where α_{s2} and β-caseins were both hydrolysed. κ-casein band, although very faint gradually decreased intensity from lanes 1- 6 confirming its hydrolysis by *Pseudomonas fluorescens.*

Microbial enzymes have been reported to show preference to hydrolyse κ-casein, which is usually found on the surface of the casein with the formation of para- κ- casein (Snoeren and Van Riel, 1979), followed by extensive hydrolysis which is non specific (Law *et al.*, 1977a). As explained in section 5.3.1.5.1, the various strains of *Pseudomonas fluorescens* have different preferences for hydrolysing caseins. The γ-caseins are a result of the activity of native enzymes (presumably plasmin) on β-caseins. As expected, the bands are fainter after 24 h than after 2 h because of the longer time of enzyme activity.

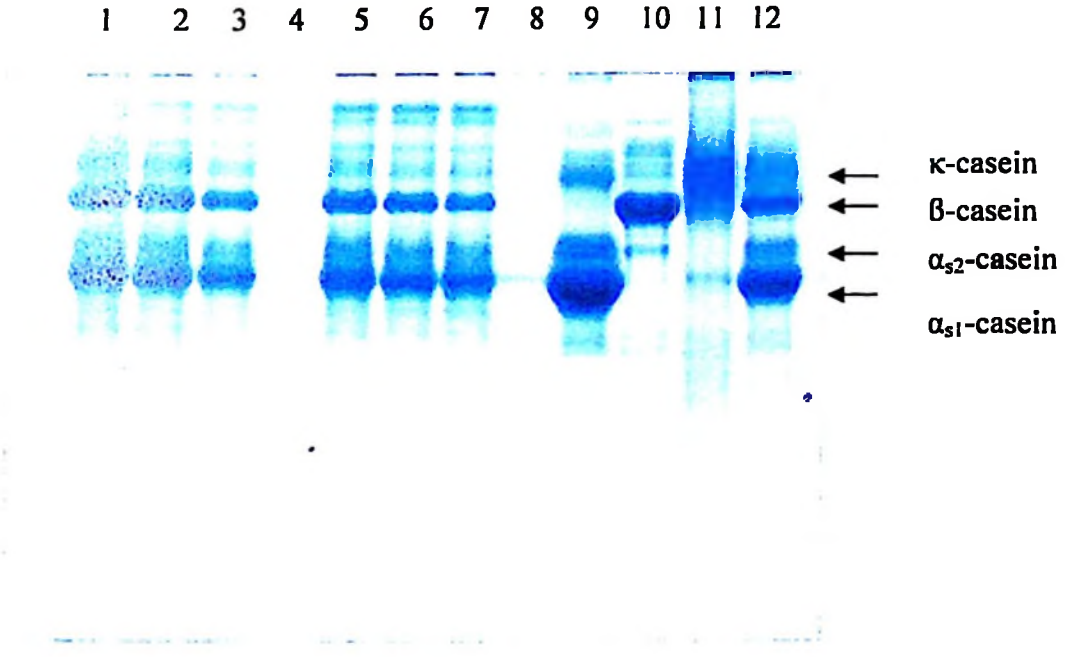

Figure 5-18: Urea PAGE electrophoretogram (T=12%, C= 4%) *Pseudomonas fl* enzymes incubated in UHT milk at 37^{0}C for 2 and 24 h. **Lanes 1-3:** *Pseudomonas fluorescens* 414 at 5% , 10% and 20% in UHT skim milk after 2 h incubation at 37^{0}C. **Lane 4:** Empty lane **Lane 5 – 7:** *Pseudomonas fluorescens* 414 at 5%, 10% and 20% in UHT skim milk after 24 h incubation at 37^{0}C. **Lanes 8:** empty lane **Lanes 9-11:** α- casein, β – casein, κ– casein and **Lane 12:** a mixture of α, β, κ caseins respectively.

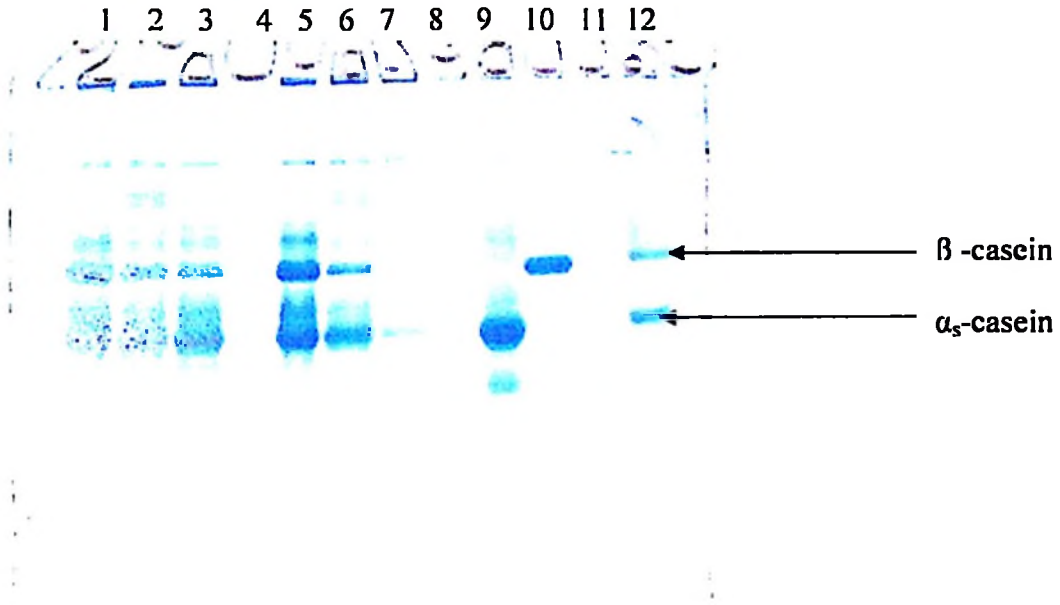

Figure 5-19 urea PAGE electrophoretogram (T=12%, C=4%) of dialysed *Pseudomonas fluorescens* (10%) enzymes incubated in UHT milk at 37^{0}C for 6 and 24 h. **Lanes 1-3:** Control (UHT milk) *Ps. fl.* enzyme 414 and *Ps. fl.* enzyme 416 at 6h **Lane 4:** Empty lane **Lanes 5-7:** Control, *Ps. fl.* enzyme 414 and *Ps. fl.* enzyme 416 incubated for 24 h. **Lane 8:** Empty lane **Lanes 9 -11:** α-($_{s2\&s1}$); β- and κ-casein respectively Lane 12: α-($_{s2\&s1}$); β- and κ-casein

Figure 5-19 shows the effect of incubation time on casein hydrolysis. As expected, with increased incubation time greater hydrolysis of caseins was observed. The β-caseins were preferably hydrolysed at 2 h incubation, whereas α-s_1 and α-s_2 casein were also hydrolysed after 24 h. It was also obvious that *Pseudomonas fluorescens* 416 enzyme had faint bands of α-s_1, α-s_2 casein and β-caseins at all incubation times, indicating more degradation and higher enzyme activity than *Pseudomonas fluorescens* 414 enzymes.

5.3.3 Proteolysis caused by commercial *Bacillus licheniformis* enzymes

5.3.3.1 Analysis by TNBS

Figure 5-21 shows proteolysis by *Bacillus licheniformis* enzyme in UHT skim milk incubated at 37^{0}C for 3 days. Detailed experimental procedure is given in section 3.3.2. The enzyme was quite active as higher absorbance values were detected from day 0, especially at higher

enzyme concentrations. The absorbance observed in the control sample is possibly due to plasmin activity during storage of the UHT milk. The TNBS protocol seems to be a reliable method to monitor proteolysis in these samples.

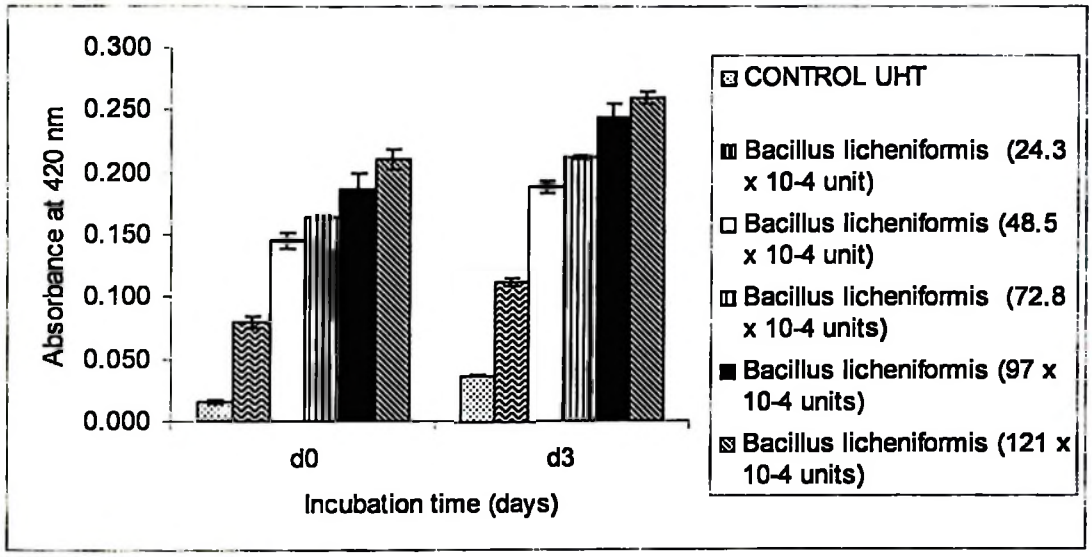

Figure 5-20 The effect of *Bacillus licheniformis* enzymes at five levels (24.3 x 10^{-4}, 48.5 x 10^{-4}, 72.8 x 10^{-4}, 97 x 10^{-4} and 121 x 10^{-4} units) in pH 4.6 soluble extracts of UHT milk after incubation at 37^0C for 3 days as determined by the TNBS method.

5.3.3.2 Analysis by HPLC

5.3.3.2.1 Markers of activity

Figures 5-21 and 5-22 show the peptide peaks eluted from *Bacillus licheniformis* enzyme using pH 4.6 and 6% TCA soluble extracts. Several small peaks are present in both figures right from the time of injection, but higher significant peaks labelled as 1-4 are in both figures and appear between 20 – 25 min. These peaks could be important markers for activity of *Bacillus licheniformis*. Peak 5 is prominent in Figure 5-21 at ~ 35 min but very low in Figure 5-22 which confirms that TCA precipitates this peptide which results from plasmin activity on the caseins.

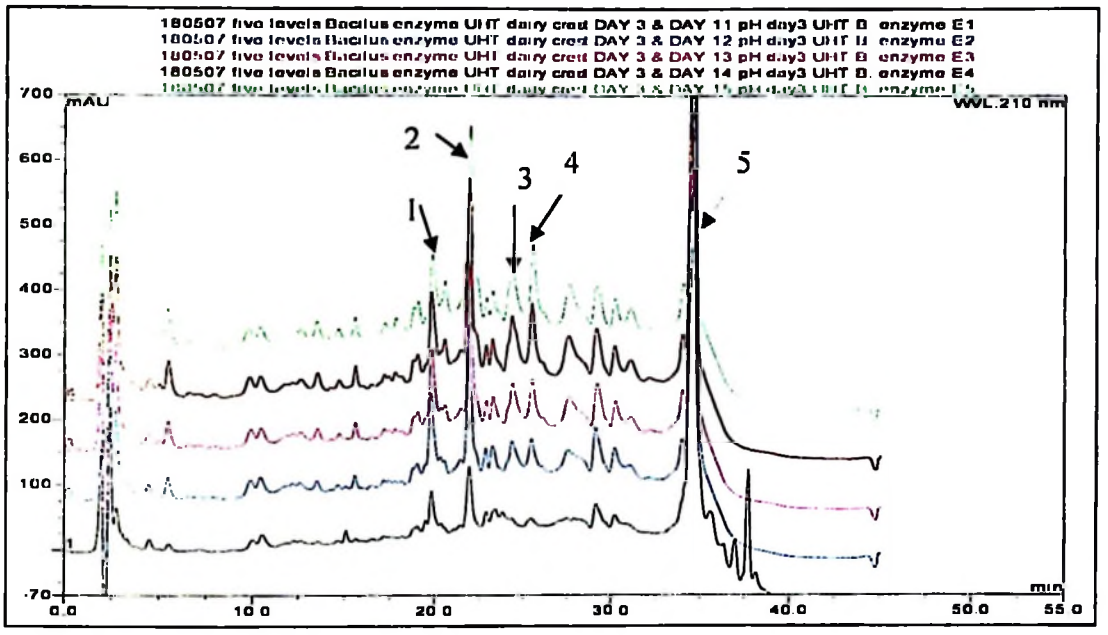

Figure 5-21 RP-HPLC chromatogram of pH 4.6 soluble extracts of *Bacillus licheniformis* enzyme inoculated at 5 levels (24.3 x 10^{-4}, 48.5 x 10^{-4}, 72.8 x 10^{-4}, 97 x 10^{-4} and 121 x 10^{-4} units) in UHT milk incubated at 37^{0}C for 3 days

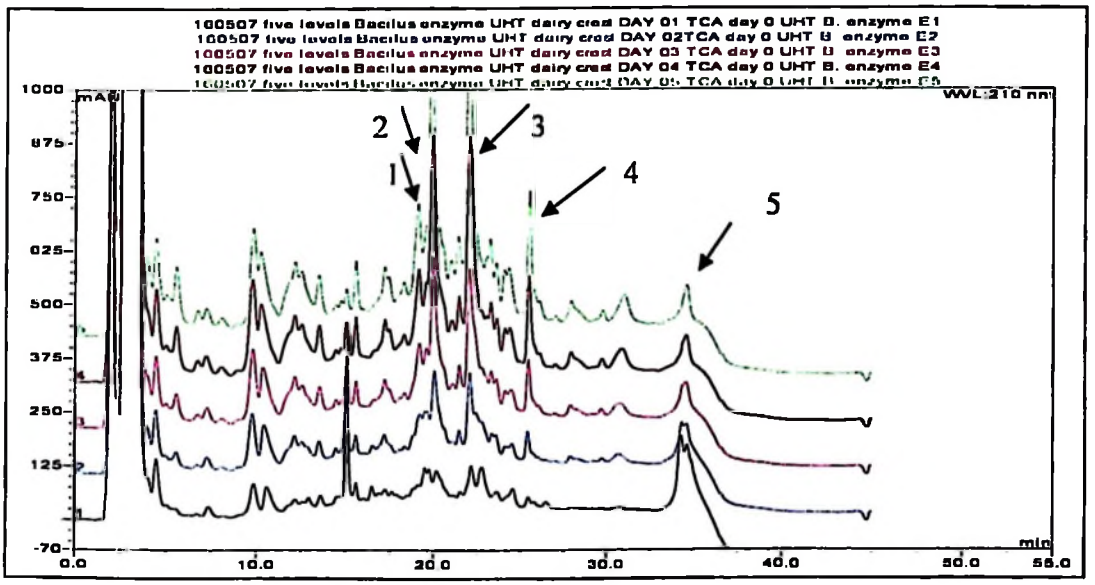

Figure 5-22 RP- HPLC chromatogram of 6% TCA extracts of *Bacillus licheniformis* enzyme inoculated at 5 levels(24.3 x 10^{-4}, 48.5 x 10^{-4}, 72.8 x 10^{-4}, 97 x 10^{-4} and 121 x 10^{-4} units) in UHT milk incubated at 37^{0}C for 3 days

5.3.3.2.2 Peak areas

This experiment was conducted to assess the suitability of the RP-HPLC procedure in monitoring lowest levels of proteolysis by *Bacillus licheniformis* enzyme. Figure 5-23 shows the effect of incubation time on the various levels of *Bacillus licherniformis* enzyme on proteolysis.

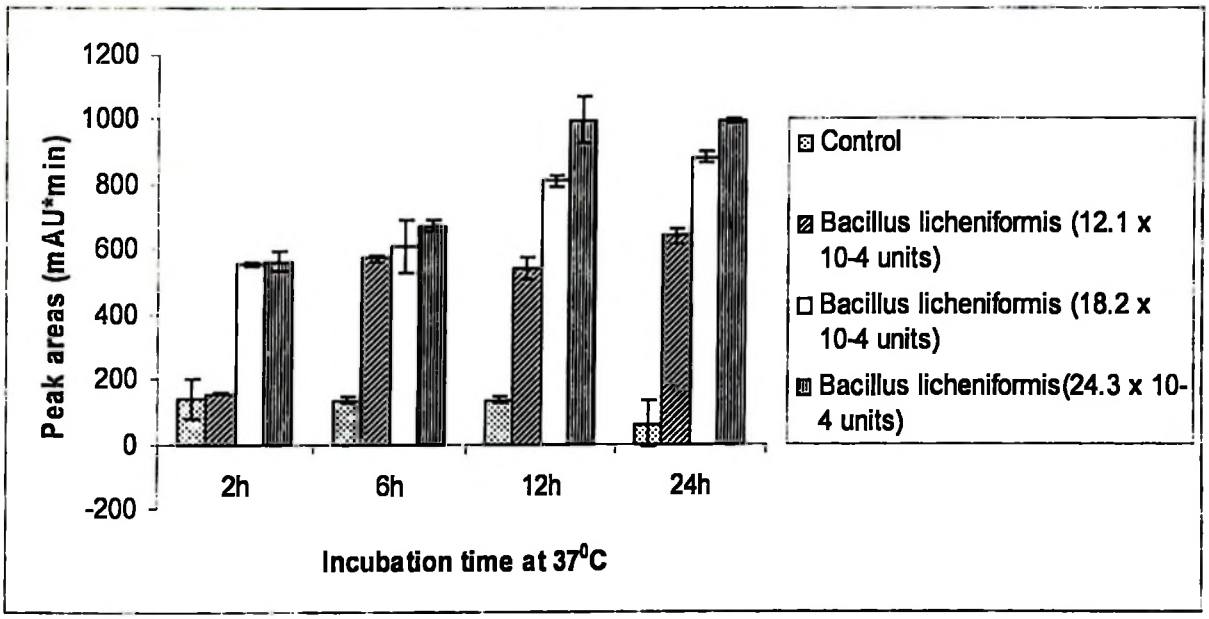

Figure 5-23 Effect of proteolysis on the various concentrations of 6 %TCA soluble extracts of *Bacillus licheniformis* enzyme inoculated in UHT milk and incubated at 37^{0}C for 24 h as analysed by the RP-HPLC method (N=8)

As expected, *Bacillus* enzyme 24.3 x 10^{-4} units had the highest activity at all incubation times. Although *Bacillus* at 12.1 x 10^{-4} units had about 200 mAU*min, the quantity had tripled at 6 h incubation. As a result, the level of activity was almost similar for all levels of *Bacillus* at 6 h of incubation. The milk at this point had some clots formed but not quite a gel. A gel was formed after 12 h of incubation of the highly concentrated sample (24.3 x 10^{-4} units). The pH (6.6±0.02) was constant throughout the experiment. By 24 h of incubation all samples had gelled except the control. *Bacillus* enzymes are known for their presence in

contaminated milk. Contamination by *Bacillus* result in gelation and milk would be of poor quality.

5.3.3.3 Analysis by urea PAGE

Analysis by Urea-PAGE provided a useful tool for monitoring breakdown products as a result of proteolysis by *Bacillus licheniformis*. Incubation was monitored for 2, 6, 12 and 24 h.

Figure 5-24 indicates that proteolysis was rapid as both α and β -casein disappeared after only 2 h of incubation. Some faint bands were observed on lanes 6 and 7 in the lower part of the gel, gradually decreasing after 12 h but completely disappeared after 24 h of incubation. These were breakdown products of α and β –caseins. Likewise, some faint bands were observed above the β –casein band. These bands progressively decreased intensity on lanes 7 and 8 until their complete disappearance on lane 9. Since these preliminary experiments indicated that the enzymes used were high as complete degradation of both α and β –caseins was evident after 2 h incubation, a lower level of 6.06×10^{-4} units was used in the next experiment.

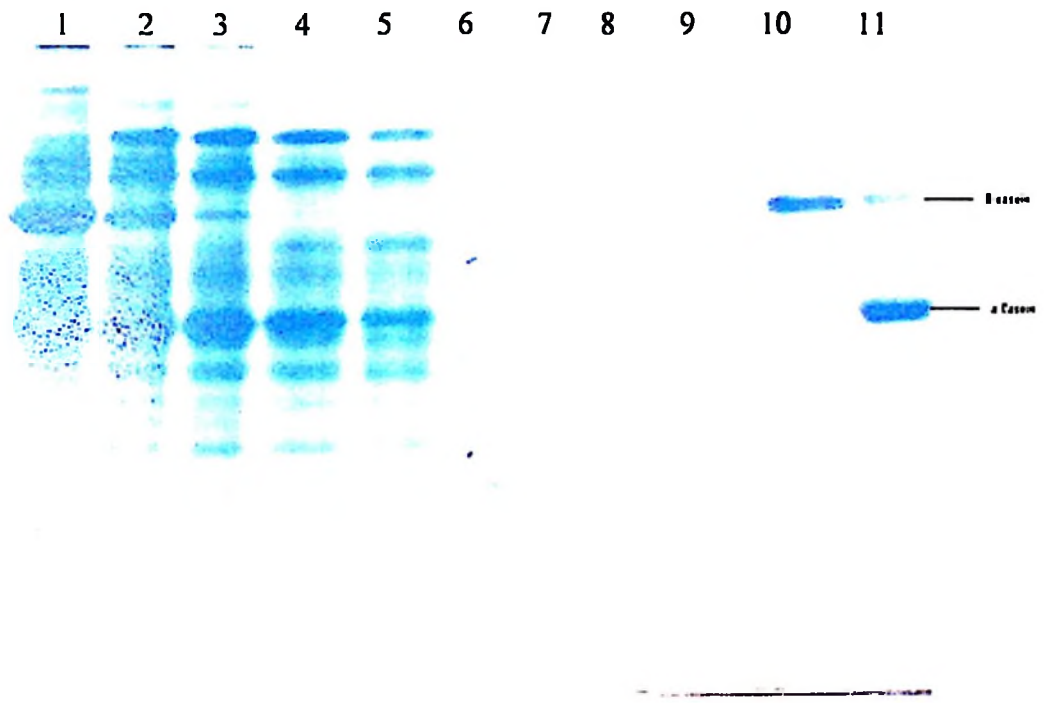

Figure 5-24 urea-PAGE electrophoretogram (T=12%, C= 4%) of UHT skimmed milk incubated with trypsin (248 BAEE units) or *Bacillus* protease (24.3 x 10^{-4} units) at 37^{0}C for 2, 6, 12 and 24 h. **Lane 1:** Control UHT milk **Lanes 2-5 :** UHT milk with added trypsin (248 BAEE units) incubated for 2, 6, 12 and 24 h **Lanes 6-9 :** UHT milk with added *Bacillus* protease (24.3 x 10^{-4} units) incubated for 2, 6, 12 and 24 h **Lane 10:** β-Casein **Lane 11:** Mixture of α β-casein
NOTE: The same gel was run for *Bacillus licheniformis* and trypsin samples; the trypsin samples however, will not be discussed here

A lower concentration of *Bacillus licheniformis* (6.06 x 10^{-4}) was used in the next experiment so as to observe changes during incubation at 37^{0}C for 0 and 3 days. Reference is made to Figure 4-8 on page 85. It is clear from the figure that proteolysis by *Bacillus licheniformis* (lanes 2 and 5) was rapid from the day of incubation. Preference for degradation of β-casein was obvious from day 0 for both raw and pasteurised milk, but no γ-caseins were formed. On day 3 however, α-casein was also degraded and β-casein (lane 8) had undergone further breakdown in raw milk. Complete disappearance of both α- and β-casein was observed on day 7 for pasteurised milk (lane 11) indicating severe proteolysis by *Bacillus licheniformis*

enzymes in pasteurised than raw milk. The same observation was made for *Pseudomonas fluorescens.*

On day 3, α-casein was also degraded and β-casein was degraded further in raw milk (lane 8) but complete disappearance of both α- and β-casein (lane 11) was observed on the same day in pasteurised milk, confirming higher proteolytic activity in the latter than the former. Higher activity in pasteurised milk could also be attributed by native enzymes particularly plasmin as pasteurisation inactivates the inhibitors in milk, resulting in higher plasmin activity. It was discussed in chapter 4 (section 4.3.1.4) that extensive proteolysis by plasmin resulted in further breakdown of γ –casein produced, that is probably the reason for its absence on lane 11.

5.4 Conclusions:

The chapter focused on the roles of *Pseudomonas fluorescens* 414 and 416 and its enzymes as well as commercial enzymes of *Bacillus licheniformis* on proteolysis of pasteurised and UHT skim milk. Dialysis was performed on *Pseudomonas fluorescens* 414 and 416 enzymes incubated in UHT skim milk to study proteolysis. Comparison was made of dialysed and non-dialysed samples. The effects on storage time and temperature as well as markers of activity in pH 4.6 and 6% TCA soluble extracts by selected methods was studied. Results demonstrated that proteolysis increased with both storage time and temperature. Dialysis proved to be extremely useful in purifying the samples since it removed breakdown products which interfered with some of the analytical procedures. This method was particularly useful for the fluorescamine procedure which reached detection limit within a short time. Non-dialysed samples had very high relative fluorescence values (over 1100 relative fluorescence units).

The RP-HPLC results indicated that both species of *Pseudomonas fluorescens* inoculated in UHT milk had similar peptide patterns in pH 4.6 and 6% TCA soluble extracts except for one peak. The exceptionally high peak was observed at 35 min in pH 4.6 soluble extracts possibly originated from plasmin was absent 6% TCA soluble extracts which precipitated large peptides. Dialysed samples of *Pseudomonas fluorescens* had lower peaks than the non dialysed samples, probably due to removal of some peptides and amino acids by dialysis technique. Significant peaks were observed between 20-30 min for dialysed samples whereas for non- dialysed sample similar sized peaks were observed from the time of injection to 30 min.

Several peaks were observed in *Bacillus licheniformis* enzyme samples from the time of injection. Significantly high peaks however were observed between 20-25 min. These peptide

peaks and the time they are eluted could serve as useful markers to identify the cause of proteolysis in milk.

Gel electrophoresis was a useful qualitative method to observe breakdown of caseins during incubation whereby both *Pseudomonas fluorescens* and *Bacillus licheniformis* showed preference for degradation of β-cascin over other caseins. The fluorescamine method as used in the current study did not seem suitable for longer proteolysis studies as the detection limit was reached within a short time. However, with the dialysis technique it was possible to obtain on-scale readings due to removal of interfering substances. Although the RP-HPLC was more accurate and sophisticated method, the TNBS method is recommended for the detection of proteolysis in milk because it is accurate, cheaper and also easier to perform.

CHAPTER 6 SUSCEPTIBILIY OF MILK PROCESSED AT HIGH TEMPERATURES TO PROTEOLYSIS DURING STORAGE

6.1 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 UHT milk (Datta and Deeth, 2001). As previously described in the introduction and literature review, proteolysis is caused by either bacterial enzymes or naturally occurring enzymes of which plasmin is significant (Grufferty and Fox, 1988b).

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 or indirectly such as directly at 142^{0}C for 4 s (Snoeren *et al.*, 1979), directly at 138^{0}C for 2-5 s (Samel *et al.*, 1971), direct and indirect respectively at 142^{0}C for 5 s and 145^{0}C for 3 s (Manji *et al.*, 1986).

Taking into account the desirable and undesirable effects of plasmin in different applications, it is crucial to understand processing stability during heat treatments. The aim of this study was to investigate susceptibility of raw milk and milk processed at various temperature-time profiles to proteolysis when stored under the same conditions.

The study was also aimed at observing changes (if any) in breakdown products at the various temperatures selected.

6.2 Materials and methods

Raw milks were processed on an APV junior UHT plate heat exchanger (APV, Crawley, UK), with two stages of heating involving hot water (80^0C) and steam ($112—142^0$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 142^0C but 15 s at 80^0C. Homogenisation took place between the heating stages at about 170 bar. These temperature- times combinations were selected based on studies of plasmin inactivation. The lowest (85 ^{0}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 2^0C for 2 days. Six batches of milk samples were treated with sodium azide (0.05%) to prevent bacterial contamination. These were then dispensed in sterile bottles in a laminar flow hood cabinet followed by incubation at 37^0C for 28 days. Sampling for analysis was done on days 0, 3, 7, 14, and 28. Clarification to obtain pH 4.6 and 6% TCA soluble extracts was carried out as given in section 3.6. The soluble extracts were analysed by TNBS, fluorescamine, gel electrophoresis and RP- HPLC.

6.3 Results and discussion

6.3.1 Proteolysis assessed by TNBS

6.3.1.1 Effect of storage on pH 4.6 soluble extracts

Milk processing is necessary for flavour improvement as well as shelf life extension. The present investigation was aimed at examining the effect of various heat processes on proteolysis during storage. Clarified samples (pH 4.6 soluble extracts as described in section 3.6) of raw milk and milk heated at various temperatures were analysed after incubation at 37^0C for 28 days.

Results from pH 4.6 soluble extracts of milk samples (Table 6-1) show that raw milk and milk sample heated at 85^0C had the highest proteolytic activities which were significantly different ($p<0.05$) from all the other samples. It was also observed that pH 4.6 soluble extract of all samples had the highest absorbance and hence increased proteolysis on day 28. The increased absorbance with time was probably due 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. Another study confirmed increased proteolysis of proteose peptones from 2.5 to 11.1 mg/ mL in whey prepared from raw milk stored for 7 d at 37^0C (Andrews and Alichanidis, 1983 b). 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.

The heat treatment at 85^0C/ 15 s was designed to mimic pasteurisation temperatures with the aim of inactivating the plasmin and plasminogen activator inhibitors. As previously

stated in chapter 4, plasmin inhibitors and plasminogen activator inhibitors are heat labile and therefore inactivated at lower temperatures compared to the other components of the plasmin system (Richardson, 1983b). About 81.1% and 35.8% of plasminogen activator inhibitors and plasmin inhibitors respectively are inactivated at 75 ^{0}C for 15 s (Prado *et al.*, 2006). This in turn increases plasmin activity as more plasminogen is also converted to plasmin (Richardson, 1983b) and hence higher proteolysis.

Table 6-1 Absorbance of pH 4.6 and 6% TCA soluble extracts of raw milk and milk processed under various Temperature – time conditions and incubated at 37^{0}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	Absorbance of 6% TCA soluble extracts of UHT skim milk at 420 nm
day 0	Raw milk	0.026±0.0023 a A	0.085±0.0108 a A
	Heated at 85°C	0.026±0.0014 a E	0.080±0.0125 a F
	Heated at 110°C	0.024±0.0008 a I	0.085±0.0110 a K
	Heated at 120°C	0.025±0.0021 a N	0.080±0.0067 a N
	Heated at 130°C	0.025±0.0020 a S	0.077±0.0075 a Q
	Heated at 142°C	0.023±0.0035 a V	0.077±0.0116 a T
day 3	Raw milk	0.090±0.0105 b B	0.301±0.0152 b B
	Heated at 85°C	0.053±0.0040 c F	0.208±0.0257 C g
	Heated at 110°C	0.031±0.0034 d J	0.093±0.0021 d K
	Heated at 120°C	0.027±0.0031 d O	0.077±0.0097 d N
	Heated at 130°C	0.027±0.0015 d S	0.081±0.0033 d Q
	Heated at 142°C	0.025±0.0023 d V	0.073±0.0091 d T
day 7	Raw milk	0.112±0.0250 e B	0.559±0.0236 e C
	Heated at 85°C	0.085±0.0111 f F	0.283±0.0085 f H
	Heated at 110°C	0.036±0.0018 g KL	0.146±0.0066 g L
	Heated at 120°C	0.033±0.0025 g PQ	0.128±0.0075 g O
	Heated at 130°C	0.026±0.0029 g S	0.125±0.0045 g R
	Heated at 142°C	0.023±0.0028 g V	0.124±0.0069 g U
day 14	Raw milk	0.164±0.0060 h C	0.949±0.0621 h D
	Heated at 85°C	0.221±0.0050 i G	0.852±0.0101 I I
	Heated at 110°C	0.042±0.0017 j L	0.177±0.0025 j M
	Heated at 120°C	0.038±0.0025 kj Q	0.141±0.0031 j P
	Heated at 130°C	0.037±0.0021 kj T	0.137±0.0036 j S
	Heated at 142°C	0.031±0.0012 kW	0.132±0.0026 j U
day 28	Raw milk	0.305±0.0087 l D	1.577±0.0592 k E
	Heated at 85°C	0.217±0.0216 m H	0.935±0.0261 l J
	Heated at 110°C	0.084±0.0078 n M	0.185±0.0047 m M
	Heated at 120°C	0.060±0.0091 o R	0.149±0.0040 m P
	Heated at 130°C	0.047±0.0056 o U	0.140±0.0020 m S
	Heated at 142°C	0.043±0.0074 o X	0.138±0.0025 m U

Different lower case letters on the same column show significant differences ($p<0.05$) per day 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)

For the pH 4.6 soluble extracts of milk heated at 110, 120, 130 and 142^{0}C for 2 s, small but significant differences in proteolysis were observed in these samples as shown in Table 6-1. Although proteolysis was initially low, it increased significantly by day 28 which was a result of accumulation of breakdown products with time. These observations are consistent with previous findings where it was stated that although enzymatic activity was decreased by UHT heat treatment, it was not inactivated (Gillis *et al.*, 1985). The small but significant differences in each of the treatments may be due to inactivation of plasmin, plasminogen and plasminogen activators which are the main actors responsible for increased activity in the samples. It may thus be concluded that lower activities in the samples were a result of higher temperature (> 85^{0}C) used which caused inactivation of enzymes responsible for proteolysis.

Research has shown that during severe heat treatments such as UHT processing, 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 a study where directly and indirectly processed whole and skim UHT milks were observed for 11 weeks (Lopez-Fandino *et al.*, 1993 a). In the study, the researchers found that after 11 weeks, significant proteolysis occurred in skim

milk (>1.0 μM glycine/ mL milk) as compared to whole milk (0.70 μM glycine / mL milk) as analysed by the TNBS method.

Samples with similar treatments were compared per day of incubation as shown on Table 6-1. On day 0, no significant differences were observed in pH 4.6 soluble extracts of the milk samples indicating that although there was some proteolytic activity, probably occurring from udder; none of the samples had undergone any significant increase in proteolytic activity. On days 3 and 7 however, pH 4.6 soluble extracts from raw milk and of milk heated at 85^0C were significantly different ($p<0.05$) from each other and from the rest of the samples. Although a study by Andrews and Alichanidis (1983 b) concluded that proteolysis progressed faster in pasteurised milk compared to raw milk, it was not exactly the case in the samples studied here. Raw milk showed higher activities than milk heated at 85^0C in the current study. Reasons for the difference in results is not clear as lower temperature was used in the previous study (72^0C / 18 s), as compared to 85^0C / 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 resulted in increased proteolysis as compared to milk heated at 85^0C in the current study. 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 first reason is more likely as the milk was of good bacterial quality. The raw milk samples analysed here had 10^3 cfu / mL psychrotrophic count which was low. It

is therefore suggested that the source of higher activity in raw milk was most likely to be due to native enzymes.

However, these two batches (raw milk and milk heated at 85^{0}C) showed the highest levels of proteolysis among all treatments analysed on each sampling day. As explained previously, the reason for increased activity in raw milk is most likely due to activity of native enzymes, most probably plasmin.

The rest of the heated samples (at 110, 120, 130 and 142^{0}C) had very low activities on both days 3 and 7 signifying inactivation of plasmin and its inhibitors.

Table 6-1 shows that on day 14, as with previous observations, pH 4.6 soluble extracts of raw milk and of milk heated at 85^{0}C were significantly different ($p < 0.05$) in proteolysis from pH 4.6 soluble extracts of all other heated samples. Similarly, the rest of the heat processed samples had significantly low activities (110, 120, 130 and 142^{0}C). The same observation was made on day 28, except the sample processed at 110^{0}C was different from other high temperature heated samples (120, 130 and 142^{0}C). Again as previously observed and discussed, pH 4.6 soluble extracts from raw milk and of milk heated at 85^{0}C had the highest activity than any other samples.

The increase in proteolytic product concentration of all pH 4.6 soluble extracts from day 0 and day 28 was as follows: raw milk (11.5 times), processed at 85^{0}C (8.3 times), 100^{0}C (3.5 times), 120^{0}C (2.4 times), 130^{0}C (1.9 times) and 142^{0}C (1.9 times). It is obvious from the trend that increase in the rate of proteolysis was inversely related to the heat treatment implying that the lower the temperature employed, the higher the enzyme activity and the greater 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^{0}C have shown the highest proteolysis 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 native enzymes.

6.3.1.2 Effect of storage on 6% TCA soluble extracts

To examine proteolysis in raw milk and milk heated at various temperature-time combinations, soluble extracts of 6% TCA were analysed following incubation at 37^{0}C for 28 days.

Table 6-2 indicates that significant differences ($p<0.05$) were found in 6% TCA soluble extracts of raw milk and of milk heated at 85^{0}C on the various days of incubation with large increases in absorbance throughout storage. For 6% TCA soluble extracts of samples heated at 110, 120 and 130 and 142^{0}C, absorbance increased slightly significantly over the 28 day period. These results clearly indicate that both raw milk and milk heated at 85^{0}C had a higher proteolysis than the rest of the samples as observed in pH 4.6 soluble extracts. Due to increased proteolytic product concentration either as a result of native enzymes or bacterial contamination or both, proteolysis increased significantly with time.

proteolytic activities. These results were similar for both extracts especially between days 0-7, implying similar activities 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 been broken down during storage and leading to the formation of smaller peptides and amino acids which were soluble in TCA. As the TNBS protocol measures groups of free amino acids resulting from proteolysis (Fields, 1971), it may be concluded that both soluble extracts analysed by this protocol have shown similar trends in proteolytic activities. The strength of association between these two soluble extracts is signified by the strong correlation (R^2= 0.955) as in Figure 6-3 which confirms that TNBS is a useful method for assessing proteolysis in milk.

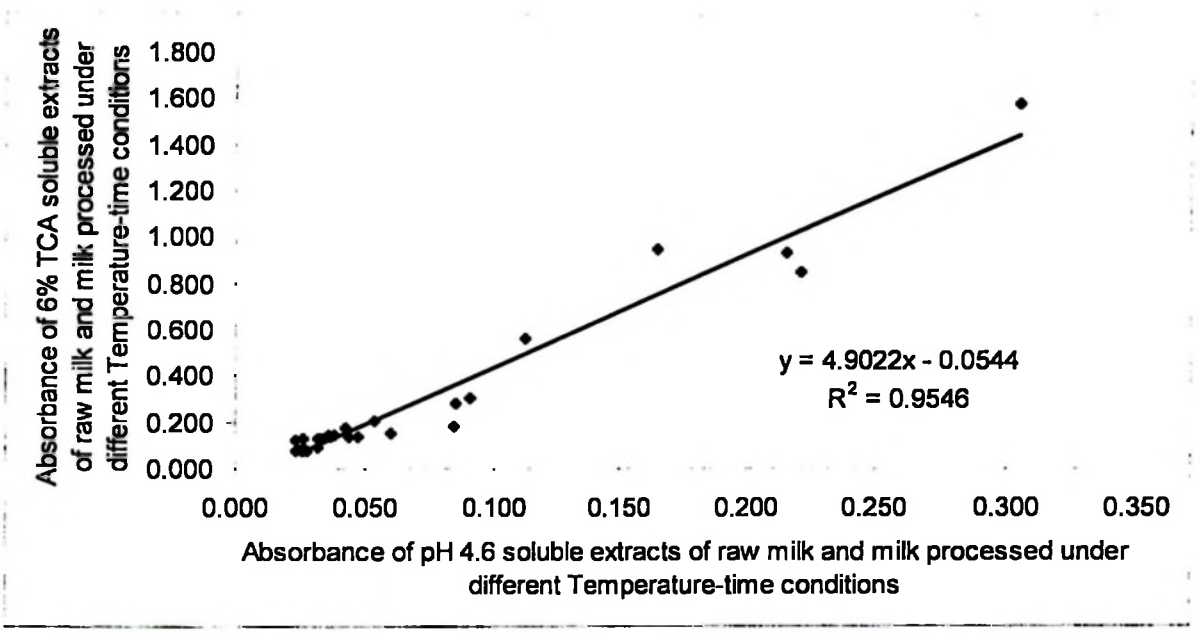

Figure 6-1 Correlation between pH 4.6 soluble extracts of milk and 6% TCA soluble extracts of raw milk and milk processed at various temperature-time conditions as analysed by the TNBS method at 420 nm

6.3.2 Proteolysis assessed by the fluorescamine method

6.3.2.1 Effect of storage on pH 4.6 soluble extracts

Fluorescamine reacts with free amino acids to form fluorescent derivatives which are measured by the spectrofluorimeter. The pH 4.6 and 6% TCA soluble extracts of raw and high temperature processed milk samples were analysed by this method after proteolysis and clarification as described in section 3.6.

Table 6-2 clearly shows that pH 4.6 soluble extracts of raw milk and that of milk processed at 85^{0}C had higher fluorescence readings than the rest of the samples for all the days of incubation. Although the pH 4.6 soluble extracts of raw milk, and of milk heated at 110^{0}C and 120^{0}C showed statistically significant differences ($p<0.05$) in fluorescence readings in all days of incubation, the three remaining heat treated milk samples did not differ significantly in fluorescence values in some days as shown in Figure 6-4. The Figure shows that samples processed at 120, 130 and 142^{0}C had more or less similar rates

of proteolysis which were lower than that of raw milk and of those processed at 85 and 110^{0}C. It was unexpected to reach the upper detection limit by day 28 in sample processed at 110^{0}C as relative fluorescence reading on day 14 was quite low.

Generally the same observation as before was made where raw milk and milk heated at 85^{0}C had the highest fluorescence readings throughout incubation. It also indicates that the rest of the samples heated at higher temperatures had lower readings. This implies that the two methods, TNBS and fluorescamine have shown similar trends of results. It has been documented that more 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, 1999a). Hence high heat treatment may cause decreased proteolytic activity due to inactivation of plasmin by thiol-disulphide interchange reaction (Kennedy and Kelly, 1997).

Table 6-2 indicates that on day 0, only pH 4.6 soluble extracts of samples heated at 85^{0}C and of those heated at 142^{0}C were significantly different ($p<0.05$) from each other whereas the rest were not statistically different from either of them. However, from day 3 to 28 high statistically significant differences in proteolysis ($p<0.05$) were observed between pH 4.6 soluble extracts of raw milk, of those processed at 85 and 110^{0}C with the rest of heat processed samples which were not statistically different from each other (Table 6-2). It may thus be concluded that as observed earlier (6.3.1.1), processing at higher temperature affects proteolysis positively by lowering its susceptibility to spoilage.

Table 6-2 Relative fluorescence of pH 4.6 and 6% TCA soluble extracts of raw milk and milk processed under various Temperature – time conditions and incubated at 37^{0}C for 28 days to examine the effect of proteolysis on storage time by the fluorescamine method at 475 nm

Incubation time (days)	Treatments	Relative fluorescence of pH 4.6 soluble extracts of UHT skim milk at 475 nm		Relative fluorescence of 6% TCA soluble extracts of UHT skim milk at 475 nm	
day 0	Raw milk	105± 2.0	ab A	48± 8.5	a A
	Heated at 85°C	114± 2.8	a F	34± 2.9	b F
	Heated at 110°C	106± 7.0	ab J	31± 2.1	b J
	Heated at 120°C	108± 5.4	ab O	31± 5.2	b N
	Heated at 130°C	105± 4.0	ab S	30± 5.5	b S
	Heated at 142°C	100 ± 4.4	b W	31 ± 3.6	b W
day 3	Raw milk	345± 44.0	c B	176± 8.6	c B
	Heated at 85°C	301± 9.8	d G	103± 6.0	d G
	Heated at 110°C	125± 5.0	e K	64± 4.0	e K
	Heated at 120°C	120± 3.1	f P	53± 3.5	f O
	Heated at 130°C	118± 1.4	f T	51± 2.1	f T
	Heated at 142°C	118± 2.8	f X	46± 5.3	f X
day 7	Raw milk	487± 45.5	g C	287± 57.4	g C
	Heated at 85°C	322± 32.1	h G	141± 44.6	h G
	Heated at 110°C	203± 4.5	i L	115± 21.5	ih L
	Heated at 120°C	138± 2.8	j Q	85± 12.7	ih P
	Heated at 130°C	132± 1.8	j U	65± 10.4	i T
	Heated at 142°C	121± 3.1	j XY	54± 10.7	i XY
day 14	Raw milk	600± 20.1	k D	418± 23.0	j D
	Heated at 85°C	563± 25.3	l H	334± 21.4	k H
	Heated at 110°C	214± 4.1	m M	127± 4.4	l L
	Heated at 120°C	143± 2.6	n Q	104±10.7	l Q
	Heated at 130°C	136± 2.2	n U	102± 7.4	l U
	Heated at 142°C	131± 3.8	n Y	67± 9.2	m Y
day 28	Raw milk	1100 *	o E	1100 *	n E
	Heated at 85°C	1100 *	o I	1100 *	n I
	Heated at 110°C	1100 *	o N	422± 9.1	o M
	Heated at 120°C	861± 6.0	p R	215± 4.5	p R
	Heated at 130°C	723± 4.9	p V	202± 11.2	q V
	Heated at 142°C	642± 10.7	p Z	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 samples ; 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.

6.3.2.2 Effect of storage on 6% TCA soluble extracts

An investigation was carried out to determine the effect of proteolysis on raw and heat processed milk samples during storage. Proteolysis was monitored in 6% TCA soluble extracts of these samples after clarification as described in section 3.6. Table 6-2 shows that 6% TCA soluble extracts of raw milk and of milk processed at 85^0C had highest level of proteolysis which was significantly different ($p<0.05$) from all other heat treatment samples. The same observation was also made on pH 4.6 soluble extracts (6.3.2.1).

The same table also shows significant differences in proteolysis between samples on the same day of incubation. From this table it may be concluded that with a few exceptions (days 0, 3 and 7), proteolysis in 6% TCA soluble extracts of raw milk and those of milk processed at 85^0C were statistically different ($p<0.05$) in proteolysis from each other and from the rest of the 6% TCA soluble extracts of milk processed at high temperatures on each day of incubation. Again, this confirms the earlier observation that heat treatment affects proteolysis negatively through reduced proteolytic activity upon processing at higher temperatures.

6.3.2.3 Comparison between pH 4.6 and 6% TCA soluble extracts

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 142^0C had higher fluorescence readings than its corresponding 6% TCA soluble extracts (Table 6-2). 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^{0}C (Table 6-2). The pH 4.6 soluble extracts of milk heated at 110^{0}C reached a detection limit on day 28 unlike its corresponding 6% TCA soluble extract which was less than half the value. This was probably due to an error during measurements.

Figure 6-2 shows correlation between the two extracts was low (R^2=0.706). This is attributed to large differences between these extracts as shown on Table 6-2. It may be concluded that correlation between the two extracts as detected by fluorescamine was much lower than that detected by the TNBS method. However, this does not mean that the method is less accurate than the TNBS method.

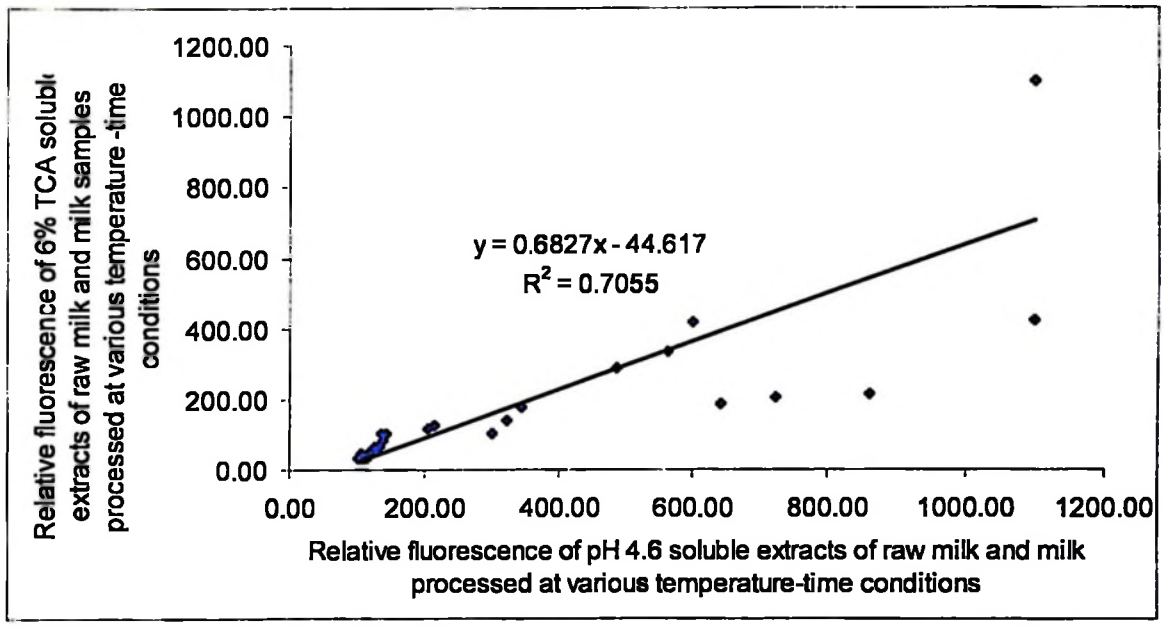

Figure 6-2 Correlation between pH 4.6 soluble extracts of milk and 6% TCA soluble extracts of raw milk and milk processed at various temperature-time conditions as analysed by the fluorescamine method

6.3.3 Proteolysis assessed 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^{0}C. The information highlighted the association of these treatments with the rate of protein breakdown which is useful for shelf life studies.

Figure 6-3 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 probably as a result of loading error, 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 b). A study whereby direct heating at 142^{0}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.

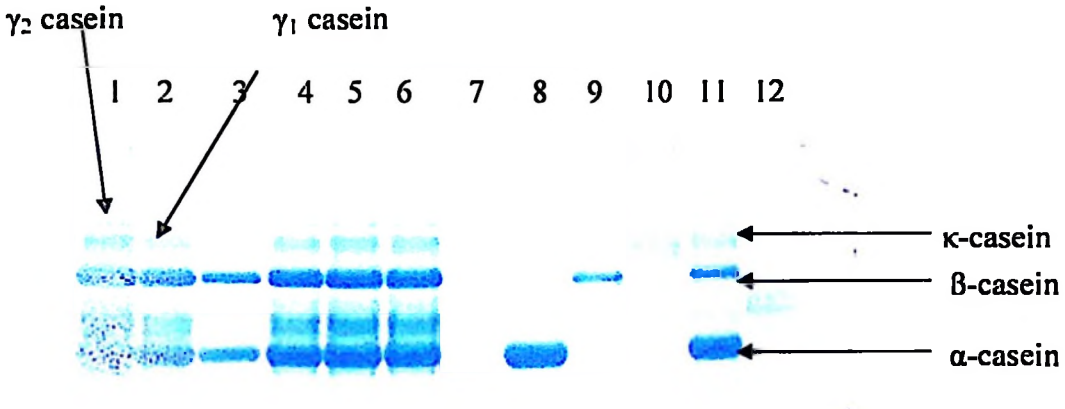

Figure 6-3 Urea–PAGE electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures on day 0. **Lane 1**: Raw milk control ; **Lane 2:** 110^{0}C for 2s; **Lane 3:** Milk processed at 85^{0}C for 15s; **Lane 4:** 120^{0}C for 2s; **Lane 5:** 130^{0}C for 2s **Lane 6:** 142^{0}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- casein. **Lane 12:** B-Lactoglobulin

Figure 6-4 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^{0}C suggest the role of native enzyme, probably plasmin whose activity on α and β-caseins results in the formation of γ-caseins as seen in Figure 6.4. It was stated by Andrews and Alichanidis (1983b) that the cleavage sites for plasmin in β-caseins were Lys_{28}-Lys_{29} whose

derivative peptide is γ_1-caseins (f29-209); Lys 105-His106 to yield γ_2-caseins (f106-209) and finally Lys107-Glu209 yielding γ_3-caseins (f108-209).

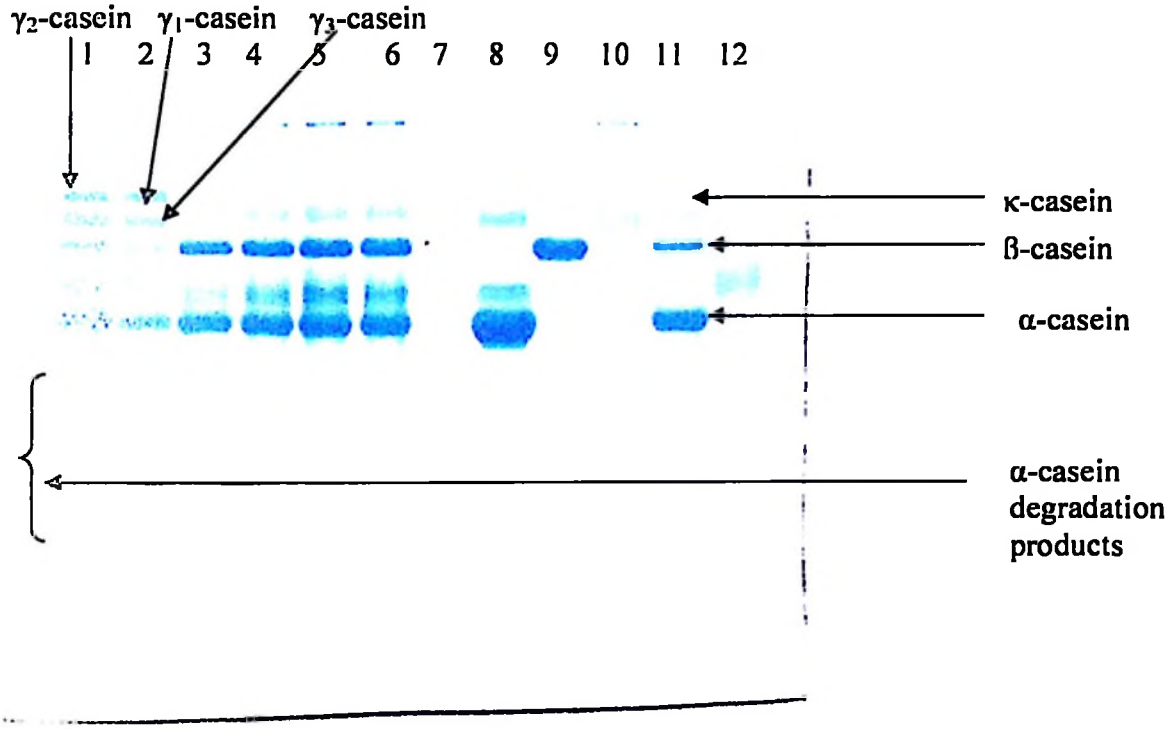

Figure 6-4 Urea–PAGE electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures and incubated at 37^{0}C for 3 days. **Lane 1:** Raw milk control ; **Lane 2:** 85^{0}C for 15s; Lane 3: Milk processed at 110^{0}C for 2s; Lane 4: 120^{0}C for 2s; **Lane 5:** 130^{0}C for 2s Lane 6: 142^{0}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- casein **Lane 12:**B-Lactoglobulin

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^{0}C which is in agreement with the finding by Andrews and Alichanidis (1983 b) who reported increased activity as being due to destruction of the plasmin and plasminogen activator inhibitors. These results however contradict the TNBS results where higher activities were found in raw milk as compared to milk heated at 85^{0}C. 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 activity in these samples was due to the higher temperatures used which inactivated plasmin and lowered proteolysis.

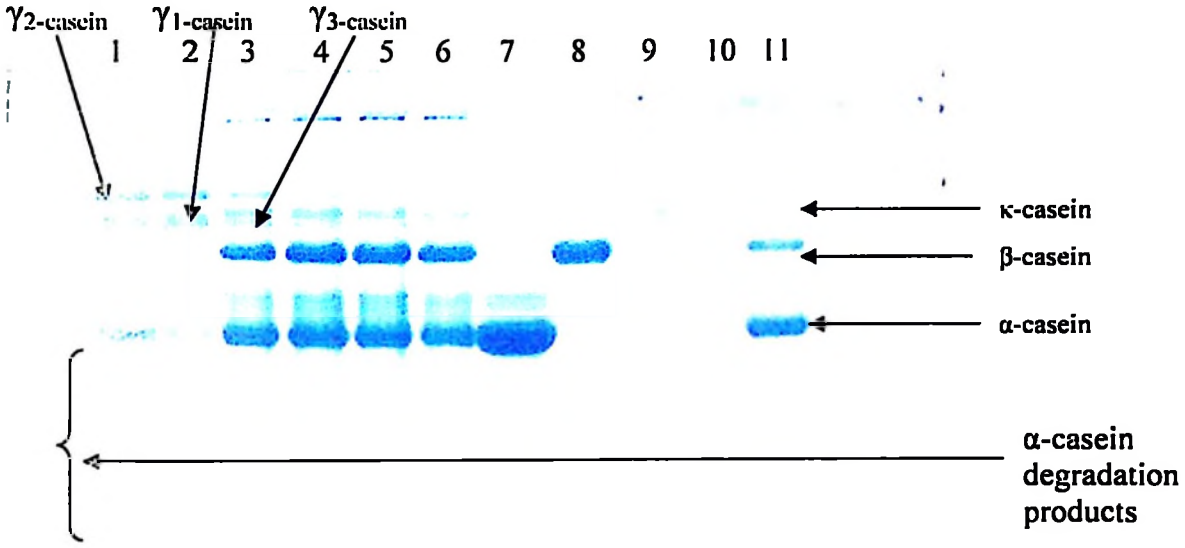

Figure 6-5 Urea–PAGE electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures and incubated at 37^{0}C for 7 days. **Lane 1:** Raw milk control; **Lane 2:** Milk processed at 85^{0}C for 15s; **Lane 3:** 110^{0}C for 2s; **Lane 4:** 120^{0}C for 2s; **Lane 5:** 130^{0}C for 2s; **Lane 6:** 142^{0}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- casein.

It is obvious from Figure 6-5 that after one week of incubation, there was progressive degradation of α and β-caseins in the order previously observed (milk processed at 85^{0}C > raw milk > milk processed at 110^{0}C > at 120^{0}C > at 130^{0}C > at 142^{0}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.

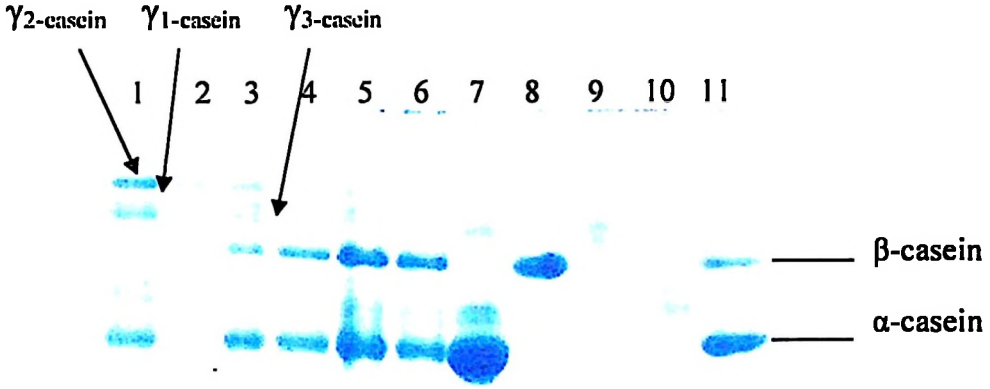

Figure 6-6 Urea–PAGE electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures and incubated at 37^{0}C for 14 days. **Lane 1**: Raw milk control ; **Lane 2:** Milk processed at 85^{0}C for 15s ; **Lane 3:** 110^{0}C for 2s; **Lane 4:** 120^{0}C for 2s; **Lane 5:** 130^{0}C for 2s **Lane 6:** 142^{0}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.

Similar trends of proteolysis as on day 7 were observed for samples incubated for 14 days as shown in Figure 6-6. In addition, the β-casein band in the raw milk sample had disappeared. Samples heated at higher temperatures (lanes 3-6) were more proteolysedas 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 6-6). Similar results were reported previously (Enright *et al.*, 1999; Kelly and Foley, 1997; Snoeren *et al.*, 1979).

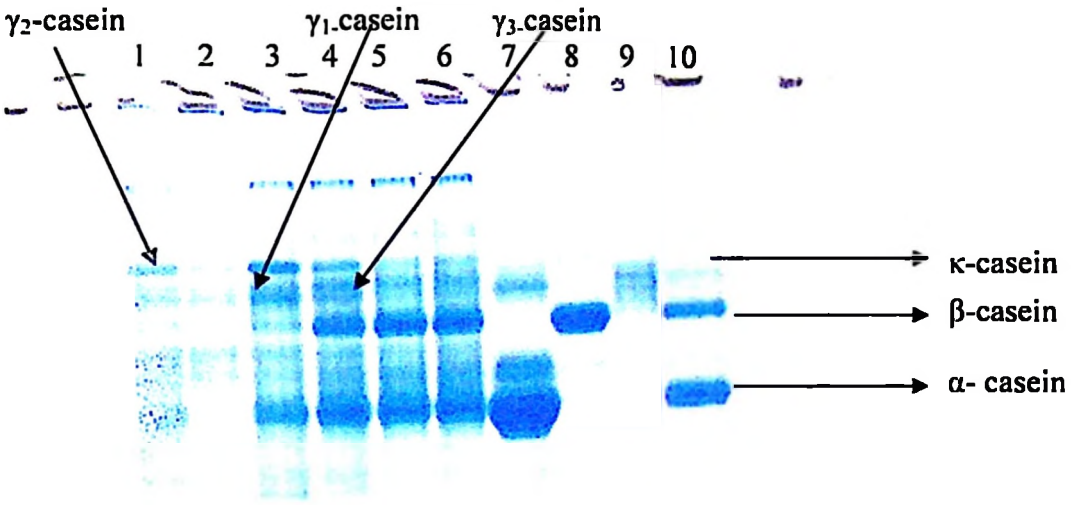

Figure 6-7 Urea–PAGE electrophoretograms (pH 8.9, T=12%, C=4%) of raw milk samples and milk processed at high temperatures and incubated at 37^{0}C for 28 days: **Lane 1:** Raw milk. **Lane 2:** Milk heated at 85^{0}C for 15s. **Lane 3:** Milk heated at 110^{0}C for 2s **Lane 4:** Milk heated at 120^{0}C for 2s. **Lane 5:** Milk heated at 130^{0}C for 2s. **Lane 6:** Milk heated at 142^{0}C for 2s **Lane 7:** α-casein. **Lane 8:** β-casein. **Lane 9:** κ -casein and **Lane 10:** α β κ –casein.

After 28 days of incubation, disappearance of β-casein was apparent from milk processed at 110^{0}C which was accompanied by the formation of γ-casein as shown in Figure 6-7. 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 ^{0}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).

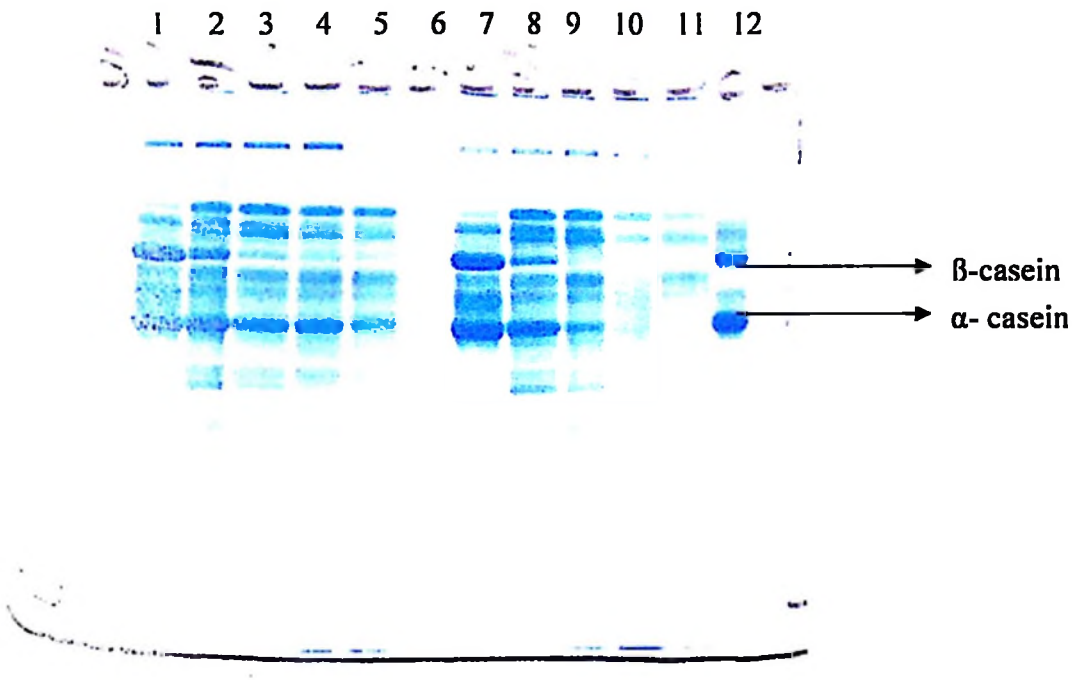

Figure 6-8 Urea-PAGE gel electrophoretograms (pH 8.9, T=12.% T, C=4%) of raw milk and milk heated at 85^{0}C/15s and incubated for 0-28 days: **Lanes 1-5:** Raw milk incubated for d 0,d 3, d 7, d 14 and d 28. **Lane 6**: Empty lane. **Lane 7-11**: : Milk heated at 85^{0}C for 2s and incubated on d 0,d 3, d 7, d 14 and d 28. **Lane 12:** α β κ –casein.

The final gel (Figure 6-8) compares the two most proteolytic samples i.e. raw milk and milk samples heated at 85^{0}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.

Conclusion from the electrophoresis study with regards to most proteolytic samples showed that milk heated at 85^{0}C for 15 s had higher proteolytic products than raw milk.

This contradicted results from TNBS and fluorescamine methods but supported by the RP-HPLC method (section 6.3.4). However, looking closely at these samples on Tables 6-1 and 6-2, their values are quite close especially Table 6-2 which recorded relative fluorescence values. Thus, although differences were observed, they could be very minor and not very significant.

6.3.4 Proteolysis assessed by RP-HPLC

6.3.4.1 Effect of storage time on pH 4.6 soluble extracts

The RP-HPLC method was used to monitor proteolysis of raw milk and of samples that had been heated at various temperature-time combinations to study its effect on storage time.

Results of pH 4.6 and 6% TCA soluble extracts from these samples are presented on Table 6-3. It is apparent that from day 0 to day 28 of incubation, pH 4.6 soluble extracts of milk heated at 85^{0}C had the highest peak area which was statistically different ($p < 0.05$) from all other samples followed by raw milk, then milk heated at 110^{0}C and finally the last three milk samples were more or less similar over the storage period.

The same table indicates that statistical differences in proteolysis ($p<0.05$) were observed for raw milk, sample heated at 85^{0}C and at 110^{0}C on each day of analysis. It implies that these samples were highly proteolytic such that significant levels of breakdown products were observed on each sampling day. The other samples did not show such a difference. Previous observations by the TNBS and fluorescamine methods also indicated that raw milk sample and sample heated at 85^{0}C had the highest proteolytic product concentrations whereas the rest of the samples had low concentrations. It is clear from the trend that the three samples (raw milk, milk heated at 85^{0}C and milk processed at

110^{0}C) exhibited higher extent of proteolysis. Proteolysis was highest in milk heated at 85^{0}C than raw milk which contradicts findings by the TNBS and fluorescamine but similar to gel electrophoresis.

Table 6-3 Peak areas of pH 4.6 and 6% TCA soluble extracts of raw milk and milk processed under various Temperature – time conditions and incubated at 37^{0}C for 28 days to examine the effect of proteolysis on storage time by the RP-HPLC

Incubation time (days)	Treatments	Total peak areas of pH 4.6 soluble extracts of milk samples (m AU*min)		Total peak areas of 6% TCA soluble extracts of milk samples (m AU*min)	
day 0	Raw milk	21.2 ±1.69	a A	32.1±2.00	a A
	Heated at 85°C	23.7±2.20	b F	33.9±1.25	b F
	Heated at 110°C	19.6±2.77	a K	29.4±1.02	c K
	Heated at 120°C	20.9±1.75	a P	29.2±1.25	c P
	Heated at 130°C	19.7±1.04	a T	27.9±1.75	c U
	Heated at 142°C	20.8±1.68	a X	25.9±1.57	d Y
day 3	Raw milk	48.4±4.77	c B	125 ±5.01	e B
	Heated at 85°C	50.5±8.18	c G	137±3.37	f G
	Heated at 110°C	36.2±2.30	d L	51.9±3.50	g I
	Heated at 120°C	23.6±0.92	e Q	36.2±3.26	h Q
	Heated at 130°C	23.2±2.36	e U	33.6±2.40	hi Y
	Heated at 142°C	21.0±0.51	e X	29.8±1.72	i Y
day 7	Raw milk	57.6±2.77	f C	134±3.89	j C
	Heated at 85°C	68.6±3.70	g H	161±6.83	k H
	Heated at 110°C	40.8±1.94	h M	60.7±3.43	l M
	Heated at 120°C	24.0±1.49	i G	47.2±2.97	m R
	Heated at 130°C	20.8±2.03	j UV	39.9±3.93	n W
	Heated at 142°C	21.6±0.52	ij X	40.8±7.77	n Z
day 14	Raw milk	70.3±7.21	k D	237±7.10	o D
	Heated at 85°C	252.9±12.12	l I	442±6.04	p I
	Heated at 110°C	44.3±4.07	m N	79.0±2.63	o N
	Heated at 120°C	26.5±2.48	n R	60.0±7.29	r S
	Heated at 130°C	23.2±1.13	n V	50.6±3.04	s X
	Heated at 142°C	21.3±1.07	n X	49.0±2.85	s Z
day 28	Raw milk	212±8.15	o E	330±9.61	t E
	Heated at 85°C	3159.43	p J	791 ±11.60	u J
	Heated at 110°C	60.4±1.89	oQ	90.9±7.93	v O
	Heated at 120°C	29.7±2.07	r S	53.3±3.92	w T
	Heated at 130°C	25.8±2.78	rs W	50.2±3.72	w Z
	Heated at 142°C	22.7±2.57	s X	49.8±2.85	w Z

Different lower case letters on the same column show significant differences ($p<0.05$) per day whereas different uppercase letters on the same column show significant differences ($p<0.05$) per sample;
The experiment was replicated 2 times (N=6); Error bars indicate standard deviations; The pH 4.6 soluble extracts were diluted (x20) whereas the 6% TCA soluble extracts were diluted (x2)

Higher proteolytic products concentration in pH 4.6 soluble extracts of raw milk were probably due to the presence of the native enzymes, whereas for samples heated at 85 and 110^{0}C, could be due to the destruction of plasmin and plasminogen activator inhibitors which increased plasmin activities in these samples. It is documented that plasminogen inhibitors are more heat labile than plasmin inhibitors (Richardson, 1983b). The destruction of the inhibitors results in increased plasmin activity, which in turn increases proteolytic activities in the samples.

Appendix 4 shows chromatograms of pH 4.6 soluble extracts of raw milk and milk processed at high temperatures which had been incubated at 37^{0}C for 7, 14 and 28 days. From each of these chromatograms, a high peak was observed at around 34 min for all samples, which was previously linked to activity by plasmin (chapter 5). The peak was highest in pH 4.6 soluble extracts from samples heated at 85^{0}C followed by those from 110^{0}C. This is due to destruction of plasmin inhibitors which increased plasmin activity on the caseins and hence increased proteolysis. The peak areas progressively decreased with increasing heat treatment applied as observed in the remaining pH 4.6 soluble extracts of samples heated at temperatures higher than 110^{0}C (appendix 4). The peaks also revealed that the highest rates of proteolysis occurred in the pH 4.6 soluble extracts of milk heated at 85^{0}C than any other sample. This is consistent with results from gel electrophoresis (Figures 6-3 to 6-8).

6.3.4.2 Effect of storage time on 6% TCA soluble extracts

The RP-HPLC method was also used to analyse 6% TCA soluble extracts of raw milk and milk samples heated at various temperature-time combinations following proteolysis and clarification by TCA as described in section 3.6.

The 6% TCA soluble extracts indicate that although there were significant differences in proteolysis on day 0, the peak areas were generally low as indicated on Table 6-3. On day3, statistical differences in proteolysis were found between the first three samples which had higher peak areas than samples processed at higher temperatures (120, 130 and 142^{0}C).

From days 7 to 14, pH 4.6 soluble extracts of raw milk samples and of samples heated at 85, 110 and 120^{0}C had higher peak areas which were statistically different from the rest of the samples. On day 28 however, only the first three samples were significantly different in proteolysis from each other and from the remaining samples which were not significantly different ($p>0.05$).

The first four milk samples in Table 6-3 show significant differences ($p<0.05$) in proteolysis at each day of incubation. Proteolysis in the last two samples was low and varied from day to day. The 6% TCA soluble extracts of milk heated at 85^{0}C had the highest activity than any other sample for all incubation days followed by 6% TCA soluble extracts of raw milk. This was also observed for pH 4.6 soluble extracts (Table 6-3) and contradicted previous findings by the TNBS and fluorescamine method. This will be discussed on 6.3.4.3.

From appendices 4 and 5, it is clear that the peak at 34 min which was apparent in all pH 4.6 soluble extracts was not so evident in these samples. This had been observed previously (Lopez-Fandino *et al.*, 1993 b; Datta and Deeth, 2003). The authors explained that unlike bacterial proteases which break down proteins into smaller peptides, plasmin forms large peptides which are precipitated by TCA and hence would not appear in 6% TCA soluble extracts, but in pH 4.6 soluble extracts. Higher peak areas were observed for

6% TCA soluble extracts of samples heated at 85^{0}C followed by raw milk sample. All the other samples (appendix 5) had quite low peak areas which progressively decreased with increasing heating temperature.

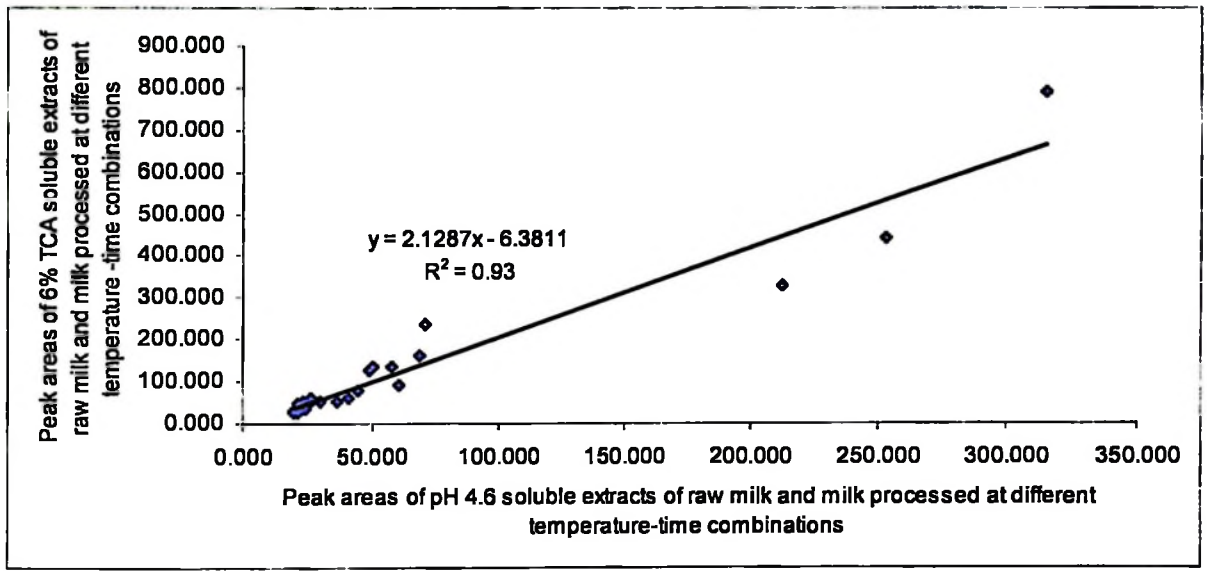

Figure 6-9 Correlation between pH 4.6 soluble extracts of milk and 6% TCA soluble extracts of raw milk and milk processed at various temperature-time conditions as analysed by the RP-HPLC method.

6.3.4.3 Comparison between pH 4.6 and 6% TCA soluble extracts

The pH 4.6 and 6% TCA soluble extracts were correlated to assess their association or relationship as shown below in Figure 6-9. The two extracts were strongly correlated (R^2=0.93) indicating that the RP-HPLC method is useful for the detection of proteolysis in milk.

The reason for the differences in results between the HPLC with TNBS and fluorescamine regarding higher proteolysis in milk heated at 85^{0}C than in raw milk is probably due to the difference in the measurement principles of the methods. Le *et al*, (2006) explained that the HPLC method measures the absorbance at a given wavelength

of separated peptides and amino acids, the response of which is based on mass and therefore the sensitivity for detection of larger peptides is greater than for small peptides. The fluorescamine method measures the free amino groups, thus the higher the amino groups the greater the fluorescence yield, irrespective of the amino acid composition (Beeby, 1980). The latter method is therefore more sensitive to small peptides than large peptides and proteins (Le *et al.*, 2006). Since the TNBS method also measures free amino groups, the same principle is involved and hence sensitivity for smaller peptides is higher than for larger ones.

6.4 Conclusion

This chapter focused on the effect of high temperature processed milk samples and their susceptibility to proteolysis during storage. Raw milk was a control. The rapid methods used to monitor proteolysis were also assessed. The experiments revealed that raw milk and milk heated at 85^{0}C for 15s had the highest proteolytic products concentration and thus it may be concluded that these two milk samples were the most susceptible to proteolysis during storage. Although higher proteolytic products concentration were from milk heated at 85^{0}C than from raw milk due to inactivation of plasmin and plasminogen inhibitors, the methods used gave contradictory results. The TNBS and fluorescamine methods showed that the raw milk sample was the most proteolytic, whereas analysis by gel electrophoresis and RP-HPLC indicated that milk samples heated at 85^{0}C had the highest concentration of proteolytic products. This was discussed as being due to the different sensitivities of the various methods used to measure peptides and amino acids released.

It was also revealed that samples heated at higher temperatures (>85^{0}C) had lower proteolytic products concentration than raw milk and of those heated at 85^{0}C due to destruction of the plasmin enzyme which resulted in low proteolysis. Thus, samples heated at above 85^{0}C were less susceptible to proteolysis during storage, than raw milk and those heated at 85^{0}C.

The pH 4.6 and 6% TCA soluble extracts were well-correlated in all methods (R^2>0.9), except for the fluorescamine method which was low (R^2=0.706). The gel electrophoresis was a useful qualitative procedure in indicating the degree of casein breakdown with time.

CHAPTER 7 THE ROLE OF IONIC CALCIUM IN GELATION AND PROTEOLYSIS

7.1 Introduction

Gelation in UHT milk is one of the most common problems encountered in the dairy industry (Datta and Deeth, 2001). A gel is a three dimensional protein matrix formed during heating by the β-lactoglobulin interacting with κ-casein to form β-lactoglobulin – κ-casein (β-κ) complexes. Although the main cause of gelation is unknown, native enzymes, particularly plasmin and bacterial proteases have been implicated (Fox, 1981a). Proteases cleave the peptide bond which anchors κ-casein to the casein micelle, facilitating the release of β-κ complexes from the micelle. This is considered as a first step of age gelation. The second step involves aggregation of β-κ complexes and formation of a three-dimensional network of cross-linked proteins (Datta and Deeth, 2001). When a critical concentration of cross linked β-κ complex is reached, a gel is produced. It is an irreversible phenomenon which occurs when viscosity increases to more than 10 mPa.s at 20^0C (Datta and Deeth, 2001).

Calcium in milk is partitioned between the serum (30%) and colloidal phase of milk (70%). 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). 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 CCP. It is essential for gelation of milk by rennet during cheese manufacture (Tsioulpas, 2005). It was stated that a minimal amount of ionic calcium is needed to initiate renneting (Hyslop, 2003).

Minimum concentration of ionic calcium is necessary for aggregation of casein micelles (Van Hooydonk *et al.*, 1986). A study revealed that removal of calcium from milk using a cation exchange resin, caused prolonged coagulation and produced a weaker gel (Sharma and Sindhu, 2001). This was accompanied by ionic calcium reduction. In the same context, it is speculated that the level of ionic calcium may have a role in gelation of UHT milk during storage. However, the amount of enzyme required and the degree of proteolysis necessary to obtain gelation have not been determined (Kohlmann *et al.*, 1991a). To examine the role of ionic calcium in gelation and proteolysis, it was reduced in pasteurised and UHT 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 and chymosin. The effect of ionic calcium reduction by *Pseudomonas fluorescens* 416 was not performed because it would cause contamination of the equipment. However, time of gelation to occur in the samples was observed. The information will highlight the relationship between the presence of enzymes and the role of ionic calcium in proteolysis and gelation.

7.2 Materials and methods

In various experiments, sequestering agents 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) were used to reduce ionic Ca in pasteurised and UHT milk. Samples were then mixed on magnetic stirrers for 30 min after which they were injected with trypsin (248 BAEE units) and chymosin (0.03%) followed storage at 25 and 4^0C. Samples were analysed by ionic Ca analyser before and after incubation. Gelation and proteolysis were observed as described below.

7.2.1 The effect of ionic calcium reduction on gelation

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 observed daily. Gelation was indicated by the high resistance of milk to flow when poured out from the container.

7.2.2 The effect of ionic calcium reduction on proteolysis

Samples mentioned above were incubated at 37^0C for 2h to induce proteolysis followed by clarification to obtain 6% TCA and pH 4.6 soluble extracts as described in section 3.6.

7.3 Results and discussion

7.3.1 Effect on UHT milk

7.3.1.1 The effect of ionic calcium reduction in UHT milk at 25^0C

This study was aimed at examining the role of sequestering agents in ionic calcium reduction and their effect on gelation. Both SHMP (8.17, 24.51 and 32.68 mM) and TSC (17 and 51 mM) were used to observe the lowest level of ionic calcium required to cause gelation in milk. A calibration curve was constructed daily by using Calcium standards as described in section 3.11.2. The concentration of free ionic calcium in milk samples was obtained from the equation of the calibration curve. A few examples of a calibration curves are presented in appendix 6.

Table 7-1 The effect of ionic calcium reduction by TSC and SHMP at 25^{0}C

Sequestering agent	Addition (mM)	Free ionic Ca
SHMP	0.00	1.383
	8.17	0.319
	24.51	0.233
	32.68	0.115
TSC	0.00	1.382
	17.00	0.640
	51.00	0.430
	68.00	0.314

Preliminary results are presented on Table 7.1. There was a significant reduction of calcium by 76.9% and 53.7% respectively by 8.17 mM SHMP and 17 mM TSC respectively. The levels of sequestering agents were reduced further to observe changes (if any) when lowest level of calcium was reached.

Table 7-2 The effect of added TSC and SHMP on ionic calcium reduction in UHT milk stored at 25^{0}C

Sequestering agent	Molarity (mM)	Free ionic Ca (mM) *trial 1*	Free ionic Ca (mM) *trial 2*
TSC	0.00	1.377	1.338
	3.40	0.727	0.963
	6.80	0.549	0.574
	10.20	0.472	0.425
	13.60	0.388	0.366
	17.00	ND	0.307
SHMP	0.00	1.377	1.338
	1.63	0.636	0.779
	3.27	0.431	0.317
	4.90	0.318	0.158
	6.54	0.189	0.142
	8.17	ND	0.110

ND Not determined

Since the reduction in ionic calcium from trial 1 (Table 7-2) was 44.4% by 3.40 mM TSC and 53.8% by 1.63 mM SHMP, an attempt was made to use lower concentrations of

sequestering agents so as to establish the lowest ionic calcium reduction that will promote gelation.

Table 7-3 The effect of added TSC and SHMP on ionic calcium reduction in UHT milk at 25^{0}C

Sequestering agent	Addition(mM))	Free ionic Ca (mM)
TSC	0.00	1.336
	0.68	1.198
	1.36	1.104
	1.70	0.933
SHMP	0.00	1.336
	0.33	1.165
	0.65	1.074
	0.82	0.956

Based on the results presented in Table 7-3, 1.36 mM TSC and 0.65 mM SHMP were selected as the lowest level of sequestering agents to be used as they caused 17.36 and 19.6% reduction of ionic calcium respectively. The effect of sequestering agents on gelation was thus studied at levels shown on Table 7-4 and 7-5. The Table below (7-4) shows some preliminary results from samples with added sequestering agents.

Table 7-4 The effect of ionic calcium reduction by TSC and SHMP stored at 25^{0}C

Samples	Free ionic Ca (mM)
UHT (No sequesters)	1.309
UHT with 1.36 mM TSC	1.196
UHT with 2.72 mM TSC	1.127
UHT with 0.65 mM SHMP	1.083
UHT with 1.31 mM SHMP	0.904
Trypsin (No sequesters)	1.704
UHT milk with trypsin and 1.36 mM TSC	1.526
UHT milk with trypsin and 2.72 mM TSC	1.416
UHT milk with trypsin and 0.65 mM SHMP	1.520
UHT milk with trypsin and 1.31 mM SHMP	1.319

Table 7-4 indicates that 1.36 mM and 2.72 mM TSC reduced ionic calcium by 8.6 and 13.9%, respectively, from control UHT milk (without trypsin). However, at the same level of TSC in trypsin, the ionic calcium was reduction was by 10.4 and 17.0%, respectively. Addition of 0.65 mM and 1.01 mM SHMP, resulted in ionic calcium reduction by 17 and 31% from the control UHT 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. 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. TSC was less effective in lowering the level of ionic calcium as compared to SHMP, which caused higher reduction in ionic calcium. Similar results were also observed from the work of Tsioulpas (2005) where it was stated that SHMP had a strong chelating effect. In one study it was revealed that TSC treated samples gelled more rapidly than either controls or samples with added SHMP (Kocak and Zadow, 1985a). 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.

7.3.1.2 The effect of pH reduction on gelation of UHT milk at 25^{0}C

The experiment was conducted to monitor pH changes during storage in relation to ionic calcium reduction by SHMP and TSC. This should give a clear understanding of the effect of pH changes in relation to ionic calcium reduction, gelation and proteolysis.

Figure 7-1 shows that the pH decreased steadily with incubation time. This was also reported previously (Kocak and Zadow, 1985a). 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. The increase was highest in samples with increasing concentration of TSC (Figure 7-1). However, pH decreased with time.

It has been previously reported that TSC increases the pH of samples (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 below:

$$3Ca^{2+} + 2HPO_4^{2-} \leftrightarrow Ca_3(PO_4)_2 + 2H^+$$ (Horne, 2003)

The same mechanism was described during acidification. 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 and Clarke, 1990).

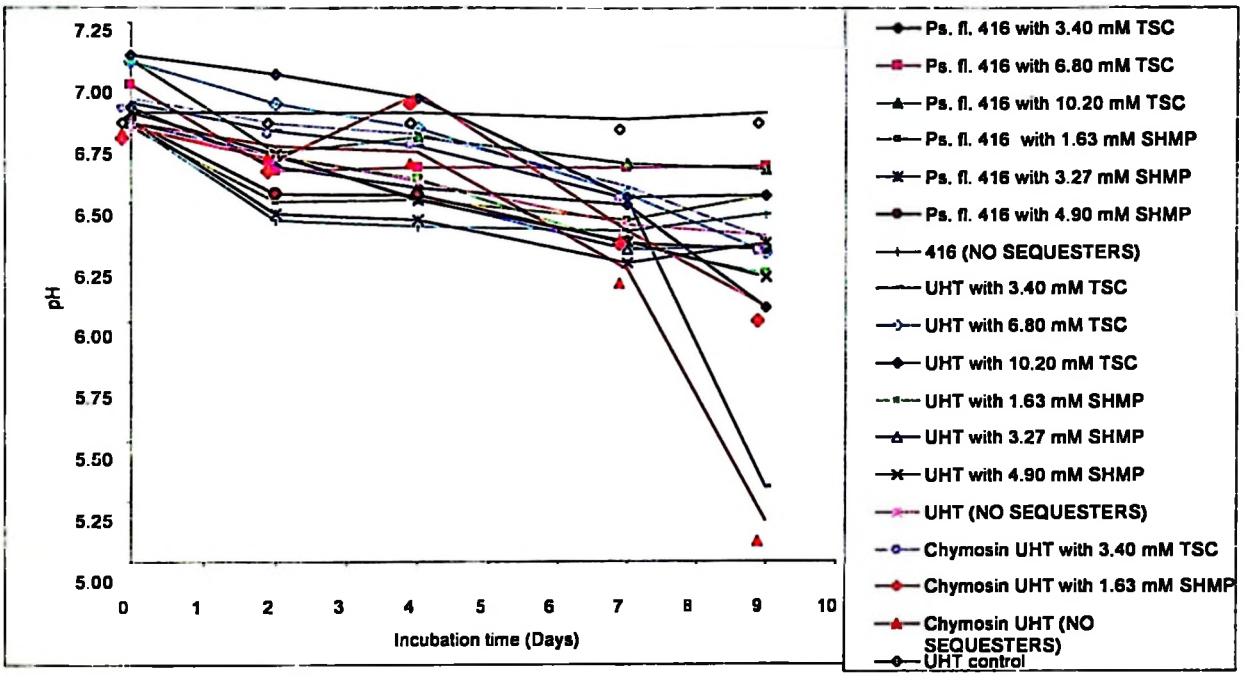

Figure 7-1 pH of UHT milk samples incubated with sequestering agents and *Pseudomonas fluorescens 416* at 25^{0}C for 9 days

7.3.1.3 The effect of ionic calcium reduction in UHT milk on gelation at 25^{0}C

To examine the effect of ionic calcium reduction by SHMP and TSC on gelation, inoculated and non-inoculated UHT milks were used to observe changes during storage at 25^{0}C. Chymosin and *Pseudomonas fluorescens* enzymes were used so as to accelerate gelation and determine the lowest level of ionic calcium responsible for gelation.

Table 7-5 shows the gelation times of various UHT treated samples at 25^{0}C. It was interesting to note that only one sample inoculated with *Pseudomonas fluorescens* 416 (no sequesters) gelled, but none of the other samples inoculated with *Pseudomonas fluorescens* 416 with added sequestering agents gelled. The most plausible explanation is the high reduction of ionic calcium which retarded gelation. Unfortunately, it was not

possible to check the ionic calcium reduction from these samples as the process would contaminate the equipment. Longer incubation times for gelation studies would be more appropriate.

The chymosin treated sample (without sequesters) gelled on the same day of incubation. This confirms the significance of the level of ionic calcium causing gelation during cheese manufacture. The two remaining chymosin samples also gelled on days 4 and 5 having 3.40 mM TSC and 1.63 mM SHMP, respectively.

Table 7-5 The effect of ionic calcium reduction in UHT milk on gelation for samples stored at 25^0C

SAMPLE	GELATION DAY
Ps. fl. 416 with 3.40 mM TSC	NG
Ps. fl. 416 with 6.80 mM TSC	NG
Ps. fl. 416 with 10.20 mM TSC	NG
Ps. fl. 416 with 1.63 mM SHMP	NG
Ps. fl. 416 with 3.27 mM SHMP	NG
Ps. fl. 416 with 4.90 mM SHMP	NG
Ps. fl. 416 (NO SEQUESTERS)	d 2
UHT with 3.40 mM TSC	d 5
UHT with 6.80 mM TSC	d 9
UHT with 10.20 mM TSC	d 9
UHT with 1.63 mM SHMP	d 9
UHT with 3.27 mM SHMP	d 9
UHT with 4.90 mM SHMP	d 12
UHT (NO SEQUESTERS)	d 5
Chymosin UHT with 3.40 mM TSC	d 4
Chymosin UHT with 1.63 mM SHMP	d 5
Chymosin UHT (NO SEQUESTERS)	d 0

NG- no gelation observed

Both UHT (without sequesters) and UHT (with added 3.40 mM TSC) gelled on the 5^{th} day of incubation. This observation may be explained by the fact that, as observed earlier (Tables 7-2 and 7-3), ionic calcium reduction by TSC was lower than that of SHMP, hence the tendency of the samples with added TSC to gel earlier than the latter.

It was surprising to observe that, UHT samples with added TSC (6.80 mM and 10.20 mM) and added SHMP (1.63 mM and 3.27 mM) gelled on the same day (day 9). However, UHT milk with 4.90 mM SHMP gelled on day 12. It can be concluded that ionic calcium in the samples that gelled on day 9 equally influenced gelation, implying no clear relationship between the levels of sequestering agents used with onset of gelation. Some researchers did not find any correlation between ionic calcium with onset of gelation (Kocak and Zadow, 1985a). The sample that gelled on day 12 however was from the highest SHMP used. As SHMP delayed gelation, it is in accordance with previous findings (Datta and Deeth, 2001).

7.3.1.4 Effect of proteolysis of UHT milk at 25^{0}C

To investigate the link between ionic calcium reduction and proteolysis, inoculated and non-inoculated UHT milk samples in which calcium had been reduced, were analysed by RP-HPLC after clarification to obtain pH 4.6 soluble extracts. 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.

Figure 7-2 shows that the degree of proteolysis increased with time for all samples except the control sample which was constant throughout the incubation time. Proteolysis in latter samples was low (less than 140 mAU*min), possibly from activities of native enzymes in milk. There was no clear relationship between the effect of proteolysis and the type or quantity of sequestering agent added in non-inoculated samples (Figure 7-2). As both sequesters showed increased proteolysis with time, this suggests that the sequestering agents do not have influence on proteolysis.

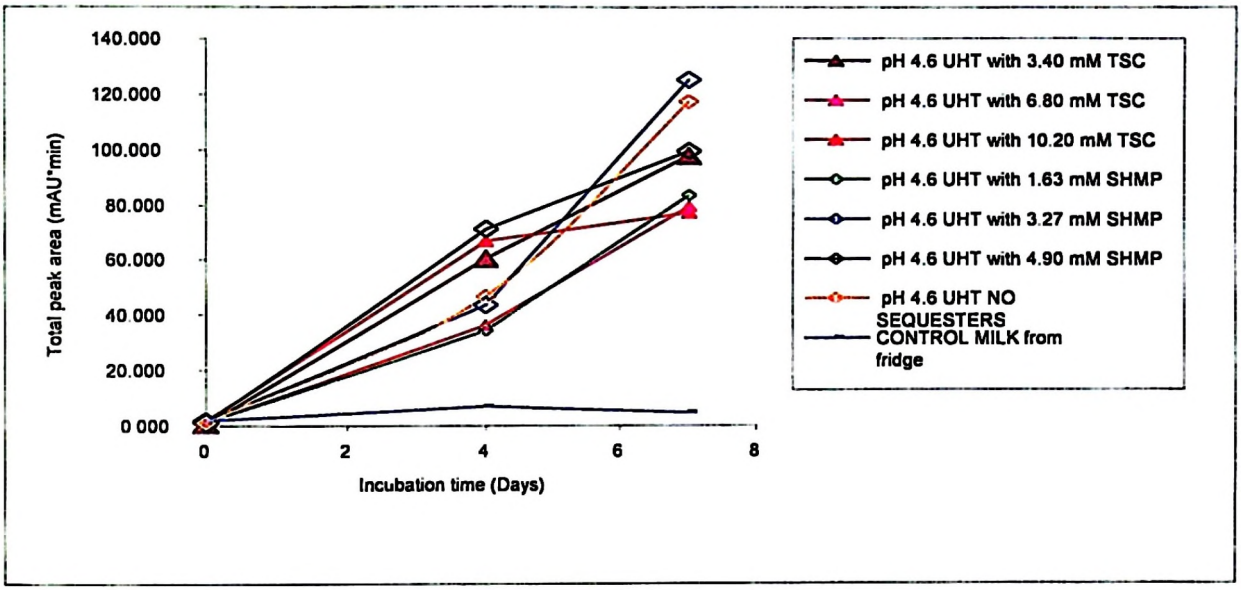

Figure 7-2 Proteolysis of pH 4.6 soluble extracts of UHT skim milk with added sequestering agents analysed by the RP-HPLC method

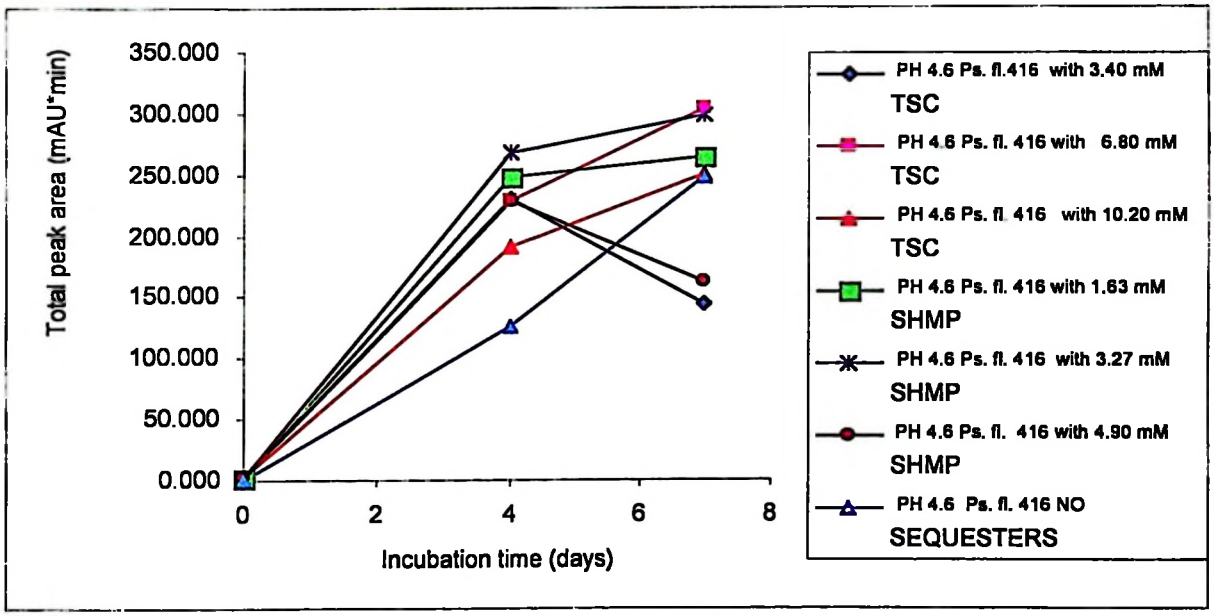

Figure 7-3 Proteolysis of pH 4.6 soluble extracts of UHT skim milk inoculated with *Pseudomonas fluorescens* 416 (with sequestering agents) analysed by the RP-HPLC method

It is obvious that proteolysis was higher in samples inoculated with *Pseudomonas fluorescens* 416 (with or without sequesters) than non-inoculated samples (with or

without sequesters) as shown on Figures 7-2 and 7-3. Although proteolysis generally increased with time in Figure 7-3, two samples were exception. In these samples (inoculated UHT with added 3.40 mM TSC and 4.90 mM SHMP), proteolysis decreased after day 4 of incubation. This trend is unclear but it may be due to complexing of SHMP or TSC with the breakdown products formed during proteolysis. This needs to be further investigated.

Pseudomonas fluorescens 416 without sequesters gelled on day 2 (Table 7-5), indicating the role of the bacterial proteases to gelation. In the same table however, inoculated samples with added sequestering agents did not gel. 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 (Law *et al.*, 1977b). It was also revealed that proteases from psychrotrophic bacteria are capable of causing gelation of UHT milk (Cogan, 1977). Therefore, it is very likely that the enzymes from the *Pseudomonas fluorescens* 416 initiated proteolysis causing gelation.

Although inoculated samples had higher rates of proteolysis than non-inoculated, 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 7-3 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 . 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.*, 1991c), indicating the significance of minor proteolysis by plasmin prior to gelation. It was also revealed that neither gelation nor proteolysis (9 months at 20^0C) 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 20-25^0C in the current study would be more useful for long term study of the effect of storage on proteolysis and gelation. As no correlation was found between proteolysis and the effect of sequestering agents, it implies that proteolytic activity is not influenced by ionic calcium concentration.

7.3.2 Effect on pasteurised milk

7.3.2.1 Calcium reduction in pasteurised milk

7.3.2.1.1 Calcium reduction in pasteurised milk during storage at 5^0C

Pasteurised milk was used to study the effect of ionic calcium reduction by TSC and SHMP. Trypsin was added to observe its influence on ionic calcium reduction and to relate it to proteolysis and gelation. The effect of storage was monitored at 5 and 25^0C.

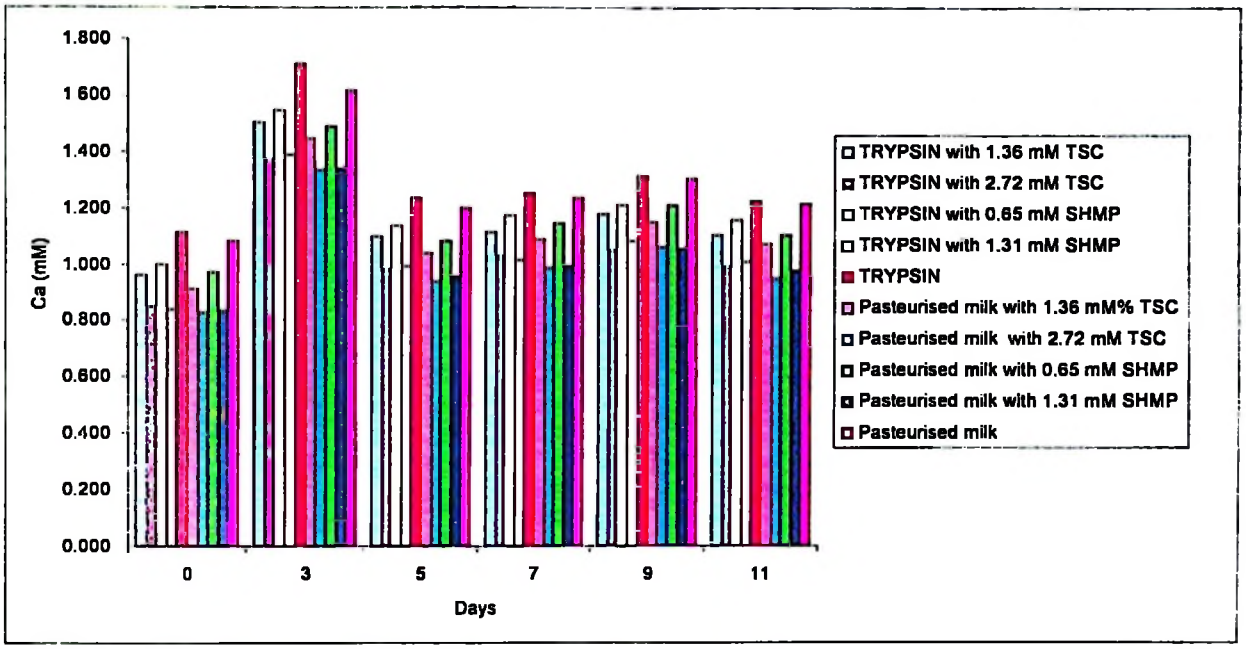

Figure 7-4 Ionic calcium levels of pasteurised milk samples incubated with sequestering agents and trypsin at 5^0C for 11days

Figure 7-4 shows the ionic calcium levels in pasteurised milk treated with two levels of TCS and SHMP. Ionic calcium reduction in pasteurised milk by 1.36 and 2.72 mM TSC was 10.2% and 22.7% whereas it was 15.6% and 23.2% by 0.65 and 1.31 mM SHMP respectively. Confirming earlier observations, SHMP reduced more ionic calcium than TSC. Although the ionic calcium was more or less constant with incubation time for all samples, it was highest on day 3. The reason for this behaviour is unknown but may be due to errors on the day of observation probably related to the equipment malfunctioning on that day. The control samples of trypsin (without sequesters) and pasteurised milk (without sequesters) had higher levels of ionic calcium than the rest of the samples, confirming the role of trypsin in casein micelle destabilisation which increased ionic calcium in the soluble phase as described earlier.

7.3.2.1.2 Calcium reduction in pasteurised milk during storage at 25^{0}C

The effect of ionic calcium reduction during storage at 25^{0}C is presented in Figure 7-5. Again control pasteurised milk (without sequesters) had the highest level of ionic calcium. The same figure shows that calcium levels were higher on day 1 and 2 than on day 0, implying that equilibrium of the reaction was reached after 1 day of storage. Although it has been established that ionic calcium is reduced with heat treatments, it is usually restored during storage although to slightly lower levels than found in raw milk.

In support of earlier observation (A.3.2.1.1), ionic calcium reduction by SHMP was higher than TSC. The levels of ionic calcium reduced progressively for each sequestering agent (Figure 7-5). Samples with higher ionic calcium were expected to gel earlier than the rest based on the assumption that ionic calcium is crucial for gelation. This will be discussed later in this chapter.

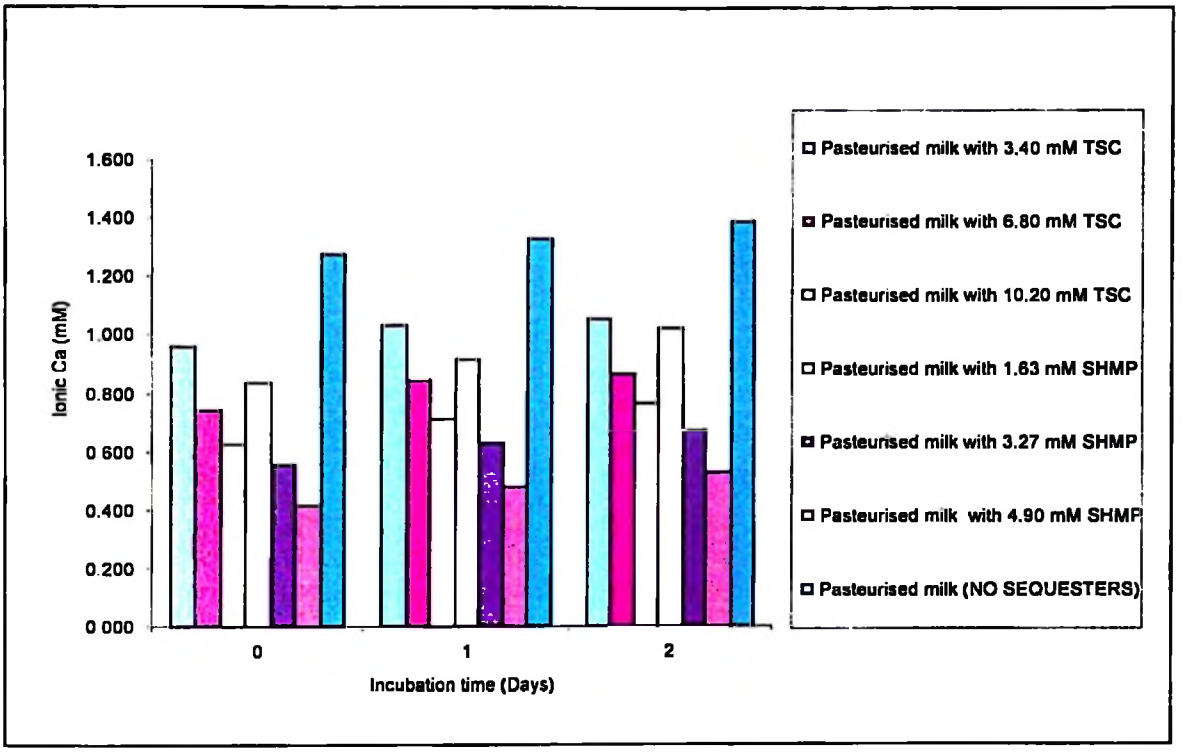

Figure 7-5 Ionic calcium levels of pasteurised milk samples incubated with sequestering agents at 25^0C for 2 days.

7.3.2.2 pH changes in pasteurised milk

7.3.2.2.1 pH change in pasteurised milk during storage at 5^0C

To examine the effect of ionic calcium reduction by SHMP and TSC, changes in pH were monitored so as to establish a relationship between the effect of sequestering agents with pH changes during storage. The effects of trypsin and *Pseudomonas fluorescens* 416 in the samples will also be studied with regard to their influence on pH.

The changes in pH during storage of pasteurised milk at 5^0C are shown in Figure 7-6. The pH was constant for the first three days after which it started to increase. However, on day 5 it decreased rapidly to the lowest level on day 7. It increased again on steadily until day 11.

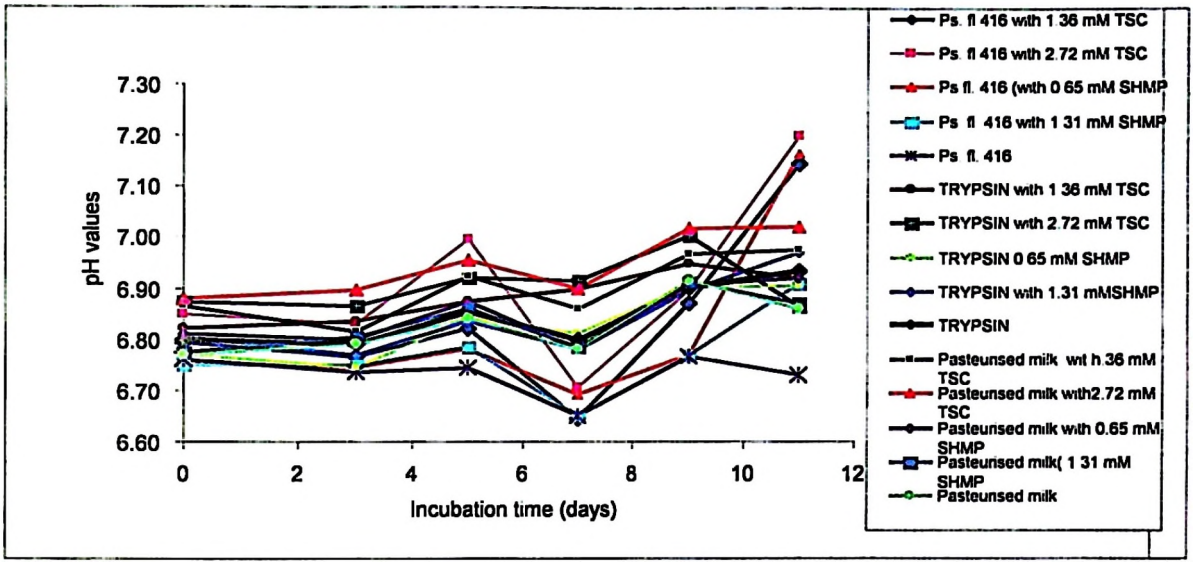

Figure 7-6 pH of pasteurised milk samples incubated with sequestering agents and trypsin at 5^0C for 11 days

From these results, it is obvious that although the pH was falling, the lowest value was above 6.6 which suggests that the milk was still of good quality.

Although pH fluctuated from day to day in some cases, however samples treated with TSC had higher pH than SHMP (Figure 7-6). Some inoculated samples have shown to raise pH with time. However, since this was not consistent for all samples studied, no conclusion can be made in this respect. Samples with added trypsin had constant pH values.

Other authors reported a decrease in pH throughout storage of UHT milk at 20^0C (Kocak and Zadow, 1985a). These differences could be due to the fact that higher levels of sequestering agents (0.1 – 0.5%) and storage temperatures were used in their experiment as compared to the current experiment where (0.04 – 0.08%) sequestering agents were used in pasteurised milk stored at 5^0C. In general addition of sequestering agents such as TSC decrease ionic calcium and raised pH (Tsioulpas, 2005).

7.3.2.2.2 pH changes in pasteurised milk during storage at 25^{0}C

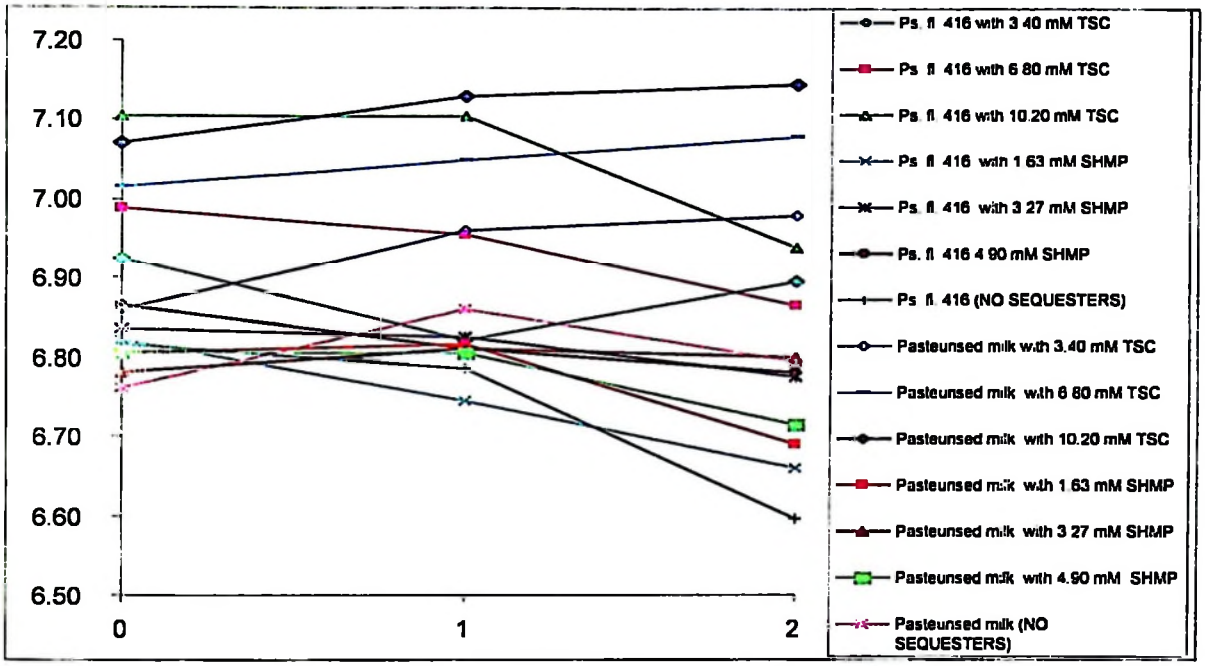

Figure 7-7 pH of pasteurised milk samples incubated with and without *Pseudomonas fluorescens* 416 and sequestering agents incubated at 25^{0}C for 2 days

From Figure 7-7, it is clear that TSC raised the pH of some samples on day 0 where the lowest pH was from a control pasteurised milk sample (without sequesters). The pH was found to be highest at 10.20 mM TSC (very close to pH 7). On day 2 of incubation, non inoculated pasteurised milk samples with added TSC (3.4 - 10.20 mM) showed highest pH values whereas the same samples with added SHMP (1.63 – 4.90 mM)) had low values. A study by Tsioulpas (2005) confirmed that an inverse linear relationship existed between ionic calcium and pH where a decrease in ionic calcium caused an increase in pH. This relationship was explained by the following equation:

$$Ca^{2+} + H_2PO_4 \leftrightarrow CaHPO_4 + H^+ \qquad \text{(Horne, 2003)}$$

For inoculated samples however, no clear relationship could be established as pH reduction was observed for some samples but increased in others. It is possible that

microbial growth and production of metabolites contributed to the pH fluctuations during incubation.

7.3.2.3 The effect of gelation in pasteurised milk during storage

7.3.2.3.1 Effect of gelation in pasteurised milk during storage at 5^0C

To investigate the relationship between ionic calcium reduction by TSC and SHMP with gelation an experiment was designed in which inoculated and non-inoculated samples were stored at 5 and 25^0C. Signs of gelation and pH were examined daily.

Samples inoculated with *Pseudomonas fluorescens* 416 (control, without sequesters) showed first clots on day 7 and actual gelation on the 8^{th} day. Inoculated sample with 1.36 mM TSC gelled on day 9 whereas 0.65 mM SHMP inoculated with *Pseudomonas fluorescens* 416 gelled on day 11. None of the other samples gelled.

The trend for gelation described above supports the suggestion that ionic calcium is responsible for gelation as control samples with higher ionic calcium gelled before the other two samples with added TSC and SHMP. To further support this fact, as ionic calcium reduction by TSC was less than SHMP (7.3.1.4), gelation occurred earlier in the former sample than in the latter.

It was also observed that although samples containing *Pseudomonas fluorescens* 416 gelled, control pasteurised milk samples did not. It may be argued that probably the presence of this bacterium (*Pseudomonas fluorescens 416*) at such high levels (10^6cfu/ mL) initiated proteolysis which led to gelation. Kocak and Zadow (1985a) described age-gelation as a two stage process, which began with proteolysis, followed by storage-induced physico-chemical changes which affect the aggregation of destabilised casein micelles. Proteolysis by plasmin and bacterial proteases have been implicated as the

cause of age-gelation in milk (Fox, 1981a). These results are also in agreement with other researchers who explained that with a high initial psychrophic count in raw milk such as 2.06 x 10^6 cfu/ mL, gelation would not be delayed in UHT milk (Snoeren *et al.*, 1979). None of the samples containing trypsin gelled. It is possible that the incubation time was not long enough to reach conclusive remarks with regards to the current experiment. However, it is thus suggested that longer incubation time be considered in the future so as to confirm the findings about the role of trypsin with or without sequestering agents to gelation.

7.3.2.3.2 Effect of gelation in pasteurised milk during storage at 25^0C

Table 7-6 The effect of ionic calcium reduction in pasteurised milk on gelation for samples stored at 25^0C

SAMPLE	GELATION DAY
Ps. fl. 416 with 3.40 mM TSC	d 2
Ps. fl. 416 with 6.80 mM TSC	d 2
Ps. fl. 416 with 10.20 mM TSC	d 2
Ps. fl. 416 with 1.63 mM SHMP	d 2
Ps. fl. 416 with 3.27 mM SHMP	d 3
Ps. fl. 416 with 4.90mM SHMP	d 3
Ps. fl. 416 (NO SEQUESTERS)	d 1
Pasteurised milk with 3.40 mM TSC	d 3
Pasteurised milk 6.80 mM TSC	d 4
Pasteurised milk 10.20 mM TSC	d 5
Pasteurised milk 1.63 mM SHMP	d 4
Pasteurised milk with 3.27 mM SHMP	d 5
Pasteurised milk 4.90 mM SHMP	NG
Pasteurised milk (NO SEQUESTERS)	d 3

NG – No gel

Pasteurised milk inoculated with *Pseudomonas fluorescens* without any sequesters gelled after overnight storage at room temperature. All inoculated samples gelled on day 2 except samples with 3.27 and 4.90 mM added SHMP which gelled on day 3. This implies that these two samples gelled a day later probably due to lower calcium levels as compared to 1.63 mM SHMP. Hence, it can be concluded that SHMP influenced gelation

in pasteurised milk by reducing its ionic calcium to levels that caused gelation during storage. In contrast, TSC had the same effect at all levels in inoculated samples which gelled on day 2. It may therefore be concluded that calcium reduction by TSC did not indicate a direct relationship with gelation in inoculated pasteurised milk at 25^{0}C. Previous researchers revealed that 0.1% added SHMP had similar affinity for ionic calcium in UHT milk with 0.3% TSC although the former delayed gelation whereas the latter accelerated gelation (Kocak and Zadow, 1985a).

For non-inoculated milk samples however, samples with added TSC consistently gelled earlier than those with added SHMP as shown on Table 7-6. Pasteurised milk with 4.90 mM SHMP did not gel at all indicating that at this level, ionic calcium reduction prevented gelation.

The observations above illustrates that the presence of *Pseudomonas fluorescens* 416 influenced gelation because gelation occurred earlier at all levels in these samples than in non-inoculated samples. Activity and metabolites produced by these bacteria may play a significant role in inducing gelation.

7.4 Conclusion:

The current chapter highlighted the role of sequestering agents in ionic calcium reduction and how this influenced gelation and/or proteolysis. Ionic calcium was reduced by using two sequestering agents TSC and SHMP at various levels. Although there were some critical problems with methodology such as excluding the use sodium azide to prevent bacterial growth and short incubation times of the samples, some useful conclusions were made.

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 both UHT and pasteurised 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 25^0C.

Trypsin, chymosin and *Pseudomonas fluorescens* 416 were used in some experiments to accelerate proteolysis and to observe the minimum level of ionic calcium required to cause gelation. It was interesting to observe that trypsin in pasteurised and UHT milk increased ionic calcium to levels higher than originally present in control non treated milk. This needs to be investigated further. *Pseudomonas fluorescens* 416 was responsible for gelation of pasteurised milk after overnight incubation at 25^0C. Although proteolysis was higher in samples inoculated with *Pseudomonas fluorescens* 416, no clear relationship was established between proteolysis and gelation in UHT milk.

Gelation was observed to occur faster at 25^0C than at 5^0C in pasteurised milk. This was also reported by previous researchers. Although TSC did not seem to influence gelation in pasteurised milk at both storage temperatures, SHMP delayed gelation in some

samples at 25^{0}C. 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.

CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

8.1 Main conclusion

Several authors have suggested the need to develop simple and rapid methods for the detection of proteolysis in milk as standardised methods that can be used as a routine in the dairy industry for quality assurance. Although extensive research has been conducted, limited information is available. Of all studies that have been conducted, none has produced conclusive ideal standard methods.

In an attempt to find suitable methods for the detection of proteolysis in milk, this study focused on investigating the effect of proteolysis by bacterial proteases, trypsin and plasmin. The study started by examining the effect of proteolysis by added trypsin and plasmin and using some selected methods- the TNBS, fluorescamine, gel electrophoresis and RP-HPLC methods to monitor proteolysis. The major aspects considered included the use of low enzyme units of trypsin and plasmin as discussed in chapter 4. This was followed by monitoring the effect of proteolysis on storage temperature and time. Correlation between the two soluble extracts (pH 4.6 and 12% TCA) for each method was calculated. The various methods used (TNBS, fluorescamine and RP-HPLC) were compared by calculating correlation coefficients of each soluble fraction separately. Gel electrophoresis was compared by observing the rate of casein breakdown between the two enzymes. The effect of pasteurisation on the inactivation of plasmin was studied by comparing it with raw milk from the same batch. It was established that raw milk had higher rate of proteolysis than pasteurised milk. It was thus concluded that, in the current

experiment, residual plasmin activity was high and pasteurisation was insufficient to inactivate the inhibitors. Gel electrophoresis indicated a high rate of proteolysis by trypsin as high degradation of both α and β-caseins were observed. The experiments further revealed that casein hydrolysis by trypsin (742.35 and 1484.7 BAEE units) at 37^0C produced γ-caseins which were further degraded until their complete disappearance on day 7. The γ-caseins disappeared from day 3, at 1485 BAEE units of added trypsin. This finding has been established for the first time in this study. The pH 4.6 and 6% TCA soluble extracts were generally well correlated in all methods (R^2 >0.93). However, on comparing the individual soluble extracts from trypsin added samples, it was revealed that the RP-HPLC and fluorescamine methods were less well correlated (R^2 = 0.800 and 0.747 respectively from pH 4.6 and 6% TCA soluble extracts) compared to the rest of the methods. The TNBS method was more correlated to both the fluorescamine and HPLC in both plasmin and trypsin experiments. It was generally observed that plasmin experiments had stronger correlations in the studied extracts in all methods than trypsin. The AMC method which is specific for plasmin was used to monitor plasmin activity during storage and was found to be very sensitive in the detection of plasmin in the samples. This method however, although rapid and sensitive was very expensive.

The fifth chapter focused on the role of bacteria on proteolysis. *Pseudomonas fluorescens* and *Bacillus licheniformis* were selected because these microorganisms are documented as being the chief causes of enzymatic milk spoilage. The effect of proteolysis by *Pseudomonas fluorescens* (414 and 416) and their enzymes as well as enzymes from *Bacillus licheniformis* on storage temperature and time was assessed. Enzymes were

extracted from *Pseudomonas fluorescens* and dialysed. All methods studied confirmed that *Pseudomonas fluorescens* 416 was more proteolytic than *Pseudomonas fluorescens* 414 where increased activity was observed with incubation time. Dialysis proved to be a useful technique as it removed interfering compounds from *Pseudomonas fluorescens* enzyme allowing it to be measured by the fluorescamine method. The gel electrophoresis technique revealed the preference for degradation of β- casein over α and κ-casein by both *Pseudomonas fluorescens* and *Bacillus licheniformis.* RP-HPLC chromatograms for both *Pseudomonas fluorescens* and *Bacillus licheniformis* indicated that prominent peaks occurred between 20-30 min for the former but 20-25 min for the latter and these could serve as useful markers to differentiate the enzyme activities. A high peak that was observed at 35 min in pH 4.6 soluble extract from both enzymes was due to native enzymes, possibly plasmin.

It was also established that, unlike added trypsin or plasmin, bacterial proteases did not form γ-caseins at any time during incubation at 37^0C. Although at the highest units of added trypsin, the γ-caseins formed began to disappear after 3 days due to extensive proteolysis, the differences in casein breakdown profiles may be used to distinguish between proteolysis by trypsin and bacteria after incubation for a few hours or less than 3 days. This could provide a preliminary conclusion to the nature of an unknown proteolysis query.

The sixth chapter assessed the susceptibility of milk subjected to various high heat treatments to proteolysis during storage. These experiments were aimed at inactivating

plasmin and or its inhibitors and observing changes in proteolysis during storage. Assessment of the methods used to monitor proteolysis was also carried out. Raw milk and milk heated at 85^0C were most susceptible to proteolysis, whereas samples heated to above 85^0C were less susceptible. These findings are important because they provide information on the processing stability of the plasmin during high heat treatments. The pH 4.6 and 6% TCA soluble extracts were well-correlated.

The final experiment linked the role of ionic calcium reduction to proteolysis and gelation. Due to time constraint, this experiment was not replicated. However, the limited data obtained revealed no clear relationship between ionic calcium reduction with gelation and the extent of proteolysis.

Thus, although all the methods employed in the analysis of proteolysis were suitable, the TNBS method was recommended in the current study. The method was found to be simple, rapid and less expensive compared to the others.

8.2 Suggestions for future work

The current study has investigated the role of native and bacterial enzymes in milk proteolysis. Although a lot of work has been done to study the effect of proteolysis by plasmin and trypsin, further investigation is necessary to address some questions that remain unanswered. The effect of incubation time on proteolysis could be investigated for an extended time of at least 3 months so as to study the long term effect of storage on proteolysis.

In order to study the effect of inactivation of plasminogen activator inhibitors, pasteurisation should be carried out over a wider temperature range and at varied times. An experiment of plasmin with added inhibitors could serve as a control for experiments with plasmin so as to confirm low or no plasmin activity in the control samples.

More experiments using the dialysis technique to investigate the effect purification of *Pseudomonas fluorescens* should also be taken into account. Optimum conditions of dialysis to remove interfering substances from *Pseudomonas fluorescens* should be established. These should include variations of conditions of dialysis such as dialysis time, volumes of water to be used for dialysis and the concentration of *Pseudomonas fluorescens* in the dialysis bags. Since enzymes from *Pseudomonas fluorescens* are not commercially available, extraction procedures for *Pseudomonas fluorescens* for enzyme production should also be optimised by varying the incubation temperature and time, as well as centrifugation conditions-(time and temperature). All these conditions will affect the final concentration of enzymes from *Pseudomonas fluorescens.*

It would be of great interest to establish the link between proteolysis, ionic calcium and gelation. This information would establish the minimum level of ionic calcium in milk that if would result in gelation. At the same time, the level of proteolysis (by both native and bacterial enzymes) should be monitored to establish if any relationship exists between the three aspects. Sodium azide should be used in all experiments to prevent bacterial growth. The same methods should be used to study proteolysis- TNBS, fluorescamine, RP-HPLC and gel electrophoresis.

Identification of the peaks from the RP-HPLC would be useful to serve as markers of protcolytic activities by trypsin, plasmin, *Pseudomonas fluorescens* and *Bacillus licheniformis.*

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APPENDICES

Appendix 1
Comparison of correlations coefficients between pH 4.6 and 6% TCA soluble extracts by TNBS, fluorescamine and RP-HPLC

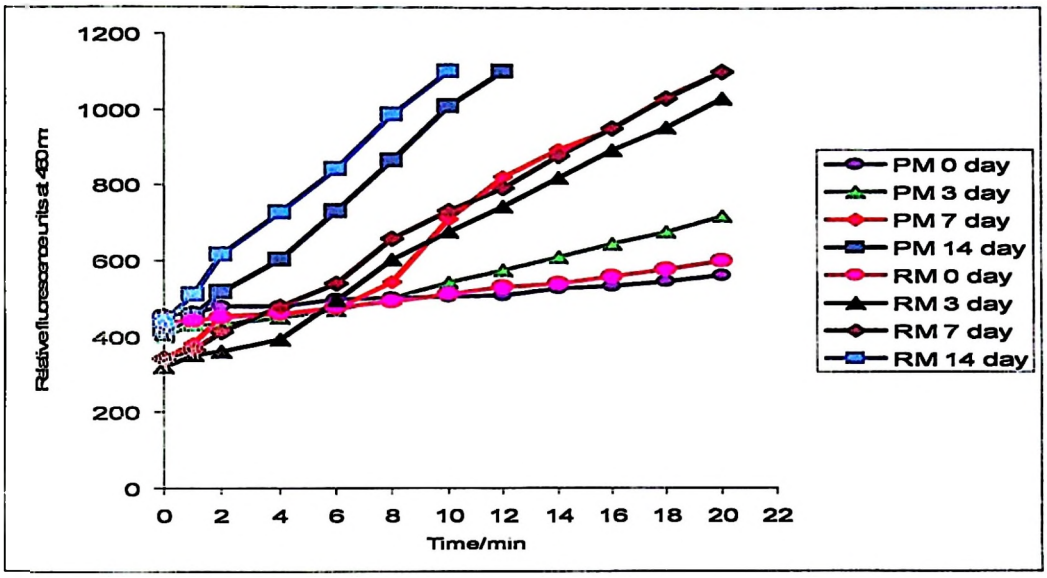
	Parameters	**TNBS**		**Fluorescamine**		**RP-HPLC**	
		pH 4.6	6%TCA	pH 4.6	6%TCA	pH 4.6	6%TCA
Trypsin	Correlation R^2	0.974		0.965		0.990	
Plasmin	Correlation R^2	0.947		0.979		0.928	

Comparison of the correlation coefficients (R^2) between TNBS, fluorescamine and RP-HPLC in either pH 4.6 or 6% TCA soluble extracts

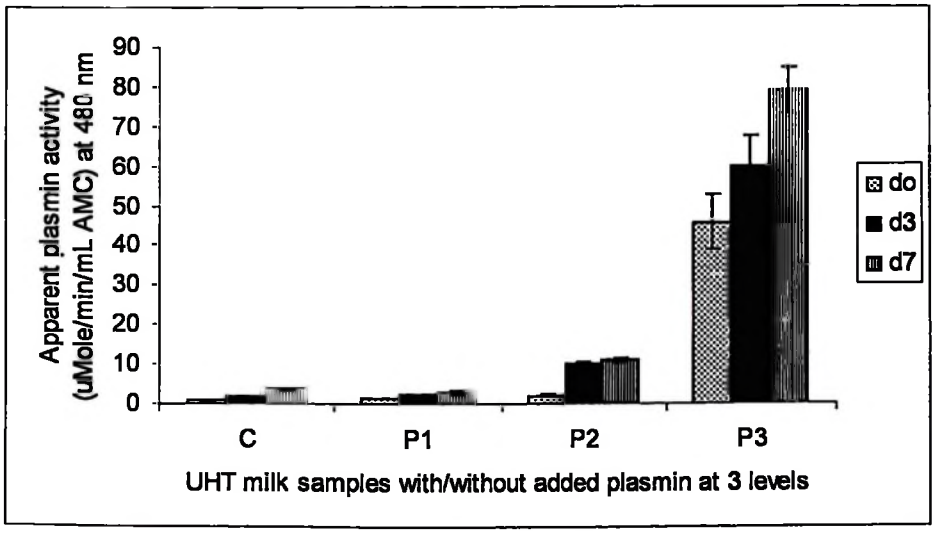
		TNBS		**RP-HPLC**	
		pH 4.6	6% TCA	pH 4.6	6% TCA
Fluorescamine	Trypsin	0.832	0.857	0.800	0.747
	Plasmin	0.981	0.980	0.868	0.967
TNBS	Trypsin	*	*	0.867	0.903
	Plasmin	*	*	0.889	0.972

Appendix 2

Relative fluorescence of control samples of raw and pasteurised milk (without any enzyme addition) incubated at 37C for 14 days as analysed by the AMC method

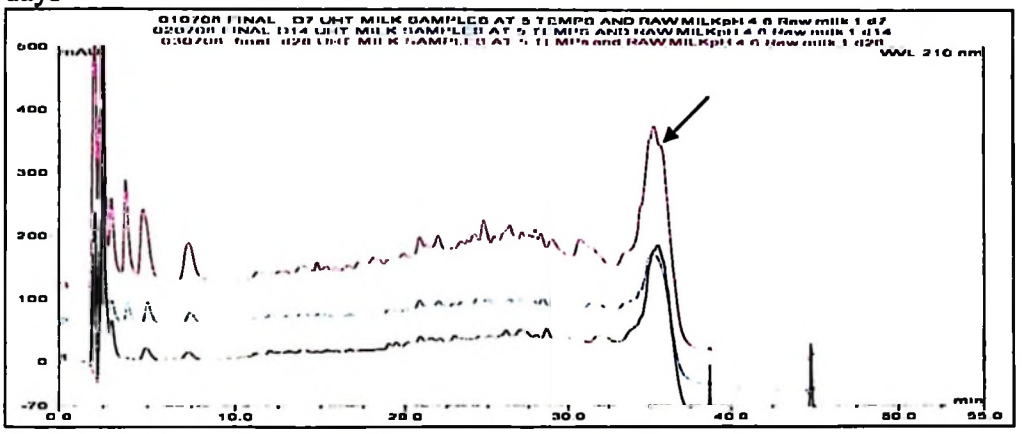


Relative fluorescence of citrate treated UHT skim milk that was treated with and without plasmin followed by incubation at 37^{0}C for 7 days as analysed by the AMC method

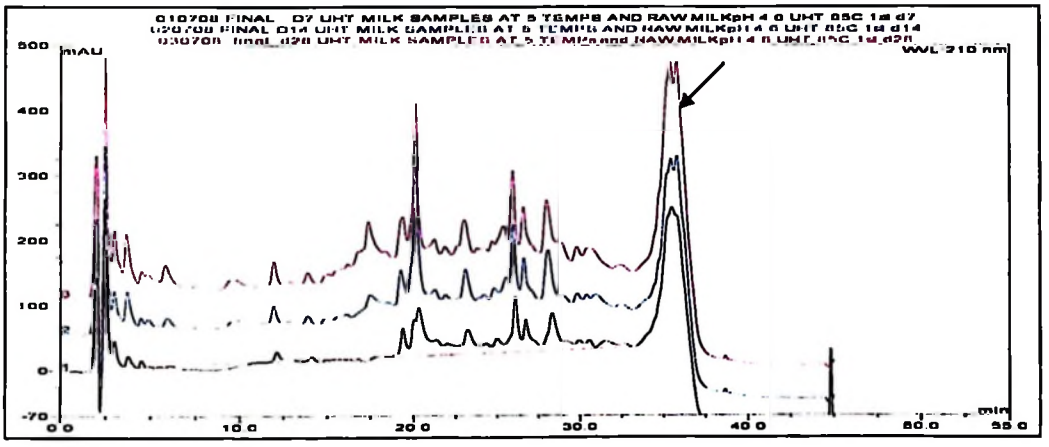


Appendix 3

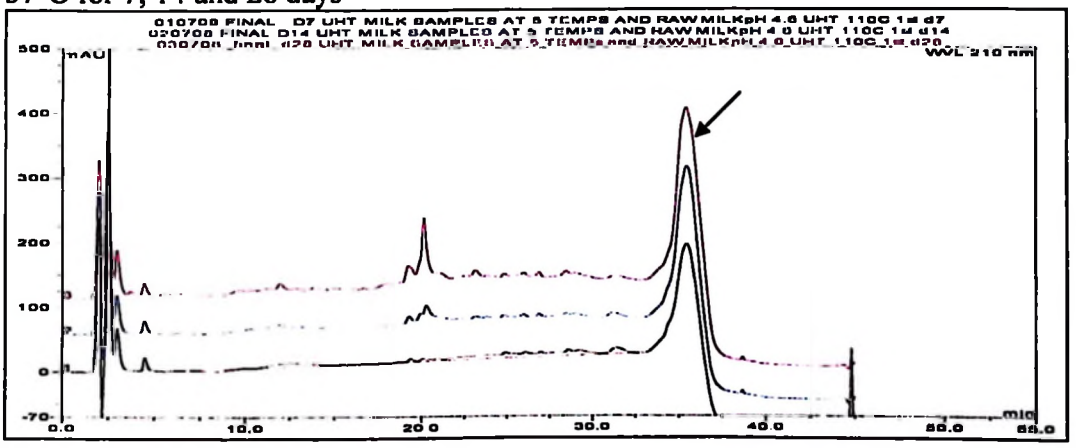
Chromatogram of pH 4.6 soluble extracts of raw milk incubated at 37^{0}C for 7, 14 and 28 days



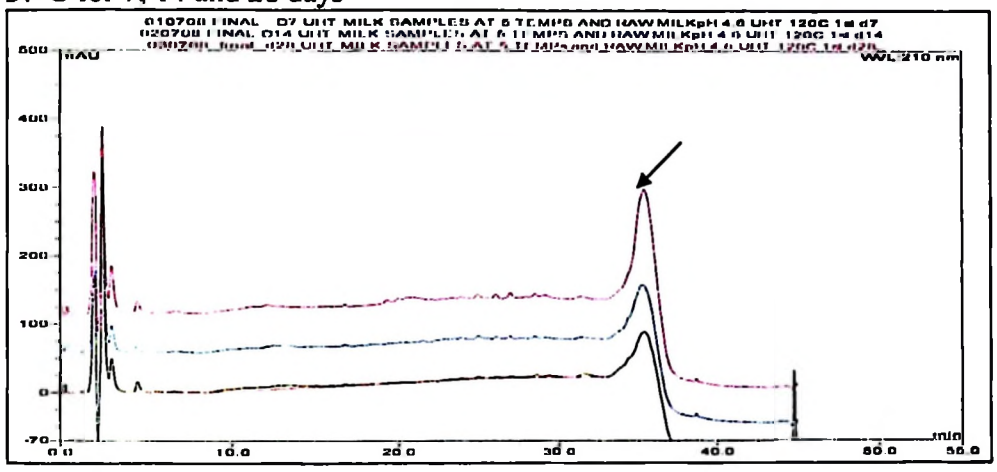
Chromatogram of pH 4.6 soluble extracts of milk processed at 85^{0}C/ 2s and incubated at 37^{0}C for 7, 14 and 28 days



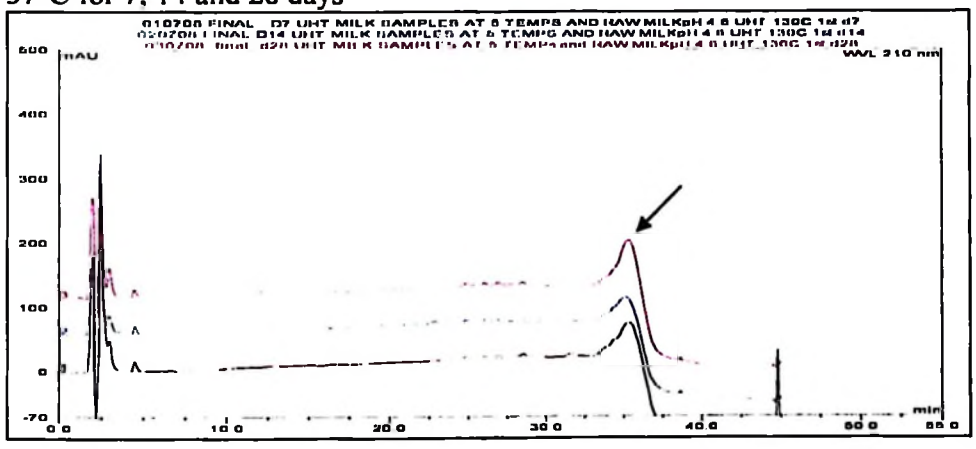
Chromatogram of pH 4.6 soluble extracts of milk processed at 110^{0}C/ 2s and incubated at 37^{0}C for 7, 14 and 28 days



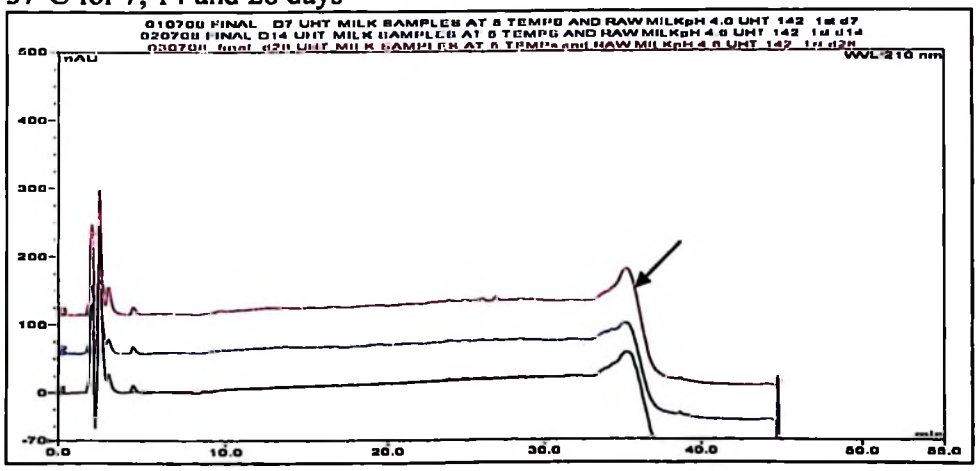
Chromatogram of pH 4.6 soluble extracts of milk processed at 120^{0}C/ 2s and incubated at 37^{0}C for 7, 14 and 28 days



Chromatogram of pH 4.6 soluble extracts of milk processed at 130^{0}C/ 2s and incubated at 37^{0}C for 7, 14 and 28 days

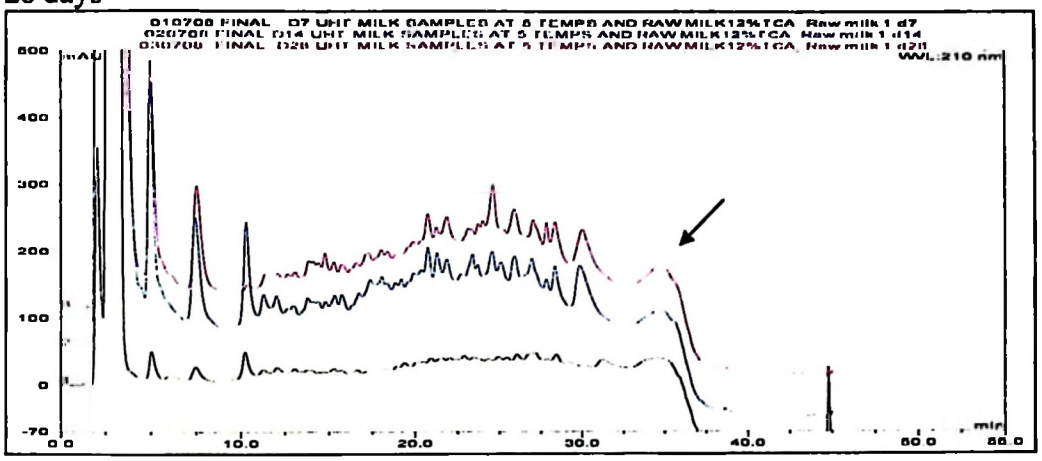


Chromatogram of pH 4.6 soluble extracts of milk processed at 142^{0}C/ 2s and incubated at 37^{0}C for 7, 14 and 28 days

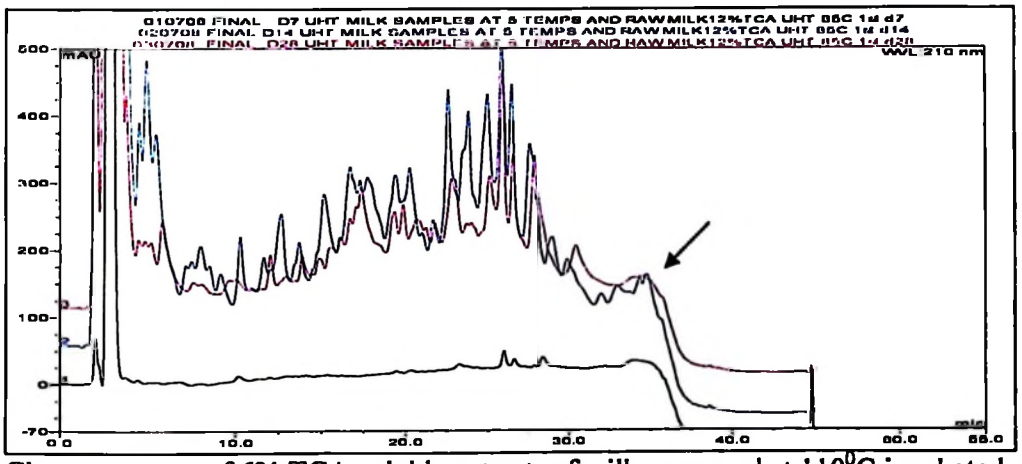


Appendix 4

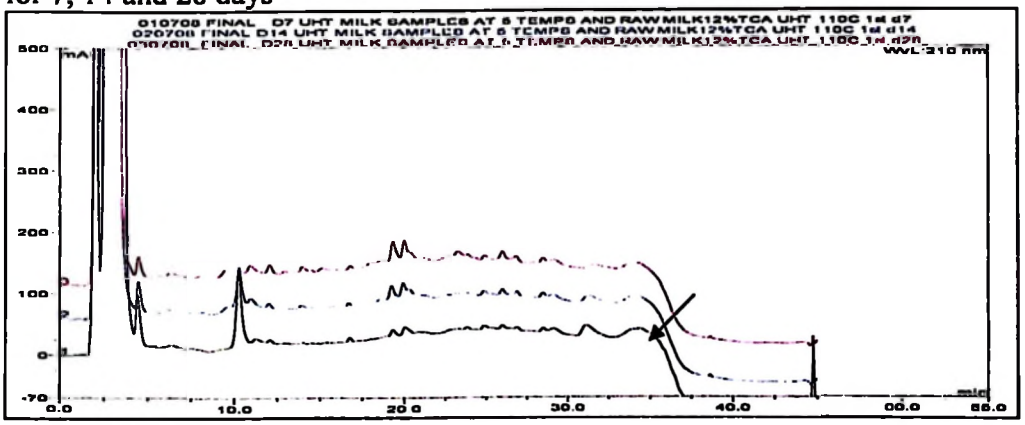
Chromatogram of 6% TCA soluble extracts of raw milk incubated at 37C for 7, 14 and 28 days



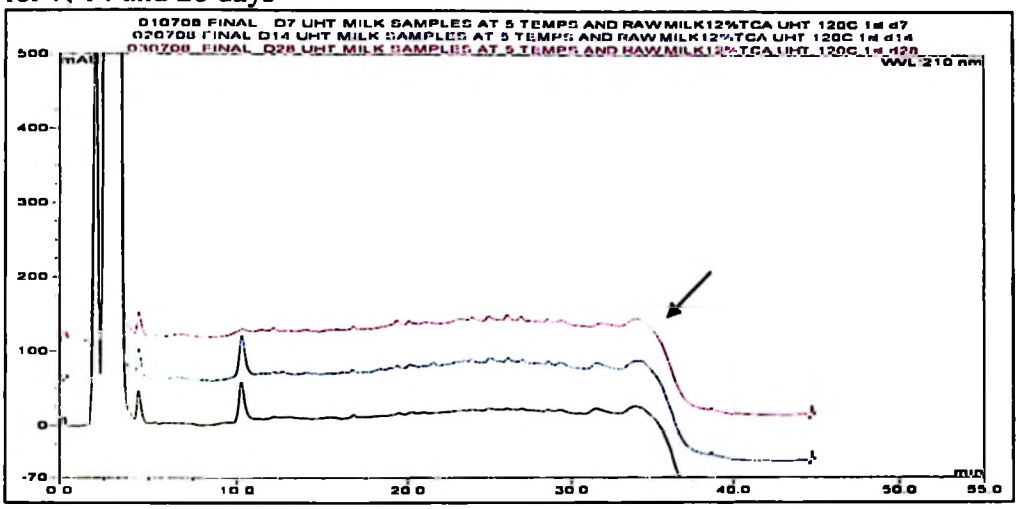
Chromatogram of 6% TCA soluble extracts of milk processed at 85^{0}C incubated at 37^{0}C for 7, 14 and 28 days



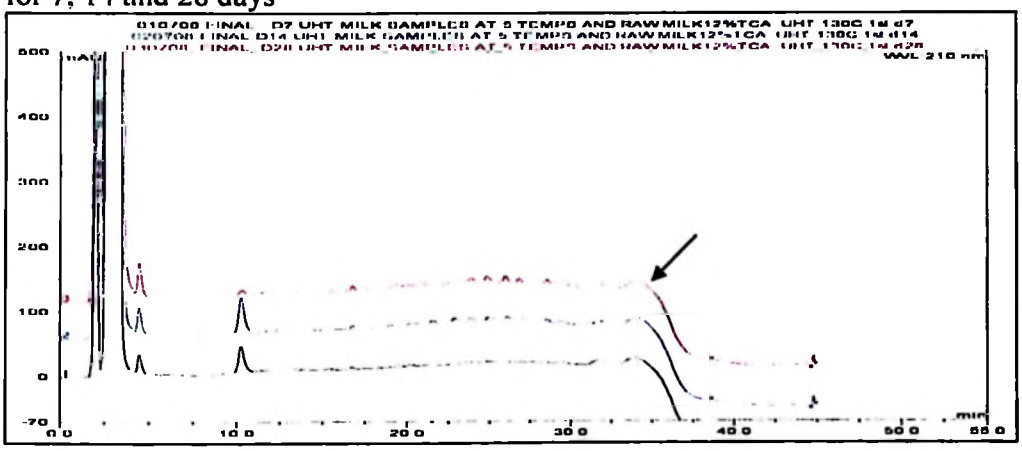
Chromatogram of 6% TCA soluble extracts of milk processed at 110^{0}C incubated at 37^{0}C for 7, 14 and 28 days



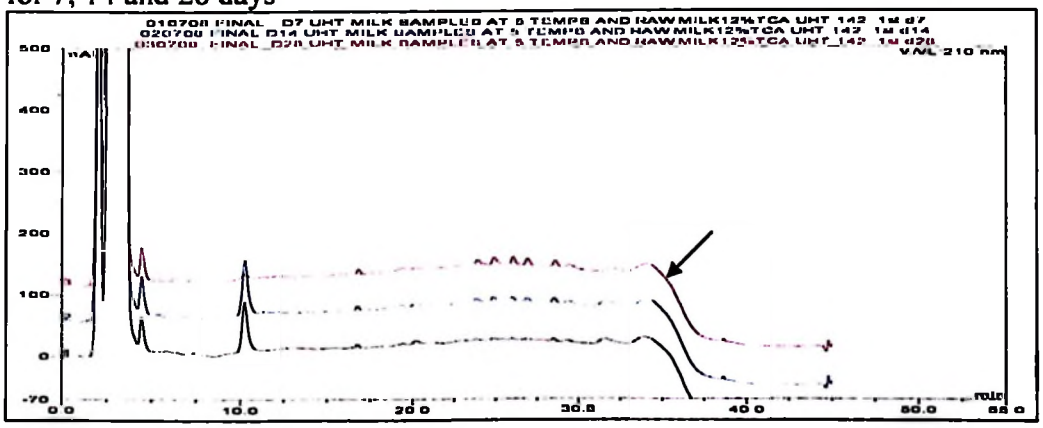
Chromatogram of 6% TCA soluble extracts of milk processed at 120^{0}C incubated at 37^{0}C for 7, 14 and 28 days



Chromatogram of 6% TCA soluble extracts of milk processed at 130^{0}C incubated at 37^{0}C for 7, 14 and 28 days

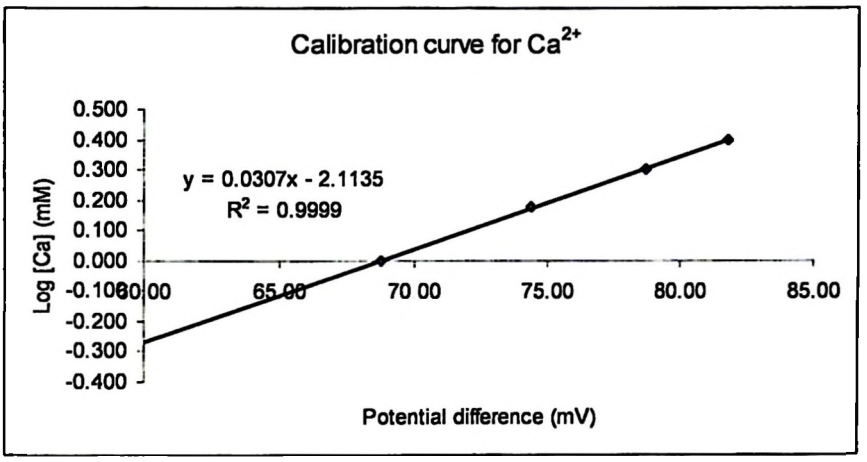


Chromatogram of 6% TCA soluble extracts of milk processed at 142^{0}C incubated at 37^{0}C for 7, 14 and 28 days

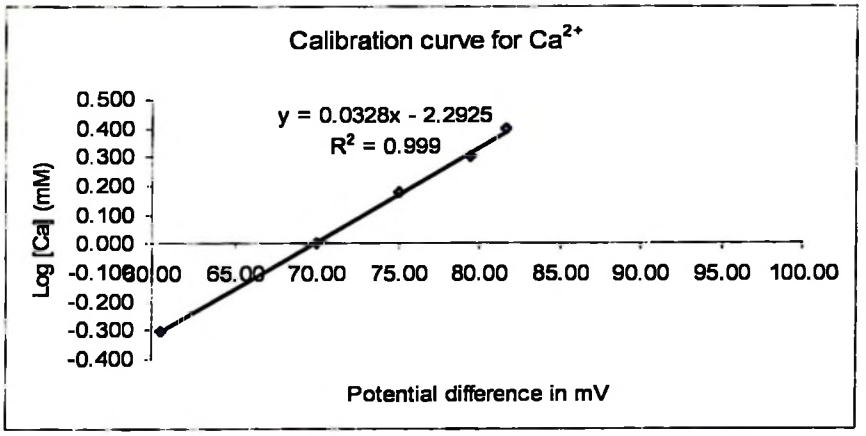


Appendix 5

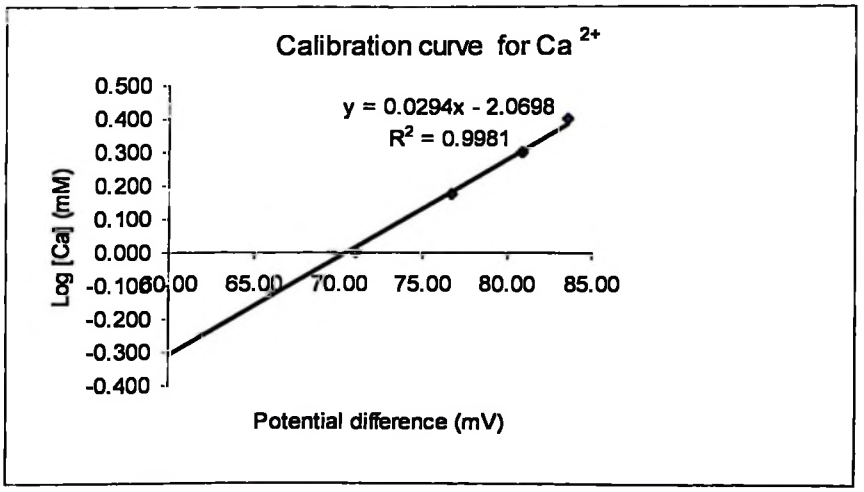
Calibration curve for free ionic calcium as analysed by the ionic calcium analyser



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



Appendix 6
Certificates of analysis for the various enzymes

Certificate of analysis for trypsin

Product Name	Trypsin from bovine pancreas, essentially salt-free, lyophilized powder, ≥10,000 BAEE units/mg protein
Product Number	T1426
Product Brand	SIGMA
CAS Number	9002-07-7

TEST	SPECIFICATION	LOT 026K7770 RESULTS
APPEARANCE	REPORT RESULT	WHITE POWDER
PROTEIN BY UV ABSORBANCE	REPORT RESULT	98% (E1% AT 280 NM = 14.4)
ENZYMATIC ACTIVITY	MINIMUM 10,000 UNITS/MG PROTEIN	10,100 BAEE UNITS/MG PROTEIN
UNIT DEFINITION	ONE BAEE UNIT WILL PRODUCE A DELTA A253 OF 0.001 PER MINUTE IN A REACTION VOLUME OF 3.2ML AT PH7.6 AT 25DEGC. (1CM LIGHT PATH)	
CHYMOTRYPSIN CONTENT	NMT 0.1BTEE UNIT/MG PROTEIN	CONFORMS
QC RELEASE DATE		FEBRUARY 2006

Certificate of analysis for *Bacillus licherniformis*

Product Name	Protease from *Bacillus licheniformis*, 7-15 units/mg protein (biuret), aqueous propylene glycol solution
Product Number	P5459
Product Brand	SIGMA
CAS Number	9014-01-1

TEST	SPECIFICATION	LOT 075K1277 RESULTS
APPEARANCE	BROWN LIQUID	HAZY BROWN LIQUID
PROTEIN BY BIURET WITH TCA PRECIPITATION	> OR = 50 MG/ML	94 MG/ML
ENZYMATIC ACTIVITY	7 - 15 UNITS/MG PROTEIN	12.9 UNITS/MG PROTEIN
UNIT DEFINITION	ONE UNIT WILL HYDROLYZE CASEIN TO PRODUCE COLOR EQUIVALENT TO 1.0UMOLE OF TYROSINE PER MINUTE AT PH7.5 AT 37DEGC. (COLOR BY FOLIN-CIOCALTEU REAGENT)	ONE UNIT WILL HYDROLYZE CASEIN TO PRODUCE COLOR EQUIVALENT TO 1.0 UMOL OF TYROSINE PER MINUTE AT PH 7.5 AT 37 DEG C. (COLOR BY FOLIN-CIOCALTEU REAGENT)
QC RELEASE DATE		JULY 2005

Certificate of analysis for plasmin

Product Name	Plasmin from human plasma, lyophilized powder, ≥3.0 units/mg protein
Product Number	P1867
Product Brand	SIGMA
CAS Number	9001-90-5

TEST	SPECIFICATION	LOT 057K1612 RESULTS
APPEARANCE		WHITE LYOPHILIZED POWDER
PROTEIN BY UV ABSORBANCE	3.0 TO 20.0%	6.8%
ENZYMATIC ACTIVITY	> OR = 3.0 UNITS/MG PROTEIN	6.4 UNITS/MG PROTEIN
UNIT DEFINITION	ONE UNIT WILL PRODUCE ONE UMOLE OF P-NITROANILIDE FROM D-VAL-LEU-LYS-P- NITROANILIDE PER MINUTE AT PH 7.5 AT 37 DEGREES C	
		PLASMA FROM EACH DONOR TESTED AND FOUND NEGATIVE FOR ANTIBODY TO HIV-1/HIV-2, ANTIBODY TO HCV AND FOR HBSAG.
QC RELEASE DATE		JUNE 2007

Calculations for plasmin addition to milk

Stock solution:
750 μg/ 10mL = 75 μg/ mL;

The protein in solution is 6.8% thus, 6.8/100 x 75 = 5.1 μg protein / mL in the stock solution

1.0 mg has 6.4 Units thus 1 μg has 6.4×10^{-3} Units, and so
5.1 μg has 32.64×10^{-3} Units
Thus, the stock solution had 32.64×10^{-3} Units

i. Adding 0.1 mg/L (0.2667 mL was added to the stock solution)
1mL of the stock solution has 32.64×10^{-3} Units
0.2667 mL of the stock solution will have 8.705×10^{-3} Units

ii. Adding 0.3 mg/L (0.8 mL was added to the stock solution)
1mL of the stock solution has 32.64×10^{-3} Units
0.8 mL of the stock solution will have 26.112×10^{-3} Units

iii. Adding 3.0 mg/L (8.0 mL was added to the stock solution)
1mL of the stock solution has 32.64×10^{-3} Units
8 mL of the stock solution will have 261.12×10^{-3} Units

Calculations of trypsin addition to milk

Stock solution: 1.5 mg/10 mL = 0.15 mg/ mL
The protein in this solution is 98%, thus, 98/100 x 0.15 = 0.147 mg protein / mL in the stock solution

1.0 mg protein has 10,100 BAEE Units,so 0.147mg protein has 1484.7 BAEE Units/ mL, Thus, the stock solution had 1484.7 BAEE Units/ mL

i. Adding 0.1 mg/L (0.1667 mL was added to milk)
1 mL of the stock solution has 1484.7 BAEE units / mL
0.1667 mL of the stock solution will have 247.45 BAEE units

ii. Adding 0.3 mg/L (0.50 mL was added to milk)
1 mL of the stock solution has 1484.7 BAEE units/ mL
0.50 mL of the stock solution will have 742.35 BAEE units

iii. Adding 3.0 mg/L (1 mL was added to milk)
1 mL of the stock solution has 1484.7 BAEE units/ mL
1.0 mL of the stock solution will have 1484.7 BAEE units/

Calculations of *Bacillus licherniformis* addition to milk

Stock solution: 10μL / 100 mL of milk = 10^{-4} mL /100 mL = 10^{-6} mL/ mL of milk
The protein in this solution is 94 mg / mL, thus the quantity in the stock solution is 94 x 10^{-6} mg protein / mL

1.0 mg protein has 12.9 units, so 94 x 10^{-6} mg protein has 12.126 x 10^{-4} units / mL,
Thus, the stock solution had 12.126 x 10^{-4} units / mL

i. Adding 0.5 mL of stock solution to UHT milk
   1 mL of the stock solution has 12.126 x 10^{-4} units
   0.5 mL of the stock solution will have 6.063 x 10^{-4} units
ii. Adding 1.5 of stock solution to UHT milk
   1 mL of the stock solution has 12.126 x 10^{-4} units
   1.5 mL of the stock solution will have 18.189 x 10^{-4} units
iii. Adding 2 mL of stock solution to UHT milk
   1 mL of the stock solution has 12.126 x 10^{-4} units
   2 mL of the stock solution will have 24.252 x 10^{-4} units
iv. Adding 4 mL of stock solution to UHT milk
   1 mL of the stock solution has 12.126 x 10^{-4} Units
   4 mL of the stock solution will have 48.504 x 10^{-4} units
v. Adding 6 mL of stock solution to UHT milk
   1 mL of the stock solution has 12.126 x 10^{-4} units
   6 mL of the stock solution will have 72.756 x 10^{-4} units
vi. Adding 8 mL of stock solution to UHT milk
   1 mL of the stock solution has 12.126 x 10^{-4} units
   8 mL of the stock solution will have 97.008 x 10^{-4} units
vii. Adding 10 mL of stock solution to UHT milk
   1 mL of the stock solution has 12.126 x 10^{-4} Units
   10 mL of the stock solution will have 121.26 x 10^{-4} units

Appendix 7 Calculations for sequestering agents added to milk at various levels

% Sequestering agents added	(g/1000 mL)	MOLARITY (mM)	
294.1		SMP	TSC.2H2O
0.04%	0.4g/1000mL	0.65	1.36
0.08%	0.8g/1000mL	1.31	2.72
0.10%	1g/ 1000mL	1.63	3.40
0.20%	2g/ 1000mL	3.27	6.80
0.30%	3g/1000mL	4.90	1.02

SHMP(%)					
	Mwt	612	g		
	1M	612	g/L		
SHMP(%)	Concentration			MOLARITY (M)	MOLARITY (mM)
0.04%	0.04g /100mL	0.4g/1000mL	0.4	0.000653595	0.65
0.08%	0.08g/100mL	0.8g/1000mL	0.8	0.00130719	1.31
0.10%	0.1 g/100mL	1g/ 1000mL	1	0.001633987	1.63
0.20%	0.2g/100mL	2g/ 1000mL	2	0.003267974	3.27
0.30%	0.3 g/100mL	3g/1000mL	3	0.004901961	4.90

TSC (2H2O)					
	Mwt	294.1	g		
	1M	294.1	g		
TSC (2H2O)	Concentration		TSC (2H2O)	MOLARITY (M)	MOLARITY (mM)
0.04%	0.04g /100mL	0.4g/1000mL	0.4	0.001360082	1.36
0.08%	0.08g/100mL	0.8g/1000mL	0.8	0.002720163	2.72
0.10%	0.1 g/100mL	1g/ 1000mL	1	0.003400204	3.40
0.20%	0.2g/100mL	2g/ 1000mL	2	0.006800408	6.80
0.30%	0.3 g/100mL	3g/1000mL	3	0.010200612	10.20