Age gelation within UHT milk

BY

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Chapter 1: Introduction

1.1. Introduction

Milk is a food product secreted by the female of all mammalian species and it contains a wide range of nutrients namely, protein, fat (which includes essential fatty acids), lactose, vitamins, minerals, enzymes, anti-bacterial agents and water. The milk's components are produced in the mammary gland. The biological function of milk is to give immunological protection to the neonate (Swaisgood, 2007). Milk gives an excellent nutritional value to both infants and adults and for this reason, it is often regarded as a nutritionally balanced food (Cilliers, 2007; Anusha and Ranjith, 2014). Worldwide, milk is one of the most commonly sold types of food and due to its profound nutritional value, it is regarded as the best by consumers (Pedras *et al*., 2012). Additionally, milk contains no anti-nutritional factors (Walstra *et al*., 2006).

Milk is a food product that is very sensitive and easily perishable (Raikos, 2010). In order to make good quality dairy products, it is very crucial that the raw milk is of good quality; raw milk being the milk that is not pasteurized or processed in any manner. However, once the raw milk is spoiled; it cannot be improved by further processing (Cilliers, 2007).

Due to the nutritional composition, high water activity and neutral pH that milk has, it becomes a suitable medium for microbial growth which then leads to the multiplication of microorganisms such as enterobacteria, lactic acid bacteria, *Pseudomonas*, *Staphylococcus*, *Listeria* as well as sporulating microorganisms such as *Clostridium*. Spore-forming bacteria are widely known for their thermal resistant characteristic and often than not they can cause milk spoilage (Pedras *et a*l., 2012). In addition, milk lacks iron which is an essential nutrient for bacterial growth and milk also contains antibacterial agents. The latter results in the growth of a number of bacteria being more or less limited in raw milk, while other bacteria can still grow and proliferate at high ambient temperatures (Walstra *et al*., 2006).

As milk is regarded as an easily perishable food product and a good medium for microbial growth, heat treatment needs to be applied for safety and acceptable shelf-life. Heat treatment

such as pasteurization or sterilization is the primary treatment used for microbial minimization of microbes in milk and such treatments are regularly applied in milk processing. Excessive heat treatment can however negatively affect the quality of milk; such effects may include off-flavors, denatured protein and a negative effect on vitamins such as folic acid and B-complex vitamins and may result in the release of sulphur compounds (Pedras *et al*., 2012).

Milk is a food product that is known to contain various indigenous enzymes which have different functions, pose different effects on stability during processing, and also have a different on the quality of dairy products. Just like in the case of lacto-peroxidase, some of the enzymes found in milk have a beneficial impact whereas some are used as indices of processing (e.g. alkaline phosphate) and others, like plasmin and lipase, have a negative impact on the quality of dairy products like plasmin and lipase. However, not all the enzyme contributes negatively to the quality of the product (Kelly and Fox, 2006).

In this particular study, the focus is on plasmin enzymes as well as the protease from psychrotolerant bacteria. Plasmin is a proteolytic enzyme that occurs naturally in the milk whilst psychrotolerant bacteria grow in milk during storage within the cold chain (Nemeckova *et al*., 2009). Resultantly, plasmin protease and that secreted by psychrotolerant bacteria induce harmful proteolytic defects in milk such as age gelation as well as bitterness in milk. Moreover, due to the plasminogen precursor (plasminogen), the activity of plasmin is often difficult to suppress or rather control and this precursor usually survives extreme heat treatments such as ultra-high temperature (UHT) processing (Bhatt, 2014).

1.2. Thesis Aim and Objectives

Aim: The overall aim of the study is to prevent or retard age gelation that occurs in UHT milk.

Objectives Chapter 3:

1: To investigate the susceptibility of UHT milk with varying fat concentrations towards proteolytic activity. In order to achieve this objective, UHT milk will be treated with *Bacillus* protease and plasmin enzyme separately and the peptides liberated during the hydrolysis will be analysed using Reverse-Phase High-Performance chromatography (RP-HPLC).

2: To investigate the impact of fatty acids on the activation of plasminogen to plasmin protease in homogenized and un-homogenized raw full cream milk. In order to achieve this objective, raw full-cream milk will be treated with *Pseudomonas* lipase enzyme and the liberated fatty acids will be investigated to see if they play a fundamental role in the activation of plasminogen to plasmin using milk agar test method and Reverse-Phase High-performance chromatography.

3: To identify the major fatty acids present in milk and to evaluate their individual and combined roles on the activation of inactive plasminogen. In order to achieve this objective, fatty acids naturally present in milk were identified using the Gas Chromatography (GC). Moreover, from the identified fatty acids, individual and the combination (cocktail) of fatty acids were tested (between C4 and C18:1) for their roles on the activation of inactive plasminogen using the milk agar plate method and Merck protease assay kit.

Objectives Chapter 4:

1: To investigate the impact of refrigeration temperature (5°C) in comparison to room temperatures (25°C) on the digestibility of casein protein. In order to achieve this objective, milk was treated with KIO_3 and plasmin protease separately; and stored in the refrigerator and at room temperature for a period of 96 h. During the incubation time, the pH of the milk samples were measured, and samples were tested using the alizarol test and Reverse-Phase High Performance chromatography for proteolytic activity.

Objectives Chapter 5:

1: To investigate the impact of additives and gases on the inhibition of proteolytic activity in the milk and to lower the susceptibility of the casein towards proteolytic attack. In order to achieve this objective, proteolyzed milk with plasmin and *Bacillus* protease was subjected to treatment with various additives namely Ethylene-diamine-tetra-acetic acid (EDTA), Calcium chloride ($CaCl_2$), Sodium hexa-metaphosphate (SHMP), and sodium citrate ($Na_3C_6H_5O_7$). Analysis was done using the Merck protease assay kit and Reverse-Phase High-Performance chromatography to identify which of the above mentioned additives have to ability to retard proteolytic activity in milk. Furthermore, various gasses such as CO_2, He and N_2 were bubbled through milk to determine their impact on the inhibition of proteolytic activity in milk. Analysis was

also done using the Merck protease assay kit and Reverse-Phase High-Performance chromatography

2: To determine how long the inhibition effects of plasmin and *Bacillus* protease remain active after N_2 pre-treatment. In order to achieve this objective, UHT fat-free milk was pre-treated with N_2 gas and stored in the refrigerator (5°C) over a period of 744 h. During time 0 h, 168 h and 744 h, milk was tested or analyzed for proteolytic activity using the Merck protease assay and Reverse-Phase High-Performance Chromatography.

1.1.2. Thesis structure

This thesis begins with the overall introduction explaining what the problem is, followed by a literature review and different chapters that address the above mentioned objectives. Just like in the case of the literature review, each chapter will begin with the introduction or a bit of background that is not addressed in the literature review. Each chapter will also include the experimental works, which are the materials and methods, discussions and Conclusions. Moreover, at the end there will be a chapter offering general conclusions and summary.

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Chapter 2: Literature Review

2.1. Composition of milk

The sole reason for the mammalian species to produce milk is to feed the mammalian young (Forsback, 2010). The concentration of constituents differs from species to species in order to meet the necessities of their specific offspring. Usually, lipids vary between 2-55%, protein 1-20%, lactose 1-10% depending on the energy required by the young (Thompson *et al.*, 2008; Anusha and Ranjith, 2014). The composition of milk includes water, fat, casein, whey protein, carbohydrates, and ash (Forsback, 2010). Moreover, the composition of the milk also varies amongst animals according to various factors, namely the stage of lactation, breeds, feed, the health of the animal, genetic factors, diet and geographic factors (Datta and Deeth, 2001; Thompson *et al.*, 2008).

- Stage of lactation

Lactation is a period from parturition to leaving the cow dry and the stage of lactation is the time that passes after the parturition (Walstra *et al.*, 2006). During lactation, the first milk that the infant receive is called the colostrum. The composition of the colostrum differs significantly from that of the normal milk. Usually, colostrum is known to contain more mineral, salts, serum protein, fat content, calcium and less lactose than normal milk (Swaisgood, 2007).

- Illness of the cow

In response to pathogenic bacteria, milk tends to have an increased somatic cell count (SCC) which is often indicative of mastitis (Walstra *et a*l., 2006). Due to high serum protein content, the milk with high SCC is more prone to gelation than milk with less SCC. Often, the more microbes there are in milk, the more enzymes will be present in that particular batch of milk. This has been attributed to increased proteolytic activity in milk which then results in increased levels of plasmin enzyme (Chavan *et al.*, 2011).

- Feed

The manner in which the cow is fed affects the composition of the milk, particularly the fat content. Usually, the fat content is reduced by either the low roughage or high-fat diets

(Swaisgood, 2007). Moreover, there are other causes which may result in the change of the milk's composition like the contamination of milk with oxygen and bacteria (Walstra *et al*., 2006).

- Season

The composition of the milk can be influenced by climatic conditions which are also known as seasonal changes. It is reported that the amount of fat and protein are influenced greatly by seasonal changes. That is, when the temperature increases, the amount of fat decrease. The season also influences the somatic cell count (SCC) and milk components. The somatic cell count is normally stable during winter and is said to increase towards the end of summer. The reasons why the somatic cell count increases during the summer duration is still uncertain but it might be associated with the udder infection, lactation stage, changes in milking practices, clinical stress and pasture-related factors (Pavel and Gavan, 2011).

2.2. Stability of milk

Milk contains of proteins and they are divided into two definite types, the casein and the whey proteins (Phadungath, 2004; Walstra *et al*., 2006). Bovine caseins represents about 80% of the total milk protein and they are precipitated at pH 4,6; at 30 °C. Casein is a phosphoprotein that has phosphoric acid that is chemically bound to the protein and it is found in milk in a form of calcium phosphate (Anusha and Ranjith, 2014). The caseins are divided into five groups, namely the alpha (α_{s1}), alpha (α_{s2}), beta (β), gamma (γ) and finally the kappa (K) casein. The beta (β) casein is made up of A1 and A2 types (Jianqin, *et al.*, 2016). In the case of whey protein, it accounts for about 16% of total milk proteins and included in this category is the α-lactalbumin (α-LA), and β-lactoglobulin (β-LG), and lactoferrin known to be heat sensitive, globular, and water-soluble proteins. The remaining are free amino acids which are made up of hydrophobic and hydrophilic regions, water and salts. Additionally, the amino acids allow the casein to act as stabilizers of foams and emulsions (Phadungath, 2004; Swaisgood, 2007; Raikos, 2010).

The casein has properties that differ greatly from those of whey proteins. They are hydrophobic and possess a high charge that is required to keep the casein in solution. It also has a few cysteine and a number of prolines residues (Anusha and Ranjith, 2014). Casein has

the tendency to associate, both through self-association and association with each other (Walstra *et al.*, 2006). Additionally, the post-translational processing of the casein like phosphorylation, glycosylation, and limited proteolysis is quite different (Bhatt, 2014).

The casein exists as micelles in milk due to the calcium phosphate present. The structure of the casein micelle is still under debate, as a result, different models of casein micelles have been proposed. The proposed models are categorized in terms of coat core, sub micelles, and internal structure models. The coat core explains the micelles as an aggregate of caseins that has an outer layer that varies in composition from that of the interior. The inner part is also not precisely clear. The sub micelle is thought to be made up of spherical uniform subunits. The internal structure model is thought to be a kind of a model that designates the manner of aggregation of different casein (Phadungath, 2004). Additionally, the stability of casein micelles depends greatly on the k-caseins which are present on the outer surface of the micelles (Anusha and Ranjith, 2014).

Swaisgood (1992) established that the casein micelle carries a unique characteristic which is their post-translational modifications. It was also established that amongst other important components of the casein, namely the α_{S1}, α_{S2}, β, and k-casein, only the $_{\gamma}$-casein (gamma) occurs naturally from finite proteolysis of β-casein by the plasmin enzyme. The casein α_{S1} is known to contain the highest charge, whilst α_{S2} is known to contain at least two cysteine residues which form disulfide bridges. The majority of k-casein is glycosylated at various degrees (Bhatt, 2014).

- Alpha s1 (α_{S1})

There is a high net negative charge in α_{S1} and a high a content of phosphates. The α_{S1} is known to associate in only two steps, at pH 6.6 and 0.05 M ionic strength. For non-associated molecules, low concentrations of the casein are required. However, this association is reduced when the ionic strength is decreased and the range of electrostatic repulsion is increased. In this association, hydrophobic interactions are also involved. Irrespective of whether or not the ionic strength and the casein concentration is high, this association decreases and eventually vanishes (Walstra *et al.,* 2006).

- Alpha (α_{S2})

The association pattern is the same as that of the α_{S1} casein. The α_{s2} casein is known to have many genetic variants and it also has a variable number of phosphoseryl residues, given that it shows a huge variability in phosphorylation. This molecule has two cysteine residues which then form a disulfide bridge and has no carbohydrate groups. They are also known to be calcium sensitive (Phadungath, 2004; Walstra *et al*., 2006).

- Beta (β-Casein)

The β-casein is the most hydrophobic casein of the five types and also consists of a large number of proline residues. The β-casein has a polar "head" and a long chain, apolar "tail". The association of the β-casein depends greatly on the temperature as well as the ionic strength. The association of β-casein does not take place below the temperature of 5°C and the molecule remains unfolded (Walstra *et al*., 2006).

- Gamma ($_\gamma$-casein)

Gamma casein is the by-product or rather the degradation product of the β-casein. It is quite soluble in ethanol. Additionally, the plasmin enzyme naturally present in milk brings about the cleavage of this molecule. The split-off parts make up most of the protease peptones in milk (Walstra *et al*., 2006).

- Kappa (k-casein)

The k-casein differs enormously from the other casein molecules in that it has two cysteine residues that make up intermolecular disulfide bonds. As a result, k-casein is present in milk as oligomers with an average molecular weight of about 120 kDa. Among the k-caseins, there are differences present solely because some of them have two ester phosphate groups and some of them have only one. This is known as micro-heterogeneity and it occurs quite often even within the individual milking of the cow. To a certain degree, the association of k-casein is more like that of β-casein (Walstra *et al*., 2006).

Importantly, β-casein is one of the most studied proteins both in oil-water interfaces and at microscopic oil-water interfaces. The durability of the β-casein stearic stabilizing layer is maintained by the cluster of five phosphoserine residues located in the tail residues. The

stabilizing layer has a structure that is sensitive to pH, ionic strength and calcium ion content (Dickison, 2010).

Federation (2007) stated that milk in its natural state exists as a colloidal suspension. The casein micelle in milk is a colloidal particle that is big enough to cause flocculation/gelation due to mutual attraction caused by Van der Waals forces. Walstra *et al*. (1999) established that under normal physiological conditions, flocculation/gelation does not occur because there are counteracting repulsive forces that prevent aggregation.

Moreover, a colloidal suspension of milk has various particles, some contain minerals and proteins and others contain fats, which are suspended in the serum. The principal protein component, the casein micelle, has various components attached to it, basically, the casein sub micelles which are glued together by means of calcium phosphate (Federation, 2007). Walstra *et al.,* (1999) added that each sub micelle has different casein molecules and not all the sub-micelles have similar compositions, thus there are two different types of sub micelles, namely those with the k-casein and those without the k-casein.

Horne (1998) established that the casein micelle has hydrophobic and hydrophilic regions and structure of the casein micelle is led by the self-associating forces between caseins, which is the balance of attractive hydrophobic and repulsive electrostatic forces as well as calcium-mediated interactions. Walstra *et al*. (1999) reported that the sub micelle has the C-terminal k-casein attached on its surface as well as the protruding hairs known as the CMP's (casein micro peptides) proteins. The CMP's are hydrophilic and they have a net negative charge which results in repulsion between micelles, pushing the micelles away from each other in the same vicinity thereby maintaining the colloidal suspension of milk.

Thus, understanding the casein protein composition and the interaction between the caseins is crucial as the casein micelles characteristics greatly impact the organoleptic properties of milk (Anusha and Ranjith, 2014).

2.3. Background on enzymes in milk

Enzymes are the biological catalysts that speed up the rate of chemical reactions without changing its equilibrium (Guinee and O'Brien, 2010). They originate from various sources such as milk itself, bacterial contamination and from the somatic cells within milk. According to Kelly and Fox (2006) the study of enzymes has been going on for years and it has been found that about 70 indigenous enzymes are present in milk and some are beneficial whilst others are detrimental to the quality of milk. It is of importance to know certain aspects when identifying the type of enzymes present in milk. Those aspects include namely, the enzymes present in milk, the level of activity they have, factors that affect their activity and the significance of the enzymes.

Limited information is available on the variations of enzymatic activity. However, Kelly and Fox (2006) established that upon arrival of milk from the farm at the processing plant, comprehensive dilutions take place as the milk is mixed with that from the other farms which may decrease the significance of variations in enzymatic activity. Moreover, how enzymes are distributed in the milk and the impact of storage and processing is continuously studied.

Some of the enzymes found in milk play a fundamental role in shielding the mammary gland against growth and invasion by bacteria. Lysosomal enzymes play the function of absorption of phagocytized bacteria. Lysozyme and lactoperoxidase (LPO) have recognizable antibacterial functions. For example, they are known to preserve raw milk and other dairy products, especially in warm climatic areas (Kelly and Fox, 2006).

The quality of milk and that of dairy products produced thereafter can be influenced by enzymes in two distinct ways. Firstly, it can be influenced by enzymes in the udder. Normally the temperature of the udder is 37°C and the incubation period ranges from 0-18 h or even beyond and these conditions are optimal for most mammalian enzymes. Under these conditions, the enzymes are more than likely to hydrolyse the casein substrate or activate plasminogen to plasmin in milk (Chavan *et al*., 2011). Although many questions arose, it's been indicated that the presence of products of enzyme activity in newly drawn milk shows that enzymes are active in the udder (Kelly and Fox, 2006).

Secondly, during the refrigerated storage, the enzyme activity may accumulate although their activities may be low at first. However, enzymes originate from psychrotolerant bacteria that accumulate and survive under low cold storage conditions. As a result, these enzymes have a

negative impact on the shelf stability of the milk and milk products (Kelly and Fox, 2006; Bhatt, 2014).

2.4. Background information on Ultra-High Temperature treated (UHT) milk

A call for Ultra-High Temperature treated and aseptically packaged milk is escalating across the globe. Consumers do not favor the addition of additives because they believe it contributes negatively to their health, and therefore they demand safe, fresh, uncompromised sensory qualities in milk especially taste and extended shelf life (Chavan *et al*., 2011). UHT processing signifies a continuous heating process at temperatures higher than 130°C, usually between 140 to 150°C for 2-10 sec followed by aseptic packaging to create a commercially sterile product. UHT processing has its own advantages and disadvantages. There are desirable and undesirable changes that occur as a result of UHT treatment. Desirable changes involve the destruction of microorganisms and inactivation of most enzymes. Undesirable changes involve browning, loss of nutrients, sedimentation, fat separation and cooked flavors (Chove *et al*., 2013).

The process of UHT treatment differs considerably from that of in-container sterilization which implicates high-temperature treatment of 110 to 120°C for 15-30 min (Datta *et al*., 2002). UHT treatment produces a good quality product in comparison to in-container sterilization. This is as a result of the continuous flow of milk and quick heating and cooling rates that brings about fewer chemical changes in milk (Dennill, 2015).

The whole concept of UHT treatment i.e. temperature and time combinations is determined by the necessity to inactivate heat-resistant bacterial endospores and the necessity to reduce chemical changes that negatively affect sensory and nutritional aspects of the milk (Datta *et al*., 2002; Tran *et al*., 2008; Dennill, 2015). The process of UHT treatment is done in two ways, namely direct heating or indirect heating. Direct heating focuses mainly on the mixing of super-heated steam with milk whilst indirect heating focuses mainly on the heat exchanger that transmits heat across a partition between the milk and heating medium (Datta *et al*., 2002).

The direct systems make use of two types, namely, steam infusion and steam injection. In the steam infusion system, milk can either be sprayed into superheated steam or allowed to fall through a steam chamber. In the steam injection system, superheated steam is injected into a stream of milk. Heating in the indirect method is conducted by heat exchangers where heat is conveyed by conduction from the heating source through the surface of the metal to the milk. In this system, superheated water or steam serves as the heating source and often, when the steam is used it induces more burn on and also flavor alteration in the product. Two types of indirect systems may be used namely, tubular or plate based on the nature of heat exchanger used (Dennill, 2015).

The shelf life of milk is determined by the time necessary for the milk to reach a preset percentage of consumer dismissal (Celestino e*t al*., 1997). After high-temperature treatments (UHT), milk is rendered completely sterile but sterility does not guarantee shelf life as other changes occur during storage which may be physical or chemical (Anema, 2017). Proteolysis is one of the limiting factors of shelf life for UHT milk due to the enzymes that somehow survive UHT treatment and cause defects in UHT milk during storage. These defects include coagulation, gelation, and bitterness (Dennill, 2015).

2.5. Destabilization of UHT milk

Destabilization of milk casein results in defects such as age gelation, flocculation and compromised flavor within UHT milk.

2.5.1. Age gelation in ultra-high temperature treated (UHT) milk

Age gelation occurs in UHT milk during prolonged storage (Anema, 2017). The phenomenon of age gelation is symbolized by strong irreversible aggregation of the micelles that occurs when particles remain close together far longer than they would when there are no attractive forces between them and ultimately form a three-dimensional protein network (Datta and Deeth, 2001; Walstra, *et al*., 2006). Age gelation occurs within weeks to months of storage at ambient temperatures and a sharp increase in viscosity is evident (Datta and Deeth, 2001).

2.5.1.1. Causes of age gelation in UHT milk

There are two main distinct causes of age gelation; firstly it is proteolysis which results in the proteolytic breakdown of the casein induced by the natural milk proteinase (plasmin) and microbial proteinases which are heat stable (Chavan *et al*., 2011; Dennill, 2015; Anema,

2017). Secondly, it is non-enzymatic/chemical processes which involve the polymerization of casein and whey protein that ultimately form a three-dimensional network. This may be due to the Maillard reactions and the formation of kappa-casein-β-lactoglobulin complexes (McMahon, 1996).

2.5.1.1.1. Plasmin protease (Enzymatic process)

Plasmin is an indigenous native milk enzyme which confers similar traits to those found in the blood serum when compared kinetically, immunologically and by partial sequencing (Korycka-Dahl *et al*., 1983; Bastian *et al*., 1996). The native enzymes are usually located in different areas in milk and often, they are associated with the fat globule membrane (Walstra *et al*., 2006).

The plasmin enzyme belongs to the family of peptidase S1 and it is a serine proteinase, meaning that at its active site, it has the amino acid serine (Fajardo-Lira, 1999). Plasmin has trypsin-like specificity and usually reacts on caseins namely the β-casein, α_{S2} casein, α_{S1} casein while k-casein is resistant (Nemeckova *et al*., 2009; Crudden *et al*., 2005; Cilliers, 2007). The whey proteins are resistant towards the plasmin action. The β-lactoglobulin that is denatured prevents the activity of the plasmin as the free sulfhydryl group of the denatured β-lactoglobulin forms a thiol-disulphide interchange with the disulfide groups of the plasminogen. The plasminogen molecule is rendered inactive by allosteric hindrance for activation by plasminogen activator (Cilliers, 2007).

Additionally, it was established by Fajardo-Lira (1999) that plasmin from bovine milk has a high specificity for peptide bonds, particularly the lysine residues and to a lesser extent the arginine. Plasmin is known to hydrolyze about seven lys and about four arg peptide bonds within α_{S1} casein. The main plasmin cleavage sites were identified to be at Arg22-phe23, Arg90-Tyr91, lys102-lys103, lys103-Tyr104, lys105-Val106, lys124-Gly125 and Arg151-Gln152. Also, it was proposed that α_{S1}-casein is initially hydrolysed by plasmin towards the center of the molecule and the 12 lys and 5 Arg bonds are very fragile to plasmin (Cilliers, 2007; Bhatt, 2014).

The hydrolysis of α_{S2} casein by plasmin appears to be similar to that of β-casein in that it creates a lot of peptides with high electrophoretic mobility. Plasmin hydrolysed about eight peptide bonds in α_{S2} casein, seven lys bonds, and only one Arg bond. It also appeared that there were more plasmin fragile bonds in α_{S2} casein than in β-casein due to the high number

of lys residues in the α_{S2} casein. However, in the case of k-casein, it is resistant to plasmin hydrolysis. This may be due to the presence of carbohydrate moieties that are attached to it to prevent its hydrolysis by plasmin. Thus far, the specificity for plasmin hydrolysis of k-casein is not yet identified. Moreover, it was established that para-casein obtained from rennet hydrolysis of k-casein is more prone to plasmin hydrolysis (Bhatt, 2014).

Plasmin, plasminogen and plasminogen activators are integrated with the casein micelle and it is hypothesized that its integration occurs through lysine binding sites on the enzyme molecule which compares to the way in which plasmin binds to fibrin in the blood (Fajardo-Lira, 1999).

2.5.1.1.2. Plasmin as a part of a complex activation system

Datta and Deeth (2001); Ismail *et al*., (2011) and Chavan, *et al*., (2011) mentioned that plasmin protease (PL) forms part of the complex activation system which consists of plasminogen activators (PA), plasminogen activator inhibitors (PAI), and plasmin inhibitors (PI). Plasminogen is found in the blood; therefore, the existence of plasmin in freshly produced milk suggests that the activation of plasminogen to plasmin takes place while the milk is stored in the lumen of the udder prior to milking. It is also suggested that the activation of plasminogen takes place earlier in the process of milk synthesis. Crudden *et al*., (2005); Cilliers, (2007); and Chavan *et al*., (2011) reported that the plasminogen found in blood and milk exists in an inactive zymogen form and that serves as an anti-clotting enzyme. The concentration of plasminogen in colostrum is higher than in late lactation milk. The ratio of plasminogen to plasmin in milk is approximately 4:1, making the concentration of plasminogen higher than that of plasmin.

Cruden and Kelly (2003) established that the plasmin enzyme functions best at the pH of 7.5 and at the optimum temperature of 37°C. However, Nemeckoya *et al*., (2009) reported that irrespective of these optimal conditions for plasmin activity, it still possesses the ability to induce proteolytic changes during the storage of raw milk at lower temperatures like 4°C. Figure 2.1 depicts how plasmin forms part of the complex system.

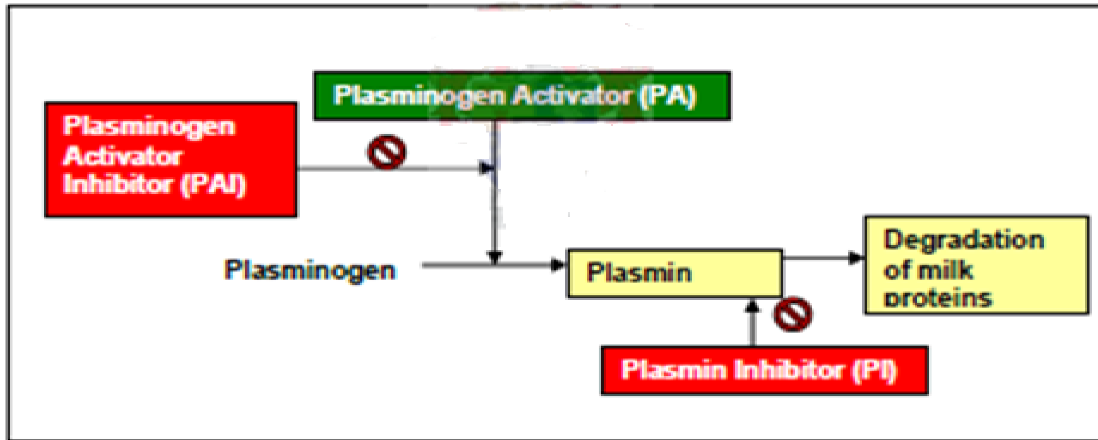

Figure 2.1: The plasmin-plasminogen system (Cilliers, 2007)

2.5.1.1.3. Plasminogen Activators

The function of plasminogen activators (PA) in the system is to induce the conversion of plasminogen into plasmin protease. Usually, the PA is indigenous to milk and they may also result from the presence of microorganisms. The PA activity is associated with the casein micelles and the SCC in milk (Chavan *et al*., 2011). There are two main types of PA that are widely known, the tissue type (t-PA) and the urokinase-type (u-PA). The activity of the t-PA is contained in the milk casein fraction and in the presence of fibrin. The activity of t-PA increases four-fold and the molecular mass of this plasminogen is 75 Kilo-Dalton (kDa). Additionally, the u-PA is contained in the somatic cell fraction and its size is 50 Kilo-Dalton (kDa) and the molecular mass is 30 kDa respectively. Nonetheless, in the presence of fibrin, the u-PA activity is not affected but it is enhanced by amiloride and urokinase (Cilliers, 2007).

The combination of the serine proteases, t-PA, and u-PA stimulate the plasminogen by the cleavage of its Arg_{561}-Val_{562} peptide bond. Therefore the developed amino acid group which is Val_{562} forms a salt bridge to Asp_{740}. The newly formed amino acid leads to the conformational changes in the active domain of plasminogen to give rise to the active plasmin (Cilliers, 2007).

In UHT milk, proteolysis usually brings about off-flavors and more often than not, it solubilizes the casein micelle and gelation results (Walstra *et al*., 2006). Figure 2.2 exhibits the impact of plasmin proteinase on the physicochemical properties of UHT milk.

Figure 2.2: Coagulation of the milk caused by indigenous plasmin enzyme (The *et al.*, 2011)

2.5.1.1.4. Microbial protease predominately *Pseudomonas* spp. and *Bacillus* spp. (Enzymatic processes)

The breakdown of proteins during storage (proteolysis) is also caused by heat-stable microbial proteinase from psychrotolerant bacteria predominantly; *Pseudomonas* spp. and *Bacillus* spp. Psychrotolerant microorganisms are known to grow at temperatures of ≤7 °C whilst their optimum growth temperature may be higher (Nielsen, 2002; Pulkkinen, 2014). These organisms are members of the Gram-negative group of bacteria for example *Achromobacter* and gram positive bacteria like *Bacillus* (Chavan *et al.,* 2011). However, *Pseudomonas* spp. and *Bacillus* spp. are of greatest importance in this study. The microbial protease produced by these psychrotolerant bacteria can incidentally elevate the plasmin levels in the casein curd (Nielsen, 2002).

Thus, while the milk waits for processing in the processing plant or on the farms, the conditions in which the milk is stored may enable the growth of psychrotolerant microbes. These microbes then secrete the heat stable enzymes that have the capacity to destabilize the casein. Under sanitary conditions, less than 10% of total microbial flora is psychrotrophs whereas under unsanitary environment about 75% of the microbial flora is psychrotrophs (Sorhaung and Stepaniak, 1997; Nielsen, 2002). While, the genus *Pseudomonas* spp. are not present in large quantities in milk initially but at the time of spoilage, it is found to dominate. After low levels of about 0.001—1 CFU/mL, *Pseudomonas* spp. have the ability to multiply to levels that can result in significant production of protease enzyme that can lead to gelation in milk. Additionally, this genus is found to be lipolytic in other words it has the ability to break down fats (Sorhaung and Stepaniak, 1997).

Another genus that is known to cause gelation of milk is *Bacillus* spp. Janstova *et al*., (2006) mentioned that *Bacillus* affects milk quality and causes significant economic loss. This genus

has vegetative cells which possess the ability to produce heat stabile extracellular enzymes after multiplication, consequently affecting the nutritional as well as sensory properties. *Bacillus* spp. survives pasteurization and it is spore forming. Spores per sé may not contribute to gelation/flocculation of milk but when the spores germinate, the vegetative cells can multiply and produce sufficient protease to cause gelation in the UHT milk.

Bacillus spp. secrete extracellular proteinases that are of comparable heat resistance to those of *Pseudomonas* spp (Chavan *et al.,* 2011). Sorhaung and Stepaniak (1997) added that the growth and the metabolic activities of *Pseudomonas* spp. or *Bacillus* spp. are also enhanced by post-pasteurization contamination. As much as UHT treatment kills almost all the heat stable enzyme, the residual protease still has the capacity to further destabilize the casein and therefore gelation result after a few weeks to a few months of storage of the UHT milk at room temperature (Sorhaung and Stepaniak, 1997).

Psychrotrophs produce proteases with wide pH and temperature ranges. The protease still remains active at lower temperatures albeit their temperature optima are between 30-45°C (Fajardo-Lira, 1999; Nielsien, 2002). Figure 2.3 exhibits the impact of proteinase produced by *Bacillus* on the physicochemical properties of UHT milk.

Figure 2.3: Coagulation of the milk caused by *Bacillus* protease in UHT milk (The *et al.*, 2011)

The heat stable proteases of these psychrotrophs destabilize all forms of casein; preferably hydrolyse first the k-casein, then β-casein and lastly α-casein. Proteinase produced by these psychrotrophs attack the k-casein forming para-s-casein and consequently destabilizes the casein micelle and coagulates milk in a way similar to that of chymosin action. The stability of the casein micelle is maintained by the k-casein and its degradation results in aggregation and gelation which is the fundamental issue in this regard (Samaržija *et al*., 2012)

According to Fajardo-Lira, (1999) and Samaržija *et al*., (2012), protease from psychrotolerant bacteria may cause the milk to have an unclean flavor. Astringent off-flavors was found to have developed in milk treated with psychrotolerant proteases and plasmin. A profound connection exists between the initial psychrotolerant count and storage life of raw milk at 2°C and 6°C (Fajardo-Lira, 1999).

Finally, the link between the proteases from psychrotolerant bacteria and the plasmin system is currently under study and little information is available. However, it is stated from the available information that some proteases produced by psychrotolerant bacteria may act as activators for plasminogen (Kelly and Foley, 1997). It was also established that the bacterial proteases had a wide range of specificity on milk caseins and less activity on whey proteins (Fajardo-Lira, 1999). Figure 2.4 depicts the mechanism of UHT milk destabilization due to heat resistant proteases during storage. The different types of casein are hydrolysed by a heat-stable protease from different species and strains of psychrotolerant bacteria. Some proteases have a preference for the cleavage site in the hydrophobic areas symbolized by red shapes in Fig 2.4, and some hydrolyse the k-casein, which induce a link between hydrophobic and hydrophilic cores (blue shapes) (Machado, 2017).

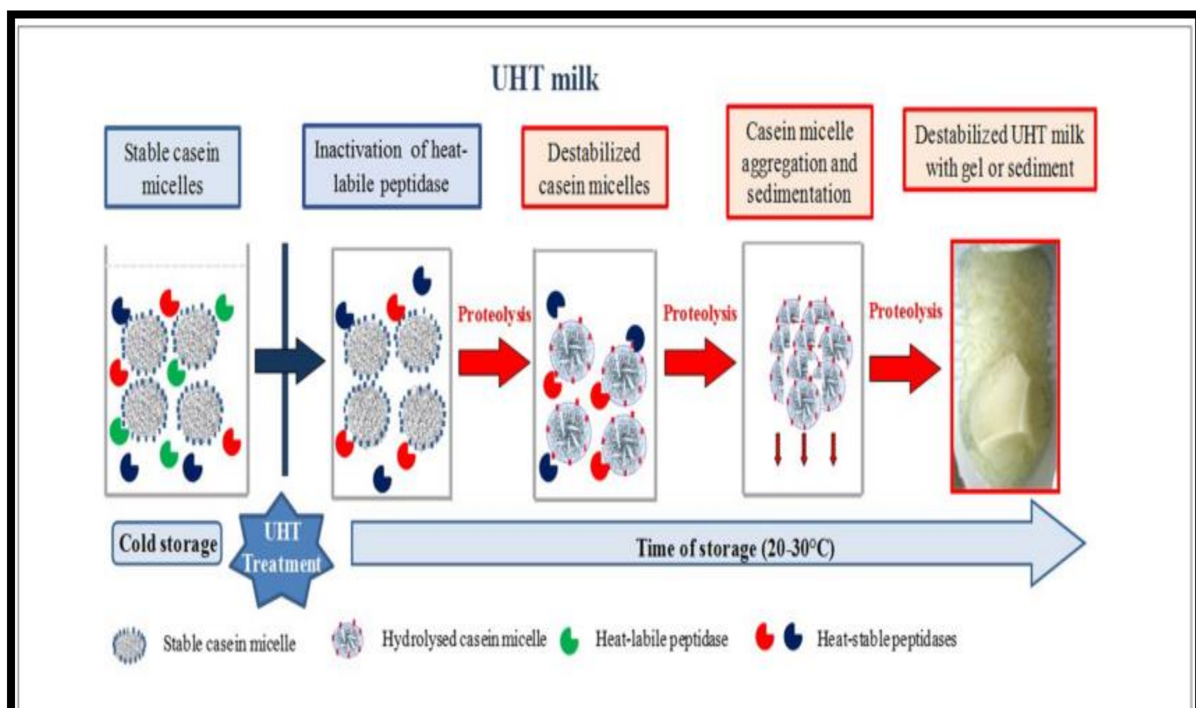

Figure 2.4: Mechanism of UHT milk destabilization due to casein micelle proteolysis by heat-resistant protease during storage at ambient temperatures (Machado, 2017)

2.5.1.1.5. Chemical processes (Non-enzymatic action)

A) Aggregation and dissociation process

The manner in which age gelation occurs chemically is based on two distinct stages, namely the aggregation stage as well as the dissociation stage. Considering the structure of the casein micelle it has the k-casein on its surface and CMP's (casein micro peptides) that have a negative charge which allows it to bind the major β-lactoglobulin whey denatured proteins which get attached to the k-casein during the UHT heat treatment forming the kappa-casein-β-lactoglobulin complexes (McMahon, 1996).

Secondly the β-kappa-complex gets removed from the casein micelle due to the breakdown of several sites that keep it glued to the casein during storage of UHT milk and as a result, there's a formation of long chains that form a 3-dimensional network which is responsible for the gelation of milk (McMahon, 1996). Above the temperatures of 55°C, the β-lactoglobulin denatures and due to this action the activity of the thiol group increases and the cysteine disulfide bonds rupture. So much so that when the heat treatment is lengthened to temperatures above 80°C, the cysteine residues will continuously be downgraded (Sawer, 1969).

Generally, the manner in which age gelation occurs chemically depend on three processes namely, the interaction between β-lactoglobulin and k-casein, the release of the β-kappa-complex from the casein particle and the cross-linking of the β-kappa-complex and associated proteins (Fig 2.5) (Chavan *et al*., 2011).

B) Polymerization

The polymerization of casein and whey proteins, on the other hand, occurs as a result of the Maillard type or other chemical reactions. The degree of polymer formation is greatly dependent on storage, time and temperature at which the UHT milk is held. The extent of polymerization of caseins and whey at higher temperatures of about 37°C is several times greater than heat-induced changes resulting from UHT treatment due to Maillard type (Andrews and Cheeseman, 1972). This is however contradictory to reports by Chavan *et al.,* (2011) that at higher temperatures of above 37°C, milk hardly form a gel. This is still subject to research since the rationale behind the concept of polymerization of casein is controversial.

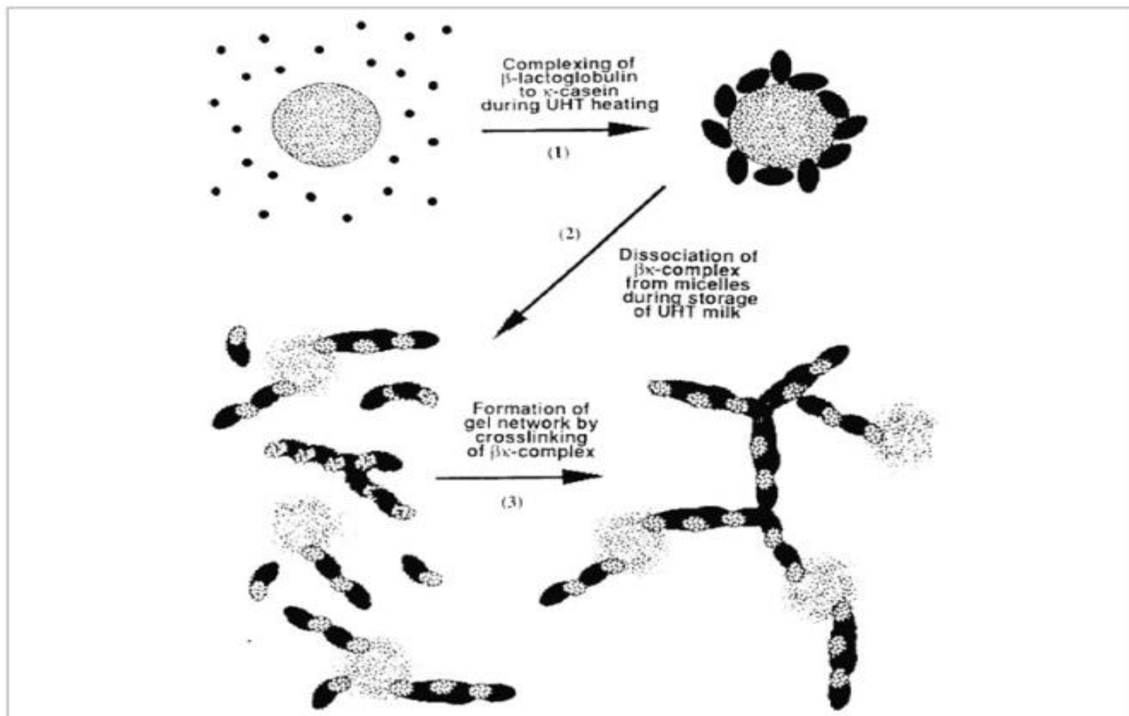

Figure 2.5: Model of age gelation in UHT milk where 1 represents the formation of the βk-complex; 2 exhibits its dissociation from micelles during storage; and 3 exhibits the ultimate gelation of the milk through cross-linking of the βk-complex (Chavan *et al.*, 2011)

2.5.1.1.6. Other factors that contribute to age gelation

A. Fat content

The shelf life of milk can be influence negatively by the fat content of milk (Dennil, 2015). It is reported by Deeth *et al*., (2002) that pasteurized skim milk has a shorter shelf life than the pasteurized full cream milk. It is assumed that this may be the result of different growth rates of psychrotolerant bacteria contaminants on different kinds of milk (Deeth *et al*., 2002). It is also mentioned that the spoilage pattern of the two kinds of milk and the occurrence of age gelation differ remarkably due to the higher amount of protease produced in skim milk than in full cream milk. In addition to reports by Janzen *et al*., (1982); Deeth *et al*., (2002), it was established that the high levels of proteolysis in skim milk may be due to the fact that there's no protective effect of the fat against proteolytic attack induced by protease from psychrotolerant bacteria.

Fat globules present in milk presumably shield the milk casein and hinder the action of the enzyme from easily digesting the casein hence gelation and flocculation is delayed (Chavan *et al*., 2011). The hydrophobic interconnections of β-lactoglobulin and k-casein to fat globules also make caseins less prone to proteolytic attack (Deeth *et al*., 2002). Additionally, a high amount of denatured whey proteins not attached to the micelle surfaces in skim milk grants weak resistance to age gelation in UHT processed skim milk (Chavan *et al.,* 2011).

Forsback, (2010) reported that the content of milk fat is affected by mastitis, however; results obtained from investigations regarding the fat content differ. Other researchers report high levels of fat content during mastitis while other literature report lower levels of fat content during mastitis. It was also indicated that the latter may be a result of decreased synthetic and secretory potential of the mammary gland (Ma *et al*., 2000; Munro *et al*., 1984 and Ali *et al.,*1980).

B. Mastitis

Mastitis is the inflammation of the mammary gland in the udder due to bacterial infection through a damaged teat. This disease causes elevated levels of the somatic cell count (SCC), altered flavor and altered milk composition. When the bacteria penetrate the teat canal, the udder quarter gets infected and the infection is usually characterized by redness, swelling, pain, and loss of function (Forsback, 2010). Milk with high microbial count is more prone to gelation than milk with a low microbial count (Chavan *et al*., 2011).

Forsback (2010), also mentioned that mastitis is divided into two types; sub-clinical and clinical mastitis. With subclinical mastitis, the SCC count is usually increased; the flavor and the composition of the milk are altered but no physical signs of mastitis are visible in the milk. Clinical mastitis, on the other hand, is denoted by fever, loss of appetite, swelling of the udder, increase in SCC and altered flavor and milk composition. Contrary to subclinical mastitis, this type of mastitis does have visible symptoms such as clots and blood in the milk.

In sub-clinical and clinical mastitis, the antigens stimulate an inflammatory response that involves the plasminogen activation system. The monocytes and macrophages move to the infected area and produce mediators of the inflammatory response which also include proteinases (Cilliers, 2007).

Somatic cells are however the primary sources of plasminogen activators in milk which implies that the higher the SCC in milk, the higher the plasmin activity. On the other hand,

leucocytes, milk coagulants, and extracellular bacterial enzymes pose no ability to activate plasminogen. In mastitic milk, the higher concentrations of plasminogen activators favor the activation of plasminogen. It was also established that plasminogen activators originate from bovine milk macrophages and blood monocytes. In mastitic milk, dissociation of plasminogen and plasmin from the casein micelle was observed and that a greater extent of plasminogen activation was associated with the casein than with the soluble plasminogen in the mastitic milk (Bhatt, 2014).

C. Heat Treatment

The onset of gelation differs based on the heat treatment applied before or during sterilization and on the degree of whey denaturation that interacts with the casein (Deeth, 2003; Chavan *et al*., 2011). The activation of plasminogen and the activity of plasmin increase during pasteurization of milk at 75°C for 15 sec due to the inactivation of heat sensitive plasminogen activator inhibitors. The inactivation of heat sensitive plasminogen activator inhibitors mostly occurs during this stage and plasmin inhibitors retain only two thirds of their activity (Ismail *et al*., 2011). In the experiment conducted by Samuelson and Holm (1966), it was reported that when sterilization temperature was increased from 142 °C to 152 °C and when the heating time was increased from 6 sec to 12 sec, the shelf life of milk was prolonged with no detection of age gelation. Zadow and Chituta (1975) on the other hand reported that the milk that was treated at temperatures between 135°C and 145°C at heating times from 3 sec to 5 sec exhibited increased gelation; consequently the method used for treating the milk contributes extensively to the quality and shelf life of milk.

Taking into consideration the intricacy of the whole milk system, proteins, particularly whey β-lactoglobulin interferes with plasmin activity and plasminogen activation during the heating process. Interaction of plasmin with whey protein considerably affects its activity. During UHT processing, the β-lactoglobulin that has one free sulfhydrl group can cause irreversible denaturation of plasmin through cysteine disulfide (S-S/-SH) interactions. Unfolding of the plasminogen molecule can also stimulate interaction with β-lactoglobulin especially at higher temperatures, consequently obstructing plasminogen activation. Even though heat can inactivate plasmin in the presence of β-lactoglobulin, any residual plasminogen or plasmin in the absence of inhibitors will result in active plasmin during storage after heat treatment (Ismail *et al*., 2011).

Moreover, even though the indirect method of UHT treatment may induce certain problems like chemical changes, the direct method is known to be the cause of bacterial induced proteolysis because the heat is not suffice to destroy the heat stable enzymes even though it does destroy the bacteria itself (Chavan *et al*., 2011; Dennil, 2015).

D. Stage of lactation

Due to the elevated permeability of the blood vessels in the mammary gland, the concentrations of plasmin, plasminogen, and plasminogen activity increases in the advanced stage of lactation. Reports indicated that during late lactation, plasminogen activation increases; and plasmin activity increase primarily because of the entry of more plasmin from the blood to the milk. The elevated levels of plasmin with advancing stages of lactation are induced by the increased levels of plasminogen together with its elevated conversion to plasmin which is connected with the somatic cell count (Bhatt, 2014).

2.5.2. Background information on flocculation in UHT milk

Flocculation is a revisable aggregation mechanism which occurs when droplets attach to one another due to unbalanced inter-atomic attractive and repulsive forces and form flocs (Sarkar, 2010). At an ambient storage temperature of UHT milk, these flocs aggregate with one another in a process called flocculation (Cilliers, 2007). According to Federation (2007), it is important to understand the definition of coagulation as well as flocculation due to the tendency of using the two terms interchangeably.

Federation (2007) concluded that the meaning of coagulation/flocculation depends greatly on the context as well as the purpose in which it is used and separating the two terms is subject to one's opinion. Flocculation is defined as a process of random interactions and aggregation reactions in the absence of denaturation and it is also symbolized by weak revisable aggregation of the micelles whereas coagulation is symbolized by strong irreversible aggregation of the micelle. In some cases, as indicated by Dickison (2010) flocculation can still be strong and irreversible depending on intermolecular forces and colloidal interactions that might be related thereof.

Dickinson (2010) established that flocculation can be explained with regards to the deviation of colloidal structure from that of a stable structure. Consequently, the dilute emulsion is regarded as flocculated when the droplets are not randomly disseminated throughout the

dispersion medium. Additionally, the word flocculation can be substituted by the term aggregation without any remarkable loss of meaning.

Thus far, flocculation is the most complicated mechanism to control of the three destabilization mechanisms shown by dispersion of protein-coated oil droplets and it is not definable in terms of one single method. These mechanisms are creaming, coalescence, and Ostwald ripening. The mechanism of flocculation is very complicated because its state can be easily affected by so many factors and it can influence the emulsion structure. The main elements that have an impact on flocculation and that need to be considered are the protein/oil ratio, oil volume fraction, homogenized fat globule, temperature, pH, ionic strength, the concentration of denatured whey proteins, calcium ion content and destabilization of casein by proteases (Dickinson, 2010).

The rate of flocculation differs significantly based on the pH values of milk (Van Aken *et al.*, 2011). Hattingh (2017) reported that when the pH of bovine milk is reduced, it results in a decrease of flocculation. Normally, the process of flocculation begins at pH 4.7-4.8. Often than not, at pH lower than 5.5, milk has a tendency to flocculate and decreasing the pH of milk below the iso-electric point (4.6) will reverse the process of flocculation.

According to Dickinson (2010), protein stabilized emulsions may be flocculated in various ways and their mechanism is explained as follows.

- Development of bridges during emulsification. When inadequate protein is present during emulsification to saturate the emulsion droplets, flocculation will occur. Due to thermal treatment, flocculation occurs as a result of cross-linking of an adsorbed protein molecule on different droplets (Dickinson, 2010).
- Decreasing the surface charge density. Flocculation is normally brought by the binding of divalent cations or decreased pH to adsorbed protein. This applies under conditions where the arrangement of casein is sensitive to macromolecular charge distribution (Dickinson, 2010)
- Increasing ionic strength. Upon addition of electrolytes such as NaCl to the aqueous phase, there's a reduction of the electrical double layer and the inter-droplet repulsion that occurs through electrostatic screening lessens (Dickinson, 2010).

In addition, Sarkar (2010) stated that the two most common types of flocculation are depletion flocculation and bridging flocculation, respectively. Often, depletion flocculation

arises as a result of a non-adsorbing biopolymer in the continuous phase of the emulsion which then encourages association of droplets in the emulsion by bringing about the osmotic pressure gradient within the continuous phase nearby the droplets. The bonds formed during depletion flocculation are usually flexible, reversible and weak. The depletion mechanism is indicated in Fig 2.6.

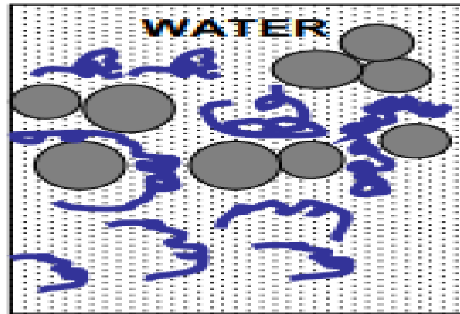

Figure 2.6: Depletion flocculation occurring in an oil-in-water emulsion. Due to the osmotic pressure gradient, the particles approach one another and push the un-adsorbed biopolymer. [Grey and dotted white depict the oil and water phases,respectively whereas the dark blue coiled structures depicts the biopolymer added for stabilizing the emulsion droplet] (Sarkar, 2010)

The other common type of flocculation is bridging flocculation. Often, bridging flocculation arises when two or more oil droplets are adsorbed by a high molecular weight biopolymer at adequately low concentrations resulting in the formation of bridges. Predominantly, high molecular weight biopolymers are anticipated to give a better shield against flocculation. However, should it happen that the biopolymer added at lower levels has a great potential point of attachment to the droplet surface; there are higher chances that the different points of attachments may come across two different droplets rather than one attaching to the surface of the same droplet. Therefore the droplets may bridge together and result in the formation of flocculation (Sarkar, 2010). The mechanism of the depletion is indicated in Fig 2.7 below.

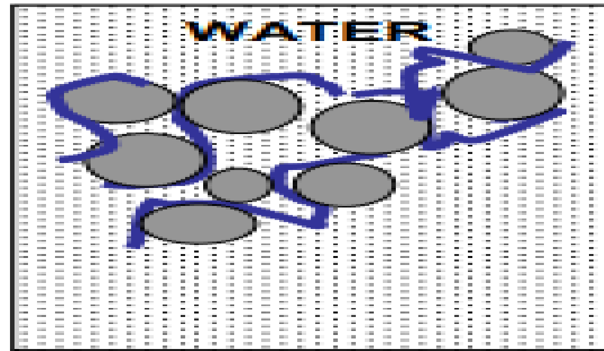

Figure 2.7: Bridging flocculation occurring in an oil-in-water emulsion. Particles approach one another due to the bridges developed. [Grey and dotted white depict the oil and water phases whereas the dark blue coiled structures depict the biopolymer added for stabilizing the emulsion droplet] (Sarkar, 2010)

2.6. Milk fat globule membrane and the impact it has on age gelation

Approximately 95 % of the total lipid in milk is in a form of a globule and their size ranges from 0.1 to 15 μm in diameter. The liquid fat droplets are normally coated by a thin membrane whose properties vary significantly from both milk-fat and plasma. The fat globule membrane (FGM) is made up of the apical plasma membrane of the secretory cell wall which frequently encloses the lipid droplets as they move through to the lumen. The principal constituents of the native FGM are the phospholipid trilayer containing associated proteins, carbohydrates and lipids derived from the membrane of the secreting mammary epithelial cell (lactocyte). The FGM reduces the lipid/serum interface to very low values, inhibiting the globules from instantaneous coalescence as well as shielding them from the enzymatic action (induced by either bacterial or plasmin proteases) which will lead to gelation of milk ("Dairy Science and Technology series, n.d").

Milk-fat is a very delicate biochemical compound in terms of auto-oxidation and is of complex composition (Muhammad *et al.,* 2018). The resistance of the milk fat globule membrane (FGM) to processing is to this day subject to research. The FGM complex structure consists of several components, for instance, the xanthine oxidoreductase which is situated in the FGM and it plays a structural role. Little information is known about the activity of the FGM enzymes and their roles. However, it is known that some enzymes may

be situated in the FGM or between the entrapped lipid droplets (crescents) and FGM (Kelly and Fox, 2006).

Moreover, not much is known about what happens to the FGM enzymes on storage, and whether or not the membrane breaks down and frees the enzymes into the milk. Nonetheless, it has been indicated by literature that plasmin enzyme can react on the FGM. Possibly, it could be through proteolysis of butyrophilin. It has also been highlighted that plasminogen and plasmin can be activated by oleic acid but there was no evidence to substantiate these findings. It is at this stage not evident whether or not this could pose a link between lipolysis and proteolysis as these findings and observations were not studied further (Kelly and Fox, 2006).

Additionally, Kelly and Fox (2006) reported that the activity of plasmin in milk may be activated or rather influenced by the products of lipid oxidation (free-fatty acids, FFA's) either by reacting instantly with the enzyme molecule or by casein micelle modification i.e. formation of chiff's bases.

2.6.1. The impact of lipase enzymes in UHT milk

Lipase is an enzyme which catalyses the breakdown of fat through the process of lipolysis. Lipolysis is a process of hydrolysis of milk lipids which results in the production of free-fatty acids as well as glycerides (Ray *et al.*, 2013). The lipolytic enzyme, lipoprotein lipase is responsible for most if not all of the lipolytic activity in milk (Olivecrona, 1980; Ray *et al.*, 2013). In blood, the lipoprotein lipase liberates the fatty acids from lipoproteins and chylomicrons. In order for this enzyme to function or rather act in the oil-in-water interface, it requires a cofactor apoprotein of certain lipoproteins to carry out its activities. This enzyme functions best at the optimum temperature of 33°C and at the optimum pH of 8.5 (Walstra *et al.*, 2006).

Lipases occur in skim milk and in whole or full-cream milk; however, the activity of lipases tends to be lower in skimmed milk than in whole milk. The higher levels of lipase in whole milk are due to the presence of their substrate that is triglycerides which are involved in lipolysis (Deeth *et al.*, 2005).

The lipids in milk exist as triglycerides and the action of lipase causes the breakdown of these triglycerides in which fatty acids are attached to the glycerol backbone. Even though milk lipoprotein lipase is destroyed by pasteurization, some bacterial lipase can withstand heat and

cause flavor defects in the heat-treated milk. It was further established that lipase from various psychrotolerant species retains about 30% of their initial activity in milk treated at 140°C for 5 sec (Dennil, 2015).

Through lipolysis, the free-fatty acids, diglycerides, and monoglycerides are released as by-products of hydrolysis and mainly the short to medium chain fatty acids are involved (Dannil, 2015). The release of particular medium chain fatty acids increased during extended storage periods at higher temperatures (Choi *et al*., 1993). These by-products (FFA) of lipase hydrolysis have a negative impact in milk; they induce strong flavours in milk. They also induce certain flavor defects such as rancid, astringent, bitter and sometimes desirable flavors in cheese and chocolates (Deeth, 2013).

Fatty acids are divided into two classes namely; unsaturated fatty acids and saturated fatty acids. The former consists of the linkage of carbon atoms in a chain by two or more double bonds in the hydrocarbon chain and in the latter; the carbon atoms are linked together in a chain by single bonds (Swaisgood, 2007).

Moreover, lipase enzymes are classified into two groups, milk lipase, and bacterial lipase. Milk lipase originates from the blood and it is present in all raw milk as a native enzyme. The main reason why lipase exists in milk is still not clear, nonetheless, it is inactivated by acid in the stomach of the neonate and it is also inactivated by conventional pasteurization (72° for 15s) treatment (Deeth, 2013). Inactivation means that the enzyme is chemically modified to such a degree that it can no longer act as a catalyst, thus reducing the activity of the enzyme (Walstra *et al*., 2006).

Thus, pasteurization prevents lipolysis, unless the milk fat globule in raw milk is physically damaged, therefore, enabling entry of lipoproteins lipase (LPL) to the milk fat triacylglycerol. Besides the natural lipolytic activity, milk may be subjected to lipase/esterase activities from contaminating bacteria (Law and Tamime, 2010).

Raw milk contains enough lipase to break down the fa,t but there are certain barriers which prevent the lipase action. Among them is the milk fat globule membrane (FGM) which protects the fat from being attacked by the lipase. Secondly, milk may lack activators that favour lipase and which can help accelerate the lipolysis process. It may also be that milk contains components which prevent lipolysis such as protease peptone components (Deeth, 2005; Walstra *et al*., 2006).

2.6.2. Lipolysis by milk lipase occurs in two ways

A. Spontaneous lipolysis

This is induced by a simple act of cooling at temperatures below 10°C immediately after the milk is has been withdrawn from the cow. Factors such as the amount of lipolytic activity, the integrity of milk fat globule membrane (FGM), and the balance between lipase activating-lipase inhibiting factors determine how prone the milk is to spontaneous lipolysis (Deeth, 2013). This form of lipolysis is known to occur in the milk of cows in late lactation, or under conditions of poor nutrition/feed, but it can be decreased by mixing milk together with the normal milk. The mixing method serves as an inhibitory effect upon spontaneous lipolysis and the levels of free-fatty acids (FFA) released are decreased contrary to the levels of FFA released by spontaneous lipolysis (Deeth, 2005; Deeth, 2013).

B. Induced lipolysis

This form of lipolysis is brought by physical damage to the FGM which would normally inhibit lipase from easily attacking the milk lipids. The physical damage includes factors such as homogenization, agitation, pumping, freezing, and thawing. These may occur on the farm or factories (Deeth, 2013). The rate at which induced lipolysis occurs depends greatly on the severity and duration of agitation as well as the temperature of milk during the agitation process. Homogenization, on the other hand, is known to activate the lipolysis of raw milk. The period of lipolysis, as well as the pressure of homogenization, has an impact on the lipolysis activity. The rate of lipolysis in homogenized milk depends greatly on the fat content, storage temperature and the length of time after homogenization (Ray *et al*., 2013). Homogenization of milk inhibits creaming by lowering the diameter and the size distribution of the fat globule. Homogenization also causes the formation of the recombined membrane. However, the recombined membrane varies significantly from the normal FGM. Homogenization is known to reduce the average diameter of the fat globule and increase the surface area significantly. Some of the FGM will stay adsorbed but there isn’t enough of it to cover the surface area that’s been created. The adsorbed layers are dominantly comprised of serum proteins and casein micelles (“Dairy Science and Technology series, n.d”).

2.6.3. Lipolysis by bacterial lipase

Microbial lipolysis is commonly caused by pre- or post-processing contamination which results in the production of off-flavors in milk. It is also indicated by literature that a lot of flavour defects are due to the high presence of psychrotolerant bacteria which secrete heat stable microbial lipase (Ray *et al*., 2013).

2.7. Inhibition of proteolytic activity responsible for age gelation in milk

Variations in distribution patterns and call for increased food quality have resulted in a desire to advance the shelf life of non-sterile dairy products. Refrigerated shelf life extension naturally requires, at a minimum, reductions in the growth rate of spoilage microorganisms and subsequent product deterioration. Lowering initial bacterial loads, increasing pasteurization regimes, and reducing post-processing contamination have all been employed with measured success (Hotchkiss *et al*., 2006). The following factors play a fundamental role in inhibiting the proteolytic activity in milk that leads to age gelation.

2.7.1. Protease inhibitors

In the plasmin activation system, there exists a plasminogen activator inhibitor (PAI) which plays a role of blocking the conversion of plasminogen to plasmin. The plasmin inhibitors (PI) block the plasmin activities which cause the degradation of the casein and later age gelation in “long life” milk. The PAI and PI are predominantly contained in the milk serum. Due to the complexes with other milk protein, the inhibitors might come in different forms. The heat stability of PAI and PI still warrants study, however thus far inhibitors are suggested to be thermally unstable. Thus, the heat treatment applied to milk such as pasteurization and UHT treatment interrupts the natural stability between the activators and the inhibitors in favor of plasmin and thereby increases the proteolytic action (Rollema and Poll, 1986; Chavan *et al*., 2011). Datta and Deeth (2001) reported that in retarding age gelation, denaturation of plasminogen activators has to be emphasized because they are three times more heat stable than plasminogen.

2.7.2. Addition of Inhibitors

Serine protease inhibitors normally added to milk inhibit the activity of the plasmin and is also known to retard the gelation processes. Examples of these inhibitors are the trypsin

inhibitor, the aprotinin protease inhibitor and Di-isopropyl fluorophosphates (Datta and Deeth, 2001).

The other two most important protease inhibitors are plasminogen activator inhibitor-1 and α-2-antiplasmin. These two inhibitors were isolated from bovine milk. However, as much as they confer the capacity to inhibit the plasmin enzyme activities in milk, they are heat sensitive. Thus for them to be functional, they need to be added aseptically after heat processing (Datta and Deeth, 2001).

Through an investigation conducted by Lu and Nielsen (1993), it was reported that the quality and the shelf life of UHT milk was extended when the plasminogen activator inhibitor-1 was added at a concentration of 125 mg/mL and at this concentration, there were no traces of plasminogen activators.

2.7.3. Bubbling gases through milk

The use of gases such as carbon dioxide, which is a naturally occurring milk component, and nitrogen can retard the growth of all psychrotolerant bacteria mainly *Pseudomonas* protease that secretes heat-stable enzymes that eventually result in age gelation (Hotchkiss *et al*., 2006). At low temperatures, these gases have the capacity to minimize the secretion or rather the action of proteinase enzymes (Sorhaung and Stepaniak, 1997). Tamime (2008) also established that carbon dioxide bubbled through milk acts as a preservative in that it has antimicrobial properties against psychrotolerant bacteria but the precise mechanism is not fully understood (Hotchkiss *et al*., 2006).

Carbon dioxide (CO_2) addition through modified atmosphere packaging or direct injection is a cost-effective shelf life extension approach and is commercially used worldwide for some dairy products and currently considered for use in other products as well. New CO_2 technologies are being proposed for the betterment of shelf life, quality, and yield of a diversity of dairy products, including raw and pasteurized milk, cheeses, cottage cheese, yogurt, and fermented dairy beverages. In addition, the largest inhibition occurs with Gram-negative psychrotrophs, particularly *Pseudomonas* spp., and the least inhibition impact is generally observed with Gram-positive psychrotrophs, particularly *Lactobacillus* spp. (Hotchkiss *et al*., 2006).

Factors such as species, substrate, and CO_2 concentration influence the effect of CO_2 on spoilage. Mechanisms by which CO_2 hinders the growth of microorganisms include the displacement of O_2; lowering of the pH in the medium or food due to the dissolution of CO_2 and formation of carbonic acid in the aqueous phase of the food in the following equilibrium (Hotchkiss *et al*., 2006).

While literature shows that CO_2 has a direct impact on the metabolic processes of certain microorganisms, the manner in which this effect is manifested is not well understood. There is evidence to support at least 3 mechanisms, including changes in membrane fluidity due to CO_2 dissolution, reductions in intracellular pH, and direct inhibition of metabolic pathways, including decarboxylation reactions and DNA replication (Hotchkiss *et al*., 2006).

2.7.4. Addition of additives in milk

2.7.4.1. Ethylene-diamine-tetra-acetic acid (EDTA)

The addition of any foreign matter in milk has to be carefully considered since it may pose some negative impact on consumer acceptance and also have legal implications. However, the addition of additives seems to be a very efficient approach in delaying age gelation and flocculation. Chavan *et al*. (2011) established that EDTA acts as a chelator and it binds metals.

The proteases isolated from *Pseudomonas* spp. are metalloenzymes, meaning that they depend greatly on the metals to be active. Thus, the addition of EDTA helps bind these metals and inactivate the enzyme. Additionally, milk contains ferritin with iron (Fe) content but unfortunately, it becomes deactivated during pasteurization processes. It was further established that additives such as sodium hexametaphosphate (SHMP) and calcium chloride had an impact on the inhibition of gelation and flocculation in milk Chavan *et al*. (2011).

2.7.4.2. Lacto-peroxidase enzyme (Antibacterial agent)

The lactoperoxidase enzyme is known to catalyse the oxidation of thiocyanate (SCN) by hydrogen peroxide (H_2O_2) to give hypothiocyanite (OSCN) and other oxyacids which prevent the growth of most bacteria. Milk has ample amounts of lactoperoxidase (about 0.4 µM) but the concentration of thiocyanate in milk is inconsistent because it depends greatly on the cyanoglucoside content of the feed (Walstra *et al*., 2006).

Sorhaug and Stepaniak (1997) established that the milk has a natural lactoperoxidase system. This system is made up of lactoperoxidase enzyme which functions as a natural antibacterial agent and its activity can be stimulated by the addition of hydrogen peroxide and thiocyanate. Walstra *et al*., (2006) added that hydrogen peroxide and thiocyanate system can be enhanced by xanthine and maybe sulfhydryl oxidase which can give rise to H_2O_2 from other substrates. Another bacteriociodal enzyme known as lysozyme hydrolyzes polysaccharides of bacterial cell walls and ultimately causes the lysis of the bacterial cell. However, the activity of lysozyme in cow's milk is very weak contrary to that in human milk.

Xanthine oxidoreductase can yield a number of antimicrobial agents, involving the reactive oxygen and nitrogen species. Based on this finding, this enzyme may be applied as an additive for infant food and it can be used to supplement the antimicrobial systems of the neonatal gut. Xanthine oxidoreductase is also known to play certain roles in the immune system and it also possesses fundamental non-enzymatic roles in the structure of FGM in milk and the secretion of the fat globule from the mammary gland (Kelly and Fox, 2006).

In work done by Barret *et al*., (1999), it was established that the quality and the shelf life of milk was increased when the milk was pasteurized at 72°C for 15 sec. The reason behind this is that lacto-peroxidase becomes completely deactivated at higher temperatures e.g. 80°C for 15s (including that of UHT treatments) whereas the residual peroxidase was only found to be 70% in the milk pasteurized at 72°C for 15s. The major antimicrobial agent produced by the lacto-peroxidase system known as hypothiocyanite was found in higher concentration in milk pasteurized at 72°C than when milk was pasteurized at 80°C. Thus, this supports the rationale that the lactoperoxidase plays a tremendous role in increasing the shelf life of pasteurized milk.

However, the use of antimicrobial additives has been discouraged predominantly due to labeling requirements and perceived toxicity risks (Hotchkiss *et al.,* 2006).

2.7.5. Low-temperature inactivation (LTI) or Thermisation of milk

Barach *et al.,* (1976) established that low-temperature inactivation at 55°C for 30-60 min is one way of inactivating the heat stable bacterial proteinase as well as plasmin in milk and their destruction at sub-sterilization temperature is feasible. This kind of treatment is inexpensive and rendered safe in reducing the defects caused by heat resistant bacterial proteinase in milk.

The degree of LTI does not depend on the proteinase concentration, it can inactivate the proteinase be it at low concentrations or much higher. LTI can be done before or after sterilization. Moreover, Chavan *et al*., (2011) established that the LTI is productive up to a period of 60 min and above that; the degree of LTI when combined with UHT treatment becomes similar to that of UHT treatment alone. The reason for this is the auto-digestion at 55°C but the changes in the inactivation characteristic are still unknown. Barach *et al.,* (1976) added that the flavor of milk was not changed even when the treatment destroyed about 65% of the proteinase in milk. The degradation of the k-casein in milk by heat or proteolytic activity leads to age gelation, but during inactivation by low-temperature treatment, there was no gelation observed in milk with a higher concentration of proteinase than that of normal milk.

2.8. Conclusions

It is evident that milk can undergo gelation and it causes an enormous financial loss in the dairy industry. Age gelation is as a result of casein destabilization during storage of UHT milk and it occurs through various mechanisms. The problem of age gelation in milk is mostly brought about the action of plasmin native milk protease and bacterial proteinase from psychrotrophs and other factors that make a contribution**.** After UHT treatment**,** most of the plasmin protease and bacterial protease is destroyed by heat but the residual protease remains which have the capacity to destabilize the casein and cause age gelation. Despite the knowledge of all the causative factors that contribute to age gelation of UHT milk, the problem still persists. The need to understand the mechanisms of this phenomenon in-depth therefore still exists. Methods meant to retard age gelation in milk have been implemented but there are disadvantages associated with them. Therefore the need to look for other options and methods to combat this problem is on-going.

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Chapter 3: The Influence of Milk Fat on Age Gelation in Milk

3.1. Introduction

Approximately all of the fat in milk is in the form of fat globules and the fat can be concentrated with the aid of gravity induced creaming. Fat is known to be susceptible to deterioration, resulting in off-flavors (Walstra, *et al*., 2006). Milk fat is secreted by the mammary epithelial cells as fat globules and it consists of an assortment of different fatty acid (FA) esters known as triacylglycerols which are generally termed triglycerides. It also consists of a mixture of compounds such as sterols esters, carotenoids, vitamins as well as free fatty acids (FFA's). The average size of the milk fat globule ranges from 1 to 18 µm (Swaisgood, 2007).

Thus far, more than 440 different types of free-fatty acids have been found in milk and the triglycerides contribute more than 98% of the milk lipids composition. The composition of the fatty acids and the fat content is greatly dependent on the diet of the cow (Forsbäck, 2010). The milk fat globule is enclosed by a lipid bilayer membrane which is consists of proteins, phospholipids, and lipoprotein, enzymes, trace elements as well as bound water. The purpose of the fat globule membrane is to keep the fat globule stable in an aqueous environment of the milk serum (Swaisgood, 2007). Triglycerides have a glyceride backbone to which the free fatty acids are attached by an ester bond (Forsbäck, 2010). No other negative impact of milk fat was reported other than the possibility of resulting off-flavors (Deeth., 2013).

3.2. Objectives:

1: To investigate the susceptibility of UHT milk with a varying fat concentration towards proteolytic activity.

2: To investigate the impact of fatty acids on the activation of plasminogen to plasmin protease in homogenized and un-homogenized raw full cream milk.

3: To identify the major fatty acids present in milk and to evaluate their individual and combined roles on the activation of inactive plasminogen.

3.3. Materials and Reagents

Objective 1: To investigate the susceptibility of UHT milk with a varying fat concentration towards proteolytic activity.

3.3.1. Milk

UHT (fat-free, low-fat and full-cream) milk was purchased from a local supermarket Checkers supermarket in Brandwag, Bloemfontein, Free State (RSA).

3.3.2. Enzymes/proteases

The commercial enzymes, Bovine plasmin (10602370001) and *Bacillus* (P5380) proteinases were purchased from Sigma-Aldrich Chemicals South Africa. The two proteolytic enzymes had an activity of 5 U/mg for plasmin protease and 11 U/mg of *Bacillus* protease, respectively. Due to the high activity of the *Bacillus* protease, the enzyme was diluted 10 times (1.1 U/ml).

3.3.3. Acidic precipitation of casein

Hydrochloric acid (HCL) and Trichloro-acetic acid (TCA) was purchased from Sigma-Aldrich chemical South Africa.

3.3.4. Merck protease assay kit

The protease assay kit (catalog no: 539125-1KIT) was purchased from Sigma-Aldrich (SA) and it was used to assay the protease activity as prescribed by the supplier.

3.3.5. Sodium azide

NaN_3 purchased from Merck (SA) was used to prevent the microbial growth.
(Gloves were used and the solution was discarded after H_20 dilution (1 in 100) and this practise was carried out throughout the experiment in the subsequent chapters).

Objective 2: To investigate the impact of fatty acids on the activation of plasminogen to plasmin protease in homogenized and un-homogenized raw full cream milk.

3.3.6. Raw full-cream milk

Raw full-cream milk was collected from a local dairy plant, Dairy Cooperation, Bloemfontein, Free-State, (RSA). The milk was homogenized using a homogenizer (Panda 200, Gea Nitro Soavi) at 50 bar in the Dairy Laboratory, Food Science Division, University of the Free-State, Bloemfontein campus (RSA).

3.3.7. *Pseudomonas* Lipase enzyme

The commercial *Pseudomonas* lipase enzyme (L9518) was purchased from Sigma-Aldrich (RSA).

3.3.8. Plasminogen (PG) from bovine plasma, ε-Aminocaproic acid-free

Plasminogen (P9156) was purchased from Sigma-Aldrich (SA).

3.3.9. Phosphate buffered saline tablets

Phosphate buffered saline tablets (P-4417) were purchased from Sigma-Aldrich (RSA).

Objective 3: To identify the major fatty acids present in milk and to evaluate their individual and combined roles on the activation of inactive plasminogen.

3.3.10. Free-fatty acids

The individual free-fatty acids were purchased from Sigma-Aldrich (RSA), namely, Butyric acid (C4), catalog no: (B103500), 2-keto-D-Gluconic acid (C6), catalog no: (K6250) , Hexanoic acid (C6), catalog no: (153745), Octanoic acid (C8), catalog no: (C2875), Decanoic acid (C10), catalog no: (W236403), DNP-ε-amino-N-Caproic acid (C12), catalog no: (D7754), Lauric acid (C12), catalog no: (W261408), Tridecanoic acid (C13), catalog no: (T0502), Myristic acid (C14), catalog no: (M3128), Palmitic acid (C16), (catalog no: W283215), Stearic acid (C18), batch no: (Batch: 32325), Linoleic acid C18), catalog no: (L1376) and Oleic acid (C18:1), (catalog no: 01257) were used.

3.4. Methods

Objective 1: To investigate the susceptibility of UHT milk with a varying fat concentration towards proteolytic activity.

3.4.1. Treatment of UHT milk with different fat concentrations by commercial proteases (*Bacillus* protease and plasmin protease)

The experiment was done using UHT fat-free, low-fat, and full-cream milk respectively. Commercial proteolytic proteases (*Bacillus* and plasmin) were added to the various batches of milk with different fat contents. A Foss MilkcoScan Minor 6 was used to determine the fat content of the various batches of milk and the fat content in fat-free milk was 0%, 1.06% in low-fat milk, and 2.73% in full-cream milk, respectively. A concentration of 10 µL plasmin protease was added to each UHT-milk (990 µL) along with 0.01 g sodium azide to prevent microbial growth. The final activity of the plasmin protease used was 0.05 U/mL. For *Bacillus* protease, 5 µL was added to each UHT-milk (995 µL) along with 0.01 g of sodium azide. The final activity of *Bacillus* protease was 0.0055 U/mL. The proteases were added independently in each milk samples.

The milk samples were divided into two sets. In the first set of samples, the reaction was stopped by heating (boiling) at 90°C for 10 min. The casein was precipitated using 0.1 N HCL to lower the pH (acidification) in order to precipitate the casein and centrifuged by a bench top Eppendorf Centrifuge (Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan), at the speed of 10 000 rpm for 20 min. The supernatant of the samples was analysed using RP-HPLC. The second set of samples were directly precipitated using 0.1 N HCl acid without stopping the reaction by heating and centrifuged at 10 000 rpm for 20 min. The supernatants obtained were analysed using a Merck protease assay kit in conjunction with a Merck protease assay using a spectrophotometer (CE 2021, 2000 series supplied by Lasec RSA).

3.4.2. Running conditions for Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

A stock solution of 0.25 g/100 mL vanillyl-alcohol in distilled water was prepared and a 100x dilution was used. Vanillyl-alcohol (50 µL of the 100x dilution) was added as a reference of retention time (standard) to the supernatant of all the samples.

The mobile phase used was acetonitrile (CH_3CN) with a concentration gradient that ranged between 0 and 65% over a period of 90 min. An automatic injector injected 10 µL of the samples per run. The column used was a 150 mm C 18 column with an internal diameter of 4.6 mm and the temperature was maintained at 40 °C. The column was eluted at 1 mL/min. The peptide profiles were detected using an ultraviolet detector set at 214 nm. The data were integrated using the HPLC data processing Shimadzu software (Kupiec, 2004).

3.4.2.1. Analysis of peptide profiles using the Reverse-Phase High Performance Liquid Chromatography

Aliquots of 200 µL clear supernatant collected in section 3.4.1 were stored in various Eppendorf tubes prior to RP-HPLC analysis at -20°C. Immediately before the RP-HPLC, the samples were thawed and transferred to RP-HPLC vials.

Objective 2: To investigate the impact of fatty acids on the activation of plasminogen to plasmin protease in homogenized and un-homogenized raw full cream milk.

3.4.3. Preparation of various stock solutions:

3.4.3.1. Plasminogen phosphate buffer stock solution

The stock solution was prepared by adding 10 mg of plasminogen (PG) to 1 mL of distilled water and 300 µL of glycerol in a 2 mL Eppendorf tube. A phosphate buffer solution was also prepared by adding 1 tablet into 200 mL of distilled water resulting in a 0.01 M phosphate buffer at pH 7,4. The plasminogen phosphate buffer solution was prepared by adding 1 mL of plasminogen stock solution to 4 mL phosphate buffer into a 30 mL cuvette tube.

3.4.3.2. *Pseudomonas* lipase enzyme stock solution

The *Pseudomonas* lipase stock solution was prepared by adding 1 mL of distilled water to 1 mL glycerol and 5 U (original activity) of *Pseudomonas* Lipase enzyme powder.

3.4.4. The treatment of raw milk with commercial *Pseudomonas* lipase enzyme

The experiment was performed using un-homogenized raw full-cream milk with a fat content of 3.22% and homogenized raw full-cream milk with a fat content 3.09% determined by using a Foss MilkcoScan Minor 6. A concentration of 10 µL commercial *Pseudomonas* lipase enzyme was added to 990 µL of homogenized raw full-cream milk and un-homogenized raw full-cream milk independently; 0.01 g sodium azide (Merck SA) was also added (to prevent

all possible microbial growth) and incubated for 6 hours at 37°C. During this step, the *Pseudomonas* lipase enzyme released fatty acids (FA) as the by-products of fat hydrolysis.

After 6 hours incubation, 50 µL of plasminogen phosphate buffer was added to each sample (950 µL) and it was carefully mixed and incubated for additional 6 hours at 37°C to enable the by-products of hydrolysis (FA) to either activate or not activate the plasminogen to plasmin protease. Subsequently, these samples were divided into two batches. In the first batch, the process was stopped by heating (boiling) at 90°C for 10 min. The casein present in samples were precipitated by adding 250 µL of 0.1 N HCl acid and centrifuged by an Eppendorf Centrifuge at the speed of 10 000 rpm for 20 min. The clear supernatant of the samples was analysed using RP-HPLC. In the second batch, the samples were directly precipitated without stopping the reaction by boiling and centrifuged at 10 000 rpm for 20 min. The supernatant was analysed by the Merck protease assay and the milk agar plate method.

3.4.5. Preparation of milk agar for protease detection

A total volume of 250 mL of milk agar (bacteriological grade) solution was prepared by using two (2X) 250 mL Erlenmeyer flasks. The first Erlenmeyer flask (250 mL) contained 50 mL of UHT low-fat milk with 0.01g sodium azide. The second Erlenmeyer flask (250 mL) contained 200 mL of distilled water and 1% nutrient agar (≈5g of agar). The UHT low-fat milk was heated at 55°C in the water bath and the flask with the agar was heated in the boiling water bath at 90°C until the agar was completely melted. Once the agar was completely melted, the flask was cooled to 55°C for 10 minutes. The solutions in the two flasks were then mixed together in a 500 mL Schott bottle and sterilized by autoclaving at 121°C for 20 minutes. After the sterilization process, the milk agar solution was cooled down to 50°C prior to plate pouring.

Aliquots of the sterile milk agar (10 mL) solution were aseptically poured into separate sterile Petri dishes. After the milk agar solution was cooled and set, the Petri dishes were incubated upside down at room temperature for 24 hours to dry. The Petri dishes were then wrapped in plastic and stored in a refrigerator at 4°C.

The supernatant (5 µL) from each sample collected in section 3.4.4 was pipetted on top of the milk agar plates and incubated for 24h to observe the formation of the clear protein

hydrolysis zones (Hattingh, 2017). This milk agar method is the modification of the method established by Fransen *et al*., 1997.

3.4.6. The detection of plasmin activity in homogenized and un-homogenized raw full-cream milk using the Merck protease assay

The protease assay kit consisted of the following reagents: The FTC-casein that contained 10 mL of 0.6% FTC casein in 50 nM Tris-HCl (pH 7.3), the incubation buffer that was 10 mL of 200 mM Tris-HCl (pH 7.8), 20 mM $CaCl_2$, 0.1% NaN_3, the assay buffer which was 120 mL of 500 mM Tris-HCl (pH 8.8) and the protease positive control that had 1 mL of 1 mg/mL trypsin in phosphate buffered saline (PBS) with 10 mg/mL bovine serum albumin (BSA).

The samples were prepared according to the following protocol: A volume of 25 µL of the incubation buffer was added followed by 50 µL of the samples supernatant (the clear supernatant obtained under section 3.4.4 and 25 µL of FTC-casein. The reagent blank was prepared by adding 25 µL of the incubation buffer followed by 50 µL of distilled water and 25 µL FTC-casein. The protease positive control was prepared by adding 10 µL of protease positive control and 90 µL of distilled water.

All the prepared samples were incubated for 24 hours at 37°C in the water bath. Five percent TCA was added to the reaction mixture in order to precipitate the remaining FTC-casein and to stop the reaction. The samples were mixed by a vortex mixer (Vortex Genie-2) and incubated for 10 min at 37°C. The samples were centrifuged in an Eppendorf centrifuge for 20 min and the clear supernatant obtained was placed into new clean Eppendorf tubes. A volume of 300 µL of the assay buffer was added and mixed well with the samples supernatant. The absorbance of the samples was read at 492 nm in a Cecil (CE 2021, 2000 Series) spectrophotometer supplied by Lasec (SA) to evaluate the total protease activity (U/mL).

The concept of the protease assay is based on the hydrolysis of the FTC-casein in the milk sample when it is incubated for 24 hours at 37°C. The bond in the FTC-casein is broken down by the protease in the milk samples resulting in the formation of the casein plus the chromophoric substance. After the hydrolysis by the proteases, there is still FTC-casein remaining that is precipitated using TCA resulting in a yellow chromophoric substance. The yellow chromophore was measured with the aid of a spectrophotometer at 492 nm. The

intensity of the color formed was directly proportional to the total protease activity in the sample (Wiesner and Troll, 1982; Twining, 1984; Hattingh, 2017).

In this experiment, the protease assay was performed to establish the proteolytic level of plasmin (PL) protease activity (U/mL) induced by PG activators (FA's) in homogenized and un-homogenized raw full-cream milk. Samples 1 and 2 in Table 3.1 were the supernatant of the milk treated with commercial *Pseudomonas* lipase enzyme while plasminogen and sample 3 is the negative control.

Table 3.1: Summary of milk samples used for protease assay

Sample	FTC-casein (µL)	Incubation Buffer (µL)	DH_2O (µL)	Protease positive control (µL)	Homogenized raw full-cream milk (µL)	Un-homogenized raw full-cream milk (µL)	Raw full-cream milk (control) (µL)
Reagent Blank	25	25	50	0	0	0	0
PPC	0	0	90	10	0	0	0
Supernatant of samples treated with Plasmin protease							
Sample 1	25	25	0	0	0	0	0
Sample 2	25	25	0	0	50	0	0
Sample 3	25	25	0	0	0	50	50

* **FTC** = Fluorescein thio-carbamoyl casein
* **PPC** = Protease Positive control

3.4.7. Analysis of peptide profiles using Reverse-Phase High Performance Liquid Chromatography

Aliquots of 200 µL clear supernatant collected in section 3.4.4 were stored in Eppendorf tubes prior to RP-HPLC analysis at -20°C. Immediately before the RP-HPLC, the samples were thawed and transferred into corresponding RP-HPLC vials. During the RP-HPLC

analysis, similar running conditions were applied as mentioned above under objective 1 section 3.4.2.

Objective 3: To identify the major fatty acids naturally present in milk and to evaluate their individual and combined roles on the activation of inactive plasminogen

3.4.8. Identification of total fatty acids composition in milk using Gas Chromatography

Raw full-cream milk (4 950 µL) was treated with 50 µL of *Pseudomonas* lipase enzyme and the sample was incubated in the water bath at 37°C for 6 h. *Pseudomonas* enzyme was chosen for this study because it is one of the predominant psychrotolerant organisms found in milk that produces lipase which causes problems in milk. Total fatty acids which are the by-products of fat hydrolysis were firstly quantitatively extracted and identified using Gas Chromatography (GC). The extraction was done by quantitatively extracting the lipids from milk samples according to the method of Folch *et al.,* (1957), using chloroform and methanol in a ratio of 2:1. An antioxidant, butylated hydroxytoluene, was added at a concentration of 0.001 % to the chloroform: methanol mixture. A rotary evaporator was used to dry the fat extracts under vacuum and the extracts were dried overnight in a vacuum oven at 50°C, using phosphorus pentoxide as a moisture adsorbent. The extracted fat was stored in a polytop (glass vial, with push-in top) under a blanket of nitrogen and frozen at –20°C pending fatty acid analyses.

A lipid aliquot (±30 mg of the milk lipid) was converted to methyl esters by base-catalysed transesterification, in order to avoid CLA isomerisation, with sodium methoxide (0.5 M solution in anhydrous methanol) during 2 h at 30°C, as proposed by Park *et al*., (2001); Kramer *et al.,* (2002) and Alfaia *et al.,* (2007). Fatty acid methyl esters (FAMEs) from the were quantified using a Varian 430 flame ionization GC, with a fused silica capillary column, Chrompack CPSIL 88 (100 m length, 0.25 mm ID, 0.2 µm film thicknesses). The analysis was performed using an initial isothermic period (40°C for 2 minutes). Thereafter, the temperature was increased at a rate of 4°C/minute to 230°C. Finally, an isothermic period of 230°C for 10 minutes followed. FAMEs n-hexane (1µl) was injected into the column using a Varian CP 8400 Autosampler. The injection port and detector were both maintained at 250°C. Hydrogen, at 45 psi, functioned as the carrier gas, while nitrogen was employed as the makeup gas. Galaxy Chromatography Data System Software recorded the chromatograms.

3.4.9. Preparation of the FFA`s stock solutions

Stock solutions for each FFA in a powder or paste form was prepared by weighing 0.02 g of each FFA and individually added to 980 µL of the phosphate buffer. The samples were mixed using a vortex mixer for 2 min and incubated in the ultrasonic water bath at 50°C for 15 min to obtain a homogenous suspension. The FFA's cocktail was prepared similarly to what has beenas described above, but in this case, 10 µL of each FFA suspension was added into a 2 mL Eppendorf tube.

3.4.10. The activation of PG by various commercial FFA's

At a volume of 50 µL, commercial free-fatty acids based on their respective carbon content namely Butyric acid (C4), 2-keto-D-Gluconic acid (C6), Hexanoic acid (C6), Octanoic acid (C8), Decanoic acid (C10), DNP-ε-amino-N-Caproic acid (C12), Lauric acid (C12), Tridecanoic acid (C13), Myristic acid (C14), Palmitic acid (C16), Stearic acid (C18), Linoleic acid C18), and Oleic acid (C18:1) was added independently and as a cocktail to 950 µL of PG phosphate buffer. The samples were incubated for 3 h at 37°C to enable the FFA's to activate PG. After the incubation period, the samples were evaluated and assayed for the activation of PG to PL using the milk agar plate, and the Merck protease assay.

3.4.11. Detection of plasmin activation using the Merck protease assay kit

The same methodology for protease assay as mentioned under section 2 (3.4.6) was followed. In this experiment, the proteolytic level of PL protease activity (U/mL) induced by PG activators (commercial FFA's) was assayed.

3.4.12. Evaluation of clear zones in milk agar

The same methodology for milk agar test as mentioned under objective 2 (3.4.5) was followed.

3.4.13. Statistical analysis

Treatments were compared with one-way analysis of variance and the means differentiated with the Tukey-Kramer multiple comparison test at $\alpha = 0.05$ (NCCS 11 Statistical Software) (2016).

*NOTE: The experiments were done in triplicates.

3.5. Results and Discussion

Objective 1: To investigate the susceptibility of UHT milk with different fat concentrations towards proteolytic activity.

3.5.1. Analysis of proteolytic profiles of UHT milk with different fat concentrations using RP-HPLC

The samples UHT milk (fat-free, low-fat and full-cream) proteolyzed by commercial *Bacillus* protease or plasmin protease were evaluated using RP-HPLC after 6 hours of incubation. This experiment was done to evaluate peptide formation as a result of protease hydrolysis.

Hattingh (2017) established that commercial plasmin and *Bacillus* protease each liberates characteristic peptide profiles at different elution times on the chromatogram shown in Fig 3.1. From this chromatographic profile, four distinct regions were identified that are associated with the action of *Bacillus* protease and only three that are associated with the action of plasmin protease. Plasmin peaks are eluted in Region A (18-20 min), Region B (23-25 min) and mainly in Region E (50-65 min). *Bacillu*s protease peaks are eluted mainly in Region C (30-35 min), Region D (45-49 min), in Region E (50-65 min) and Region F (70-75 min). Hachana *et al.*, (2010) established that peptides released by bacterial hydrolysis (*Bacillus*) are eluted much earlier on the RP-HPLC chromatogram and they are less hydrophobic while peptides liberated by plasmin hydrolysis are eluted much later and they are more hydrophobic.

The software package generally used by RP-HPLC for peak integration was programmed to integrate all the peaks occurring within the above mention regions and express them as a total area of the whole region. Thus, when milk was proteolyzed by plasmin protease only the total area of region A (18-20 min), B (23-25 min) and E (50-65 min) of various chromatographic profiles (samples) were compared with one another. In the case of *Bacillus* protease, only the total area of region C (30-35 min), D (45-49 min), E (50-65 min) and G (70-75 min) of various chromatographic profiles (samples) was compared to one another. This method made it possible to determine the total area for each enzyme activity.

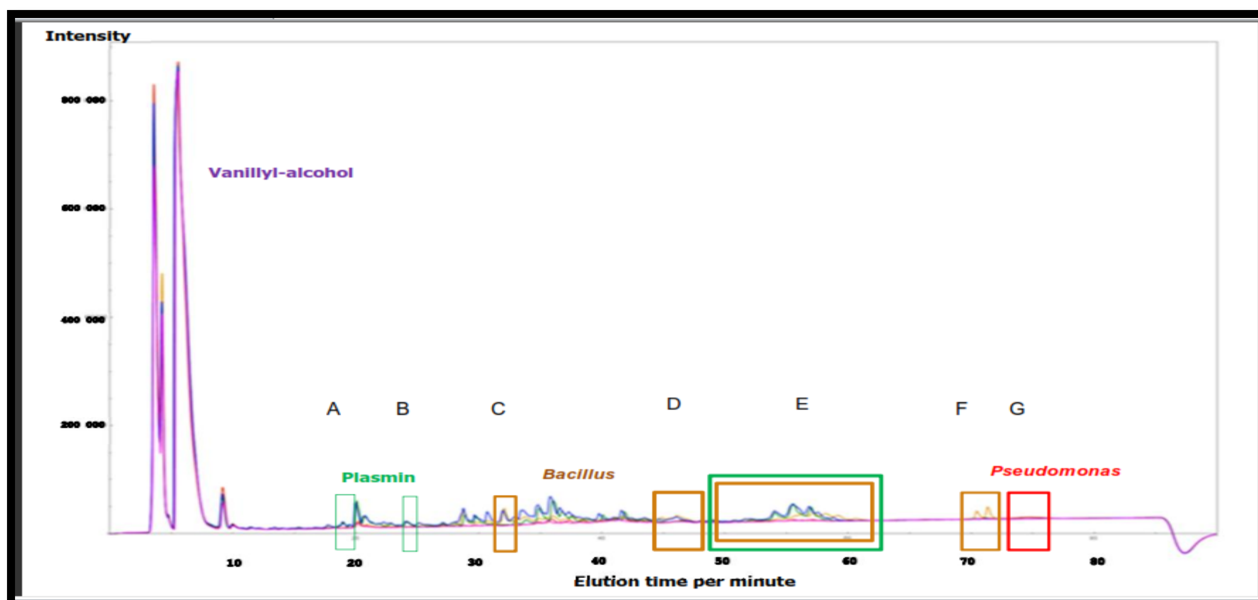

Figure 3.1: MILQC software created chromatograms of peptides liberated by Plasmin, *Bacillus* protease and *Pseudomonas* protease. The green blocks represent distinct conserved areas for Plasmin protease, orange for *Bacillus* protease and red for *Pseudomonas* protease (Hattingh, 2017)

3.5.1.1. Bacterial (*Bacillus* protease) hydrolysis in milk

The number of peptide peaks liberated indicates that the protease was able to digest the casein substrate, and as a result, releasing the peptides as the by-products of the bacterial/plasmin hydrolysis. The peptide profile formation Fig 3.2a was in region C, and E, clearly indicates that there are higher peptide peaks liberated in UHT fat-free milk (profile 4) and UHT low-fat milk (profile 3) than in UHT full-cream milk (profile 6). This indicated that the *Bacillus* protease digested the casein substrate unhindered or easier in UHT fat-free milk and UHT low-fat milk than in UHT full-cream milk. This can only be attributed to the number of fat globules present in each type of milk. UHT fat-free milk and UHT low-fat milk do not have enough fat globules to shield the casein substrate whilst the fat globules in UHT full-cream milk act as a steric hindrance against proteolytic *Bacillus* protease attack. Chavan *et al.*, (2011); Deeth *et al.*, (2002) also reported that UHT processed skim milk is more susceptible to gelation than UHT whole milk induced by psychrotolerant protease. This work was done to prove that amongst the species of pyschrotrophs, protease from *Bacillus* specifically, which is a problem in UHT milk, gives the same pattern of results. The susceptibility of various UHT milk types was not investigated on plasmin protease.

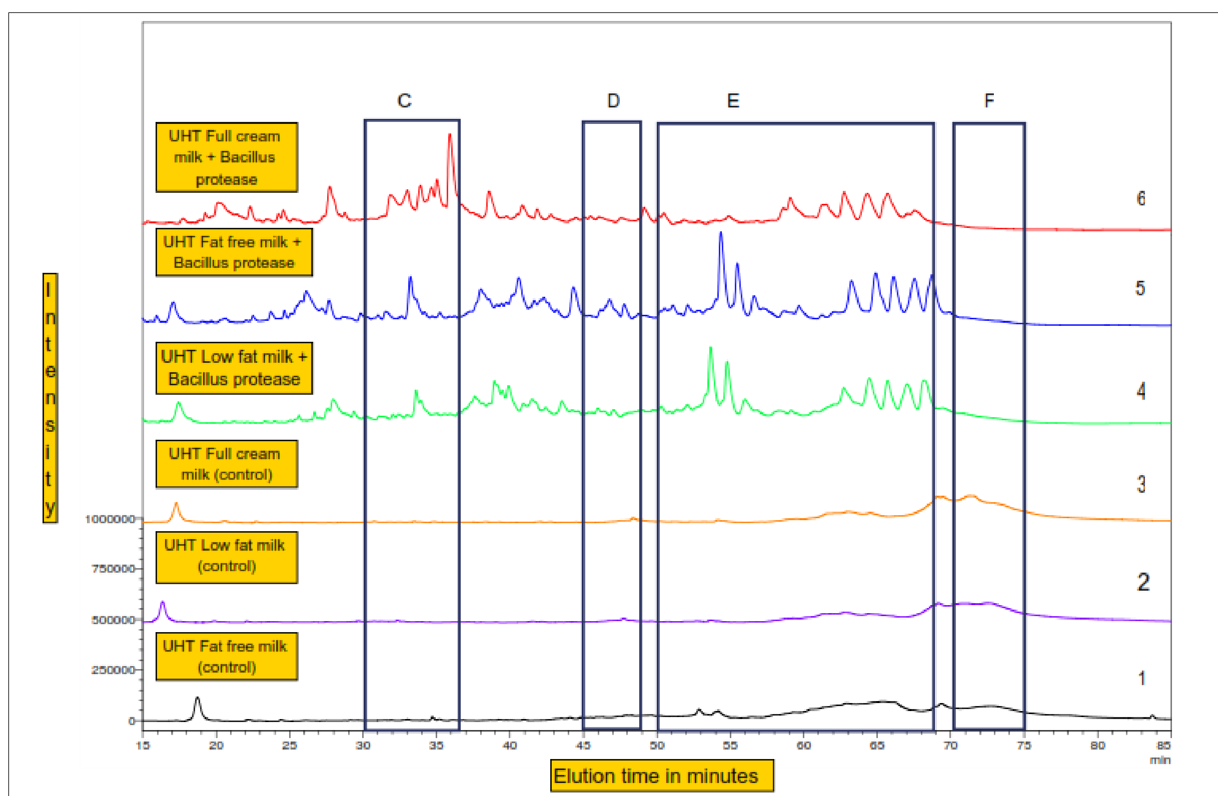

Figure 3.2a: RP-HPLC Chromatograms of UHT milk (fat-free, low-fat, and full-cream) hydrolyzed by commercial *Bacillus* protease (1) Fat-free milk (control); (2) Low-fat milk (control); (3) Full-cream milk (control); (4) Low-fat milk hydrolyzed by *Bacillus* protease; (5) Fat-free milk hydrolyzed by *Bacillus* protease and (6) Full-cream milk hydrolyzed by *Bacillus* protease

Thus, the extent of proteolytic activity from the chromatographic profiles was quantified in percentages. Therefore, the calculations were based on the total integrated area (retention time) characteristic for *Bacillus* protease (as mentioned in section 3.5.1). **The following equation was used;**

* TA = Total area

Percentage (%) = [(TA) of UHT milk proteolyzed by *Bacillus* protease / (TA) of UHT milk (control)] × 100

UHT fat-free milk was more susceptible to proteolytic attack when hydrolyzed by *Bacillus* protease and the bacterial activity was 363.7% in comparison to UHT full cream milk with the bacterial activity of 256.7% shown in Fig 3.2b. UHT low-fat milk was also susceptible to proteolytic attack when hydrolyzed by *Bacillus* protease and the bacterial activity was 360.4% in comparison with which UHT full-cream milk with the activity of 256.7%.

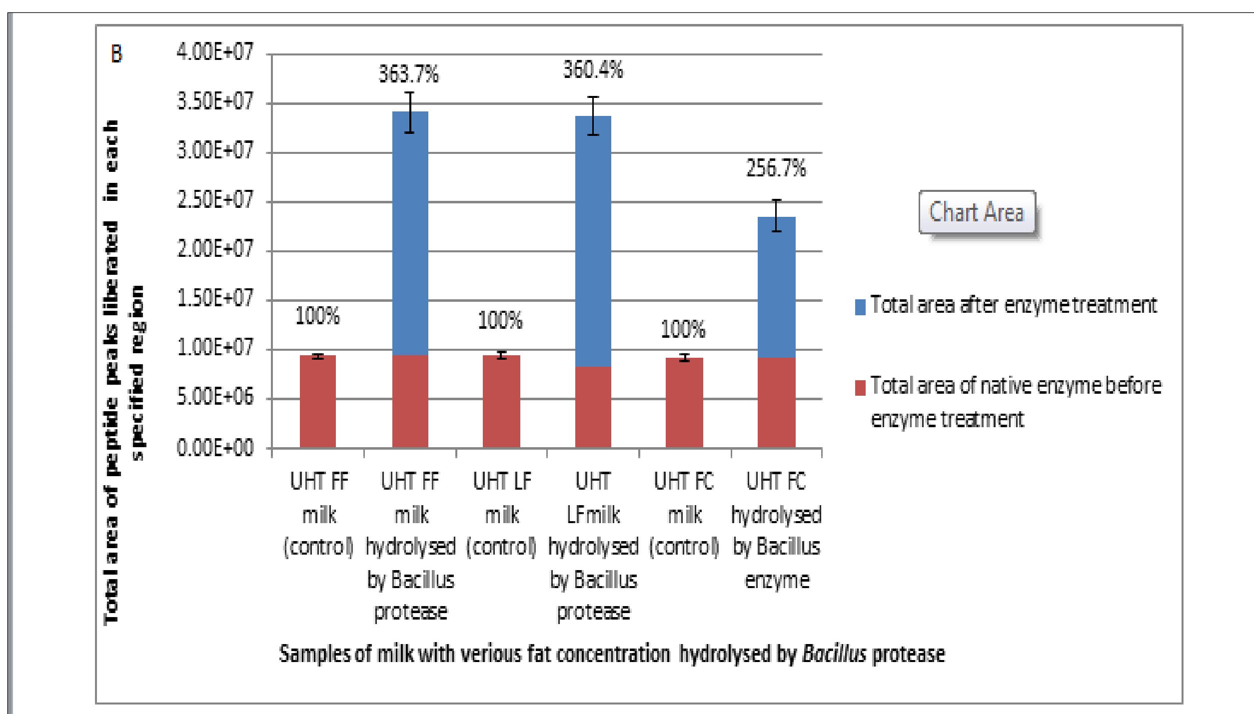

Figure 3.2b: Effect of fat content on UHT milk treated with *Bacillus* protease

***FF** = Fat free**, LF** = Low fat and **FC**= Full cream milk

3.5.1.2. Plasmin protease hydrolysis in milk

It was established in Fig 3.3a that the peptide peaks liberated as a result of plasmin hydrolysis in UHT fat-free, low-fat and full-cream milk were basically the same as shown in Fig 3.3a. There were no distinct differences in the area of peaks liberation. In work done by Chavan *et al.*, (2011); Deeth *et al.*, (2002), it was reported that UHT processed skim milk is more susceptible to gelation than UHT whole milk but nothing was stated about hydrolysis induced by plasmin protease in UHT milks. It is evident from the current work that the impact of plasmin hydrolysis is the same in UHT fat-free, low fat and full-cream milk.

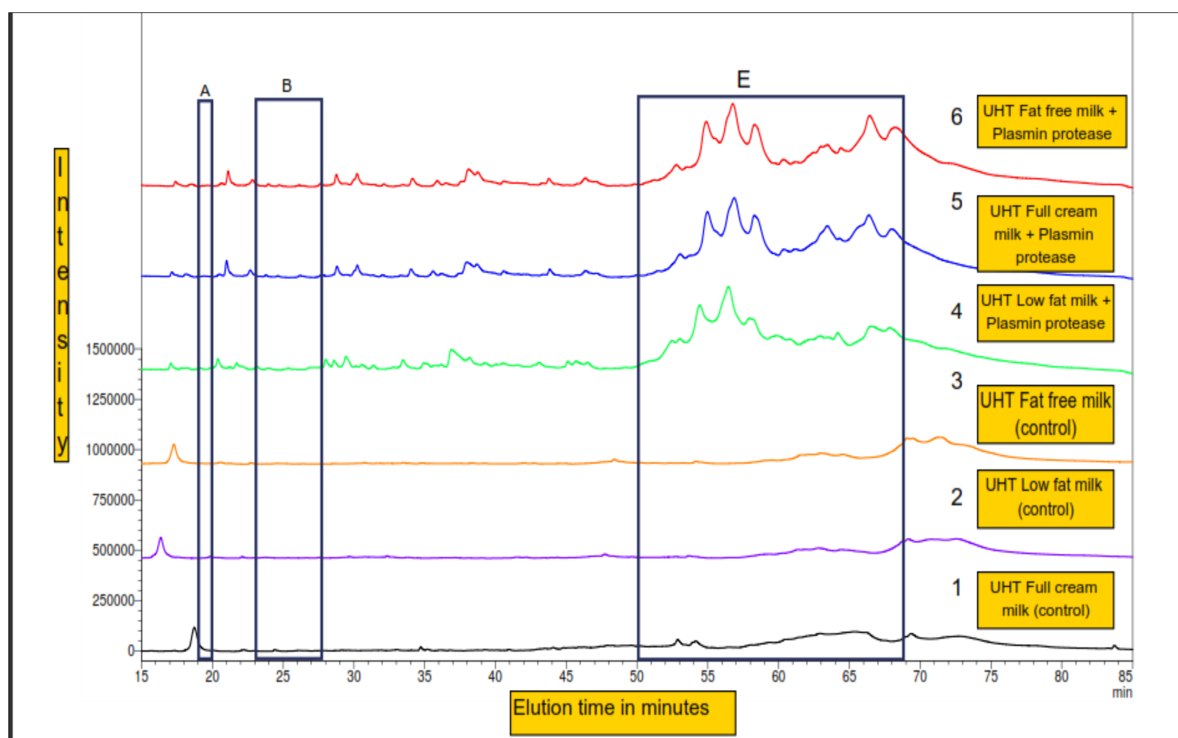

Figure 3.3a: RP-HPLC created Chromatograms of UHT milk (fat-free, low-fat, and full-cream) hydrolyzed by commercial plasmin protease. (1) Full-cream milk (control); (2) Low-fat milk (control); (3) Fat-free milk (control); (4) Full-cream milk hydrolyzed by plasmin protease; (5) Low-fat milk hydrolyzed by plasmin protease and (6) Fat-free milk hydrolyzed by plasmin protease

The extent of proteolytic activity was quantified in percentages. The calculation was based on the total integrated area (retention time) characteristic for plasmin protease (refer to section 3.5.1). **The following equation was used;**

Percentage (%) = [(TA) of UHT milk proteolyzed by PL protease / (TA) of UHT milk (control)] $\times$ 100

The plasmin protease activity was 937.4% in UHT fat-free milk, 940.7% in UHT low-fat milk and 938.2% in UHT full-cream milk as shown in Fig 3.3b. This meant that the number of fat globules did not play any role in hindering the access of plasmin protease towards digesting the casein substrate.

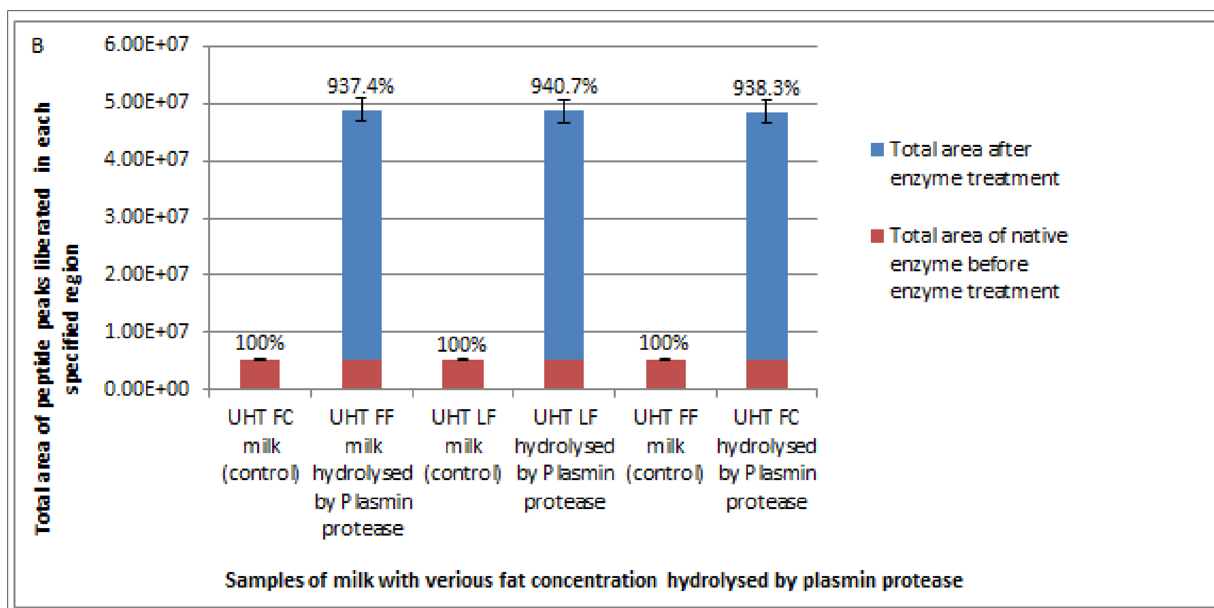

Figure 3.3b: Effect of fat content on UHT milk treated with plasmin protease

Objective 2: To investigate the impact of fatty-acids on the activation of inactive plasminogen to plasmin protease in homogenized and un-homogenized raw full-cream milk.

3.5.2. Evaluation of clear zones using milk agar plates

Milk agar plates is by far the most cost-effective and yet very simple, sensitive and rapid test used in dairy laboratories and dairy plants to evaluate the impact proteolytic enzymes have in milk (Vijayaraghavan *et al.*, 2013). This test was done to determine whether or not the liberated fatty acids by lipase from *Pseudomonas* in homogenized and un-homogenized milk play any role in the activation of plasminogen to plasmin protease. T

It was evident from Fig 3.4 that when milk was not pre-treated with *Pseudomonas* lipase enzyme (A) there was no activation of PG to PL taking place and therefore no formation of clear zones. However, when un-homogenized raw full-cream milk and homogenized milk were pre-treated with the lipase from *Pseudomonas* lipase enzyme (B, C, D, and E), the by-products of fat hydrolysis (FA's) activated PG to PL (zone indicative of protease activity). Therefore, plasmin protease hydrolyzed the casein present in the milk agar resulting in the formation of clear zones. The sharp edges of the zone perimeters are indicative of typical plasmin hydrolysis (Hattingh, A. (2017).

Figure 3.4: **Milk agar plate to prove PG to PL activation. 'A' depicts Raw milk with no enzyme; 'B' and 'D' depicts un-homogenized milk with *Pseudomonas* lipase enzyme pre-treatment; 'C' and 'E' depicts homogenized milk with *Pseudomonas* lipase enzyme pre-treatment**

3.5.3. Analysis of proteolytic activity in homogenized and un-homogenized milk using the Merck protease assay

The protease assay was done on the supernatant of different milk samples (Un-homogenized raw full-cream milk and homogenized raw full-cream milk) treated with *Pseudomonas* lipase enzyme.

Table 3.2 below exhibits the activity values (U/mL) obtained after *Pseudomonas* lipase pre-treatment. Comparing the un-homogenized raw full-cream milk (0.400 U/mL) and homogenized raw full-cream milk (0.403 U/mL) to the control sample (0.359 U/mL), it is clearly indicated that there was proteolytic activity in milk samples pre-treated with *Pseudomonas* lipase enzyme. The latter meant that the FA's released as the by-products of fat hydrolysis activated PG to PL in milk. However, there were no pronounced differences in the activation level between homogenized and un-homogenized raw full-cream milk.

Table 3.2: Proteolytic activity (U/mL) evaluated using Merck protease assay

Sample name	Proteolytic activity U/mL			
	1	**2**	**3**	**Proteolytic activity**
Raw full cream milk (control)	0.358	0.359	0.356	0.359 ± 0.001
Un-homogenized raw full cream milk	0.401	0.400	0.399	0.400 ± 0.001
Homogenized raw full cream milk	0.403	0.403	0.404	0.403 ± 0.001

3.5.4. The RP-HPLC proteolytic peptide profile after *Pseudomonas* lipase enzyme treatment of milk

The chromatographic profile in Fig 3.5a exhibits the peptide peaks eluted at various distinct regions associated with the action of plasmin protease. It was evident that there were similar high levels (integration area) of peptide peaks liberated from un-homogenized (profile 2) and homogenized raw full-cream (profile 3) in comparison to raw full-cream milk that was not pre-treated with *Pseudomonas* lipase enzyme.

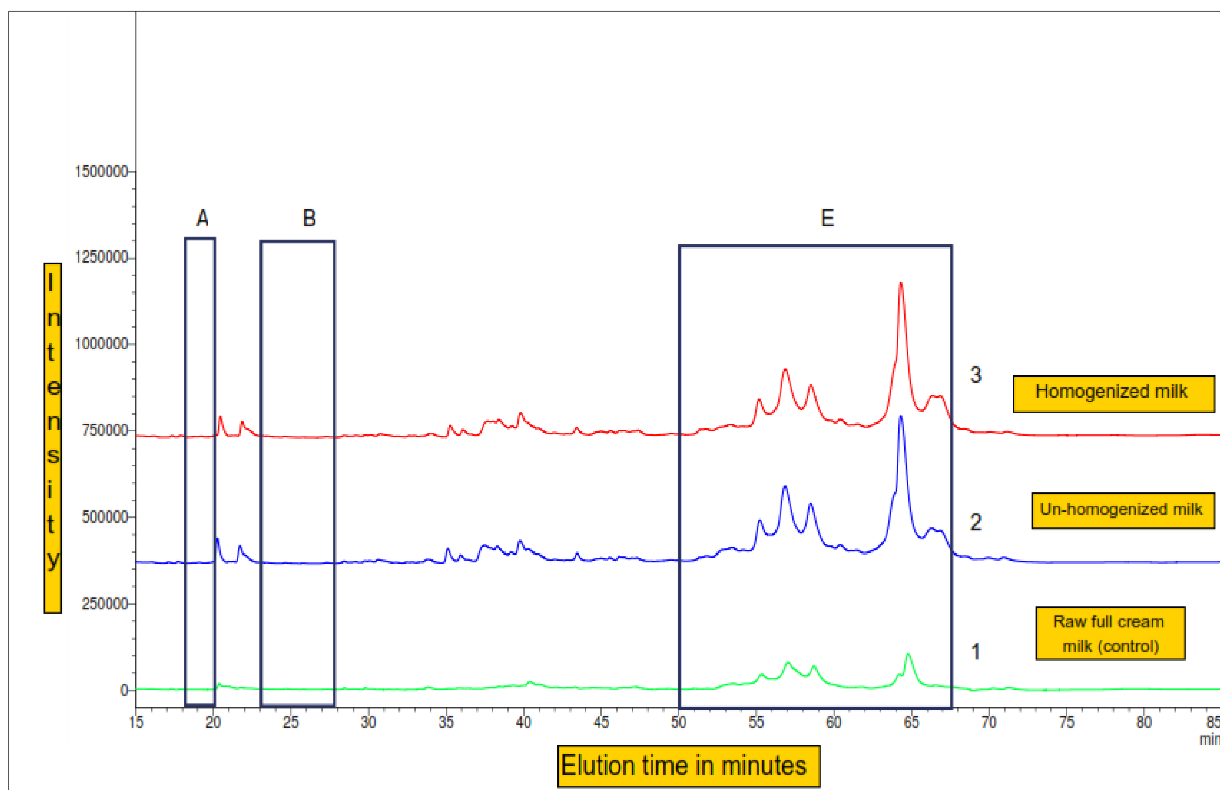

Figure 3.5a: RP-HPLC chromatogram of peptides in raw milk. (1) Raw full-cream milk (control); (2) un-homogenized raw full-cream milk and (3) homogenized raw full-cream milk

It was established that the by-products of lipase hydrolysis activate plasminogen to plasmin in both milk types (homogenized and un-homogenized raw full-cream milk) when compared to untreated raw full-cream milk. The plasmin proteolytic activity present in un-homogenized raw full cream milk was 290.2% and 290.7% for homogenized milk respectively as shown in Fig 3.5b.

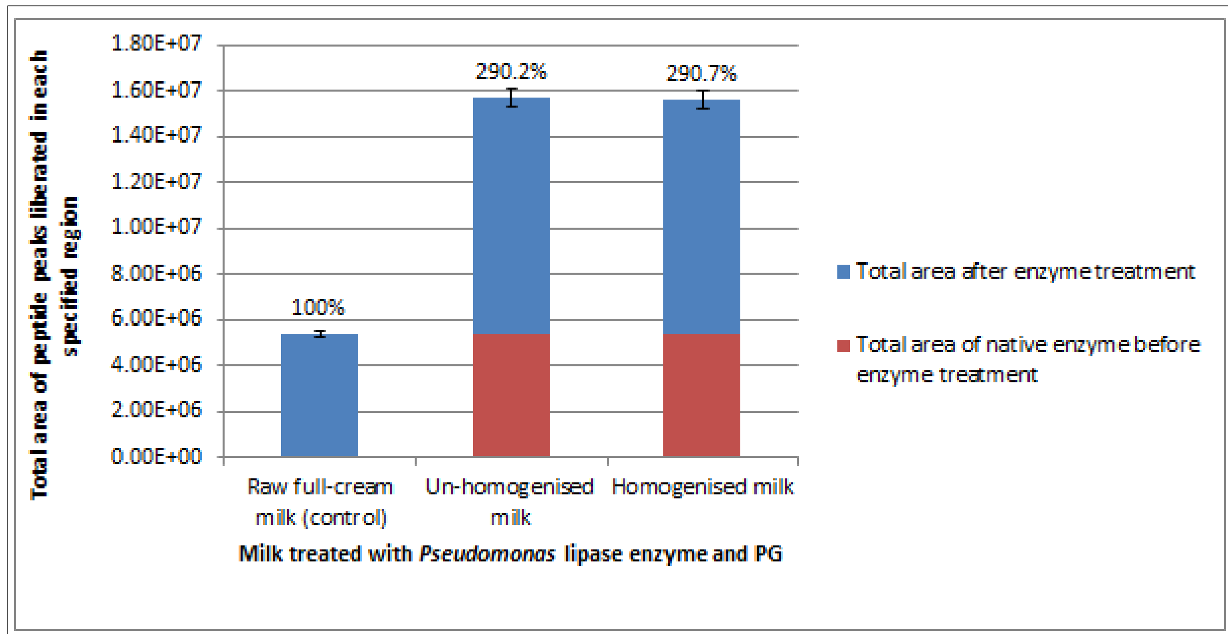

Figure 3.5b: Activation of plasminogen to plasmin protease in milk by plasminogen activators (FA)

Objective 3: To identify the major fatty acids present in milk and to evaluate their individual and combined roles on the activation of inactive plasminogen.

3.5.5. Identification of the total fatty acids composition in milk using Gas Chromatography

Milk is a good source of short and medium chain fatty acids that are not common in other animal fat (C12 and shorter). Table 3.3 shows the total fatty acid composition in raw full-cream milk. The composition of FA's corresponds to the FA's composition data established by Lopez *et al*., (2014).

Table 3.3: Composition of total fatty acids present in raw full-cream milk

FA's common name and abbreviation	FA composition (%)			Average fatty acid composition
Butyric (C4:0)*	0.89	0.14	0.15	0.39 ± 0.43
Gluconic acid (C6:0)*	0.56	0.56	0.63	0.80 ± 0.35
Octanoic acid (8:0)*	0.78	0.78	0.85	0.85 ± 0.08
Decanoic acid (C10:0)*	2.64	2.45	2.63	2.57 ± 0.11
Lauric (C12:0)*	3.16	3.16	3.25	3.29 ± 0.11
DNP-ε-amino-N-caproic acid (C12)*	4.61	4.41	4.58	4.50 ± 0.01
Tridecoic (C13:0)*	0.05	0.06	0.07	0.06 ± 0.01
Myristic (C14:0)*	12.12	12.02	12.44	12.19 ± 0.22
Myristoleic (C14:1c9)	0.92	0.88	0.93	0.91 ± 0.03
Palmitic (C16:0)*	35.32	35.70	36.25	35.76 ± 0.47
Palmitoleic (C16:1c9)	1.37	1.31	1.37	1.35 ± 0.03
Stearic acid (C18:0)*	12.23	12.36	12.54	12.38 ± 0.16
Oleic (C18:1c9)*	21.87	22.77	21.73	22.12 ± 0.57
Vaccenic (C18: 1c7)	0.92	1.61	0.89	1.13 ± 0.41
Nonoadecanoic (C19:0)	0.40	0.44	0.41	0.42 ± 0.02
Linoleic (C18:2c9,12 (n-6))	2.58	2.52	2.51	2.53 ± 0.04
Eicosenoic (C20:1c11)	0.15	0.09	0.09	0.09 ± 0.01
α-Linolenic (C18:3c9,12,15 (n-3))	0.40	0.38	0.39	0.90 ± 0.01
Conjugated linoleic acid (C18:2c9t11 (n-6) (CLA)	0.28	0.26	0.30	0.28 ± 0.02
Arachidonic (C20:4 4c5, 8, 11, 14 (n-6)	0.9	0.10	0.10	0.1 ± 0.001

Fatty acids marked with a star (*) were further evaluated in the experiments (present study)

3.5.6. Analysis of plasmin protease activity induced by various FFA's using the Merck protease assay kit

Table 3.4: Proteolytic activity (U/mL) evaluated using Merck protease assay

Sample name	**Proteolytic activity U/mL**			
	1	**2**	**3**	Proteolytic activity
Butyric acid (C4)	0.925	0.92	0.922	0.92^{h} ± 0.01
2-keto-gluconic acid (C6)	0.537	0.53	0.522	0.53^{d} ± 0.01
Hexanoic acid (C6)	0.74	0.742	0.699	0.73^{g} ± 0.01
Octanoic acid (C8)	0.377	0.399	0.398	0.39^{b} ± 0.01
Decanoic acid (C10)	0.499	0.492	0.497	0.50^{c} ± 0.01
DNP-ε-amino-N-caproic acid (C12)	0.497	0.494	0.492	0.49^{c} ± 0.01
Lauric acid (C12)	0.338	0.33	0.339	0.34^{a} ± 0.01
Tridecanoic acid (C13)	0.355	0.356	0.352	0.34^{a} ± 0.01
Myristic acid (C14)	0.498	0.497	0.499	0.50^{c} ± 0.01
Palmitic acid (C16)	0.713	0.699	0.711	0.71^{g} ± 0.01
Stearic acid (C18)	0.4	0.412	0.401	0.40^{b} ± 0.01
Linoleic acid (C18)	0.401	0.4	0.399	0.40^{b} ± 0.01
Oleic acid (C18:1)	0.622	0.616	0.612	0.62^{f} ± 0.01
Cocktail (FFA's)	0.56	0.571	0.562	0.56^{e} ± 0.01
Positive control (KIO3)	0.472	0.485	0.47	0.48^{c} ± 0.01
Negative control	0.332	0.343	0.338	0.34^{a} ± 0.01
Significance level	<0.001			

Means with different superscript in the same column differs significantly (the superscripts mean that the fatty acids differ from one another significantly and the same superscripts mean that the fatty acids don't differ significantly).

From the results evaluated using the Merck protease assay in Table 3.4, it was evident that there were significant differences in plasmin proteolytic activity in all the samples with the exception of samples treated with Lauric acid (C12) (0.336 U/mL), and Tridecanoic acid (C13) (0.354 U/mL), that showed no significant differences when compared to a negative control (0.338 U/mL). Most significant plasmin activity was observed in samples treated (decreasing order), Butyric acid (C4) (0.922 U/mL), Hexanoic acid (C6) (0.727 U/mL), Palmitic acid (C16) (0.708 U/mL), Oleic acid (C18:1) (0.612 U/mL), cocktail of all the free-fatty acids (0.577 U/mL), 2-keto-D-Gluconic acid (C6) (0.530 U/mL), Decanoic acid (C10) (0.496 U/mL)), Myristic acid (C14) (0.498 U/mL), DNP-ε-amino-N-Caproic acid (C12) (0.494 U/mL), Linoleic acid (C18) (0.402 U/mL), Stearic acid (C18) (0.404 U/mL), and Octanoic acid (C8) (0.391 U/mL), Table 3.4 above when compared against a negative control (0.338 U/mL) (plasminogen without FFA's activators). This activity indicated that PG was activated significantly to PL by the FFA's mentioned above. The positive control (KIO_3)

indicated that indeed, PG can be activated to PL protease and showed PL activity of 0.48 U/mL. Thus far, Kelly and Fox (2006), established that plasminogen (PG) in milk is activated to plasmin (PL) protease by the free-fatty acid oleic acid but there was no evidence to substantiate the findings.

3.5.7. Evaluation of clear zones using the milk agar test

This test was done to determine whether or not the commercial FFA's have the ability to activate the inactive PG to PL protease. Thus, after the incubation of plates for 24 hours at 32°C, clearing zones were visible as shown in Fig 3.6.

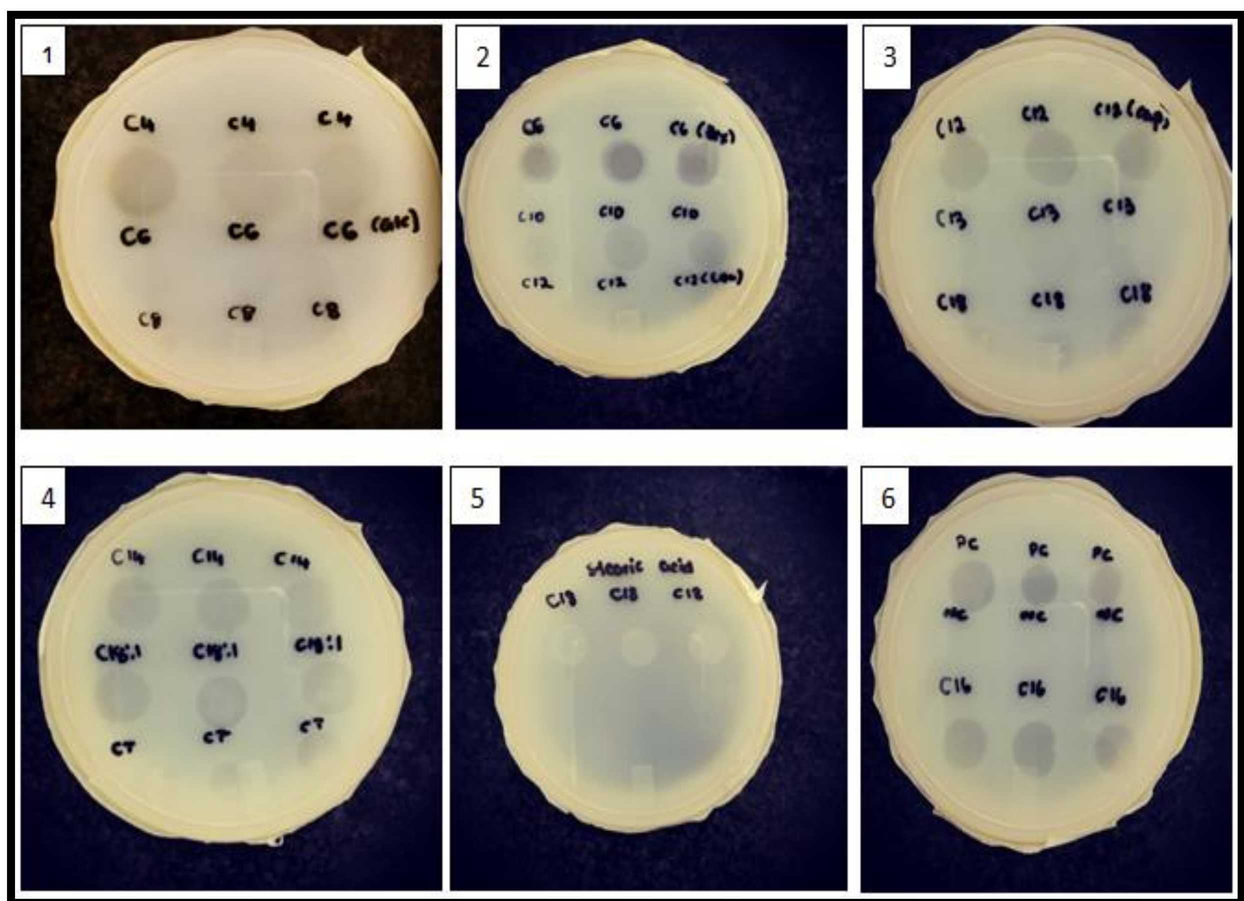

Figure 3.6: Casein digestion as a result of PG activation by various FFA's to PL protease

***CT**= Cocktail of FFA`s**, PC** = Positive control**, NC** = Negative control

The individual and combined roles of FFA's were evaluated on the milk agar plate. It was established that there were clear zones formed as a result of PG activation to PL-induced by FFA's namely, Butyric acid (C4, plate 1), Hexanoic acid (C6, plate 2), Decanoic acid (C10,

plate 2), DNP-ε-amino-N-Caproic acid (C12, plate 3), Linoleic acid (C18, plate 3), Myristic acid (C14, plate 4), Oleic acid (C18:1, plate 4), Palmitic acid (C16, plate 6), Stearic acid (C18, plate 5) and the cocktail of all the free-fatty acids (CT, plate 4) as shown in Fig 3.6 above. Also, it is important to note that PG can only be activated to PL and no other compound, the formation of zones with the sharp edge confirmed that the activity was due to PL protease action. Thus, the formation of clearing zones by plasmin was compared against a negative control (PG in the absence of FFA's activators) (NC, plate 6). The FFA's namely, Lauric acid (C12), and Tridecanoic acid (C13) did not exhibit zones of hydrolysis in the plates. It is also important to note that some of the zones could not be captured by a camera (8 megapixels, Samsung Galaxy S4 mini) which was 2-keto-D-Gluconic acid (C6, plate 1), and Octanoic acid (C8, plate 1). The results obtained in the milk agar test are in correspondence with the results obtained using the Merck Assay method.

3.6. Conclusions

It was confirmed that there were no differences in the hydrolysis of UHT milk in either fat-free, low-fat, or full-cream milk when hydrolysed by plasmin protease. Contrary to the above findings, UHT fat-free milk is more susceptible to proteolytic attack than UHT full-cream milk when subjected by bacterial protease (*Bacillus*). This indicated that fat globules in UHT full-cream milk hindered the access of proteolytic bacterial proteinase more than in the case of plasmin protease.

Due to the fact that plasmin protease is indigenous in milk and can be a major cause of proteolysis in milk, one of the factors (FFA's) thought to be responsible for the activation of plasminogen to plasmin were investigated in homogenized and un-homogenized raw full-cream milk. This was done to evaluate whether the FFA's released during fat hydrolysis by the *Pseudomonas* lipase enzyme activates inactive PG to PL protease. It was evident that whether milk was homogenized or not, the by-products of fat hydrolysis (FFA's) by the bacterial lipase enzyme activate plasminogen to plasmin equally in both milk types (homogenized and un-homogenized raw full-cream milk).

However, it was deemed necessary to determine which of the FFA's liberated by *Pseudomonas* lipase enzyme were responsible for the activation of PG to PL protease, therefore further investigations were performed to study the individual and combined roles of

free-fatty acids on plasminogen activation. It was evident that the short, medium and long chain free fatty acids have the ability to activate plasminogen to plasmin protease namely, Butyric acid (C4), Hexanoic acid (C6), 2-keto-D-Gluconic acid (C6), Octanoic acid (C8), Decanoic acid (C10), DNP-ε-amino-N-Caproic acid (C12), Myristic (C14), Palmitic acid (C16), Stearic acid (C18), Linoleic acid (C18), Oleic acid (C18:1) and the cocktail (combination of FFA's). The FFA's namely, Lauric acid (C12), and Tridecanoic acid (C13), however, showed no significant differences in plasminogen activation when compared to the negative control.

3.7. References

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Chapter 4: The Impact of Temperature on the Digestibility of the Casein Protein

4.1. Introduction

A major limiting factor for the shelf life of milk is proteolysis induced mainly by proteases. Proteases play a bigger role than any other enzymes in dairy products (Höök, 2015). The occurrence of proteolysis in milk makes either a negative or positive contribution depending on the product meant to be processed (Ismail, *et al*., 2011). It is good for cheese ripening and bad in UHT milk because it results in destabilization of the casein protein, i.e. age gelation (Datta and Deeth, 2001). Proteolysis in milk is attributed to the indigenous milk enzyme, plasmin protease and proteases secreted by psychrotolerant microorganisms during storage of the raw milk (Nielsen, 2002). Plasmin protease is heat stable and it is known to play a negative role in the firmness and safety of milk (Kelly and Foley, 1997) and may result in gelation and formation of off-flavors (Hachana *et al*., 2010). Proteolysis is also increased when plasmin (PL) protease is converted from its inactive form, plasminogen (PG). Plasminogen in milk is activated by plasminogen activators (PGA) such as the oxidizing agent, potassium iodate (KIO_3) due to its ability to prevent the β-lactoglobulin-plasmin complexation. Plasmin and plasminogen are both heat stable in the absence of β-lactoglobulin (Kelly and Foley, 1997). The activation of PG to PL occurs when the bond Arg557-Ile558 in PG is cleaved whilst the milk is located in the lumen of the mammary gland and also during storage (Höök, 2015).

In addition to plasmin protease, psychrotolerant microorganisms dominate the microbial flora of milk during cold storage and they are accountable for many problems associated with dairy products. The number of psychrotrophs that evolve after milking is determined by storage temperatures, time and sanitary conditions (Nielsen, 2002). The optimal temperature for bacterial protease synthesis is 20-30°C but substantial amounts of protease are secreted at lower temperatures 5°C (Nielson, 2002). The increase in enzyme activity is associated with the lactation stage, lactation number as well as the extent of mastitic infection (Hachana *et al.,* 2010).

4.2. Objective:

1: To investigate the impact of refrigeration temperature (5°C) in comparison to room temperatures (25°C) on the digestibility of casein protein.

4.3. Materials and Reagents

4.3.1. Milk

Raw full-cream milk supplied by Dairy Cooperation, Bloemfontein, Free-State (RSA) was used. The fat composition was 3.22% as measured by a Foss MilkcoScan.

4.3.2. Commercial Bovine Plasmin protease

The commercial enzymes, Bovine plasmin (10602370001) was purchased from Sigma-Aldrich chemical (RSA). The protease had an activity of 5 U/mg.

4.3.3. Chemicals (Potassium Iodate KIO_3)

Potassium Iodate (KIO_3), with a molar mass of (214 g/mol) was supplied by Sigma- Aldrich (RSA).

4.3.4. Acidic precipitation of casein

Hydrochloric acid (batch no: 10108772) was purchased from Sigma-Aldrich Chemical (RSA) and a 0.1 N solution was used to lower the pH (acidification) in order to precipitate the casein of all the samples.

4.3.5. Alizarol test

The 68% Alizarol solution was purchased from Selectech South Africa.

4.3.5. Sodium azide

NaN_3 purchased from Merck (SA) was used to prevent the microbial growth.

4.4. Methods

4.4.1. Treatment of raw full-cream milk with Potassium Iodate (PG activator to PL) and commercial Plasmin protease

Raw full-cream milk (100 mL) was measured and 0.01 g sodium azide was added to prevent bacterial growth during incubation (Kelly and Foley, 1997). The pH of the raw-full cream milk was measured before treatment and it was 6.66. Potassium Iodate (KIO_3) stock solution of 0.2 M KIO_3 was prepared and that involved the addition of 0.214 g KIO_3 to 5 mL of distilled water. Raw full-cream milk (980 µL) was treated with 10 µL of KIO_3 along with 10 µL of plasminogen phosphate buffer (PG phosphate buffer stock solution preparation was the same as in chapter 3, section 3.4.3.1). The milk samples were incubated at varying temperature conditions (5°C and 25°C) up to 96 h.

The commercial enzyme, Bovine plasmin was used. A concentration of 10 µL was added to 990 µL of raw full-cream milk. The final activity of the plasmin protease used was 0.05 U/mL. The milk samples were incubated under different temperature conditions (5°C and 25°C) up to 96 h. The experiment was done in triplicates.

4.4.2. The incubation of samples treated with KIO_3 and commercial Plasmin protease independently

The samples were incubated in the refrigerator 5°C and at room temperature 25°C for a period of 96 hours. After time 1 h, 24 h, 48 h and 96 h of incubation at 5°C and 25°C respectively, the samples were subjected to heating in the water bath at 90°C for 10 min to stop the enzymatic reaction induced by plasminogen activation (KIO_3) to plasmin protease and the direct action of the commercial (pure) plasmin protease. The casein in the samples was precipitated with 0.1 N HCl acid and centrifuged by a bench top Eppendorf Centrifuge (Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan), at the speed of 10 000 rpm for 20 min. The supernatants of the samples were analysed using RP-HPLC with the same conditions as indicated in chapter 3.

4.4.3. The determination of milk quality by the alizarol test

For the test, 200 µL of alizarol test solution was mixed with 200 µL of milk in Eppendorf tubes. The mixture was carefully mixed by inverting the Eppendorfs twice. The samples

were evaluated by observing the color change and the presence of precipitates (flakes). Table 4.1 shows the guideline used to interpret the alizarol test.

Table 4.1: Interpretation of the alizarol test (Kurwijila, 2006)

Guidelines	Normal milk	Slightly acidic milk	Strongly acidic milk	Alkaline milk
pH	6.6—6.7	6.4—6.5	6.3 or lower	6.8 or higher
Colour	Red-brown	Yellowish-Brownish	Yellowish	Violet
Appearance	No formation of flakes	No flakes formed	The occurrence of flakes, yellowish in colour	The occurrence of flakes, violet in colour; clots and flakes are formed

4.4.4. The milk agar test for protease detection

The same methodologies as stipulated in Chapter 3, section 3.4.5 were applied.

4.4.5. Running conditions for Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

The same RP-HPLC running conditions as stipulated in Chapter 3, section 4.4.2 was performed.

4.4.5.1. Analysis of peptide profiles using Reverse-Phase High Performance Liquid Chromatography

Aliquots of 200 μL clear supernatant collected in section 4.4.2 were stored in various Eppendorf tubes prior to RP-HPLC analysis at -20°C. Immediately before the RP-HPLC, the samples were thawed and transferred into corresponding RP-HPLC vials.

4.5. Results and Discussion

4.5.1. Assessment of proteolysis in milk incubated at 5°C and 25°C respectively

4.5.1.1. pH of the milk

Before the milk was tested for quality using the alizarol test, the pH of the milk was measured. Table 4.2 shows the pH of the milk after treatment with KIO_3 and PL protease independently incubated at 5°C and 25°C. It was evident that the pH of the milk increased as a function of time in both temperature conditions when compared to time zero of treatment.

Table 4.2: The pH levels in milk treated with KIO_3 and PL protease incubated at 5°C and 25°C

Milk samples	pH values	
Normal milk	**6.6**	**6.6**
Treatment of milk samples	**KIO_3**	**PL**
Raw full-cream milk stored at 5°C for 0 h	**6.6**	**6.6**
Raw full-cream milk stored at 25°C for 0 h	**6.6**	**6.6**
Raw full-cream milk stored at 5°C for 48 h	**6.9**	**6.9**
Raw full-cream milk stored at 25°C for 48 h	**6.8**	**6.9**

4.5.1.2. The Alizarol test

The Fig 4.1a shows that raw full-cream milk tested alizarol negative at time 0 after treatment with KIO_3 for milk incubated at 5°C shown by Tube 3. The same alizarol results were obtained for milk incubated at 25°C shown by Tube 4. Milk samples treated with KIO_3 incubated at 5°C Tube 1 and 25°C Tube 2 for 48 h tested alizarol positive. The colour of the sample changed to violet and fine flakes were observed in raw full-cream milk Tube 1 and Tube 2.

Figure 4.1a: Raw full-cream milk tested by the alizarol test for quality at varying times after treatment with KIO_3. Tube 1; raw full-cream milk sample incubated at 5°C for 48 h; Tube 2; raw full-cream milk sample incubated at 25°C for 48 h; Tube 3; raw full-cream milk sample incubated at 5°C for 0 h and Tube 4; raw full-cream milk sample incubated at 25°C for 0 h

The same trend was observed for milk samples treated with plasmin protease shown in Fig 4.1b. The alizarol test proved that raw full-cream milk tested alizarol negative at time 0 after treatment with PL protease for milk incubated at 5°C shown by Tube 3. The same alizarol results were obtained for milk incubated at 25°C shown by (Tube 4). Milk samples treated with PL protease incubated at 5°C Tube 1 and 25°C Tube 2 for 48 h tested alizarol positive. The colour of the sample changed to violet and fine flakes were observed as well as precipitates of the casein in raw full-cream milk Tube 1 and Tube 2.

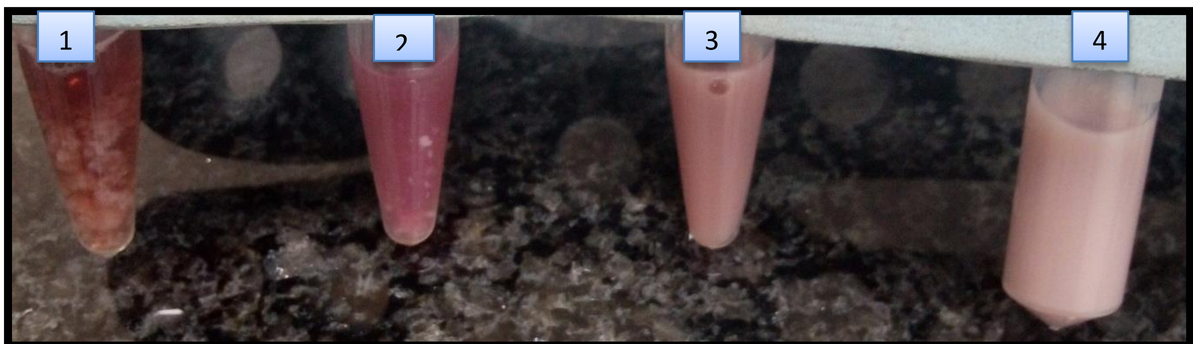

Figure 4.1b: Raw full-cream milk tested by the alizarol test for quality at varying times after treatment with plasmin protease. Tube 1; raw full-cream milk sample incubated at 5°C with PL for 48 h; Tube 2; raw full-cream milk sample incubated at 25°C with PL protease for 48 h; Tube 3; raw full-cream milk sample incubated at 5°C with PL for 0 h and Tube 4; raw full-cream milk sample incubated at 25°C with PL for 0 h

4.5.2. Evaluation of clear zones using milk agar plates

4.5.2.1. Potassium Iodate (KIO_3) treatment and plasmin treatment

It was evident through the use of the milk agar test in Fig. 4.2 that at time 0 (plate 1), there were no halos observed on the plates which meant that at time 0 of incubation, KIO_3 did not activate plasminogen to plasmin. Only after incubation at 5°C and 25°C for 48 h and 96 h (plate 2 and 3), raw full-cream milk samples exhibited clear zones (hydrolysed the casein protein). The same trend of results in the milk agar plate was seen in milk treated with commercial plasmin protease (PL).

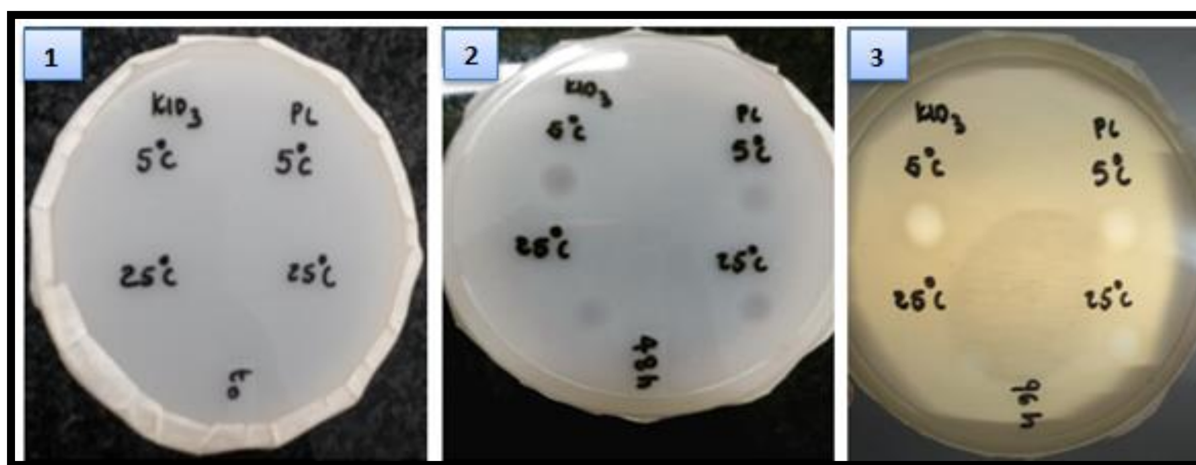

Figure 4.2: The action of the enzyme induced by activation of PG to PL (KIO3) and commercial PL protease under refrigeration temperature and room temperature

4.5.3. Analysis of proteolytic profiles of raw full-cream milk stored at 5°C and 25°C using RP-HPLC

4.5.3.1. Hydrolysis by PL protease induced by PG activator, KIO_3

It was evident through RP-HPLC data that inactive plasminogen in raw full-cream milk was activated more by KIO_3 to plasmin protease during storage at refrigeration temperature (5°C) than at room temperature (25°C). This was shown by the chromatographic profile below (Fig 4.3a and 4.3b). At the elution times 50 min to 65 min, the characteristic plasmin protease peaks were eluted (Hattingh, 2017). The area of the peptide peaks liberated at time 0, 24 h, 48 and 96 h for milk stored at 5°C were much higher and more in comparison to peptide peaks liberated in milk samples stored at 25°C.

*** Important note: The scale on the y-axes which represents the intensity is not 100% correct due to the profile overlaying (RP-HPLC GC software) used.**

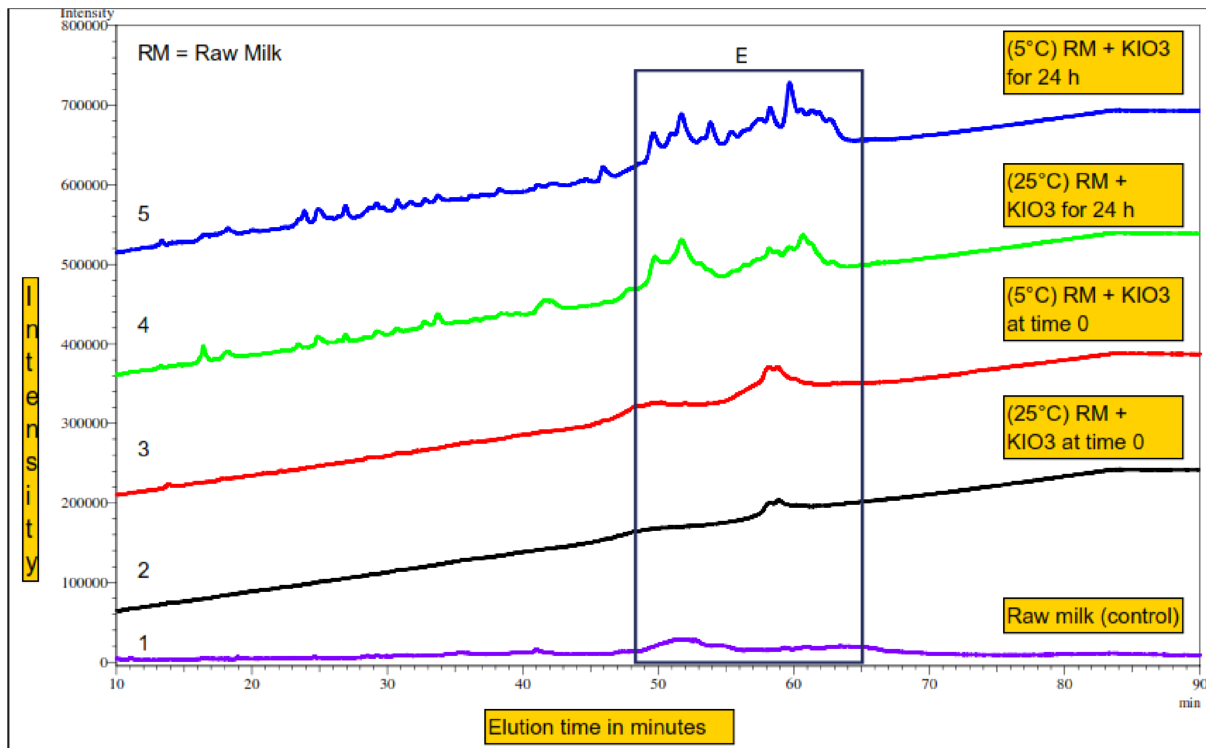

Figure 4.3a: RP-HPLC chromatogram peptide profiles in milk samples treated with KIO_3 (0-24h) and Region E includes the characteristic plasmin protease peaks. (1) Raw full-cream milk before any treatment; (2) Raw full-cream milk stored at 25°C, treated with KIO_3 at t_0; (3) Raw full-cream milk stored at 5°C, treated with KIO_3 at t_0; (4) Raw full-cream milk stored at 25°C, treated with KIO_3 for t_{24} h; and (5) Raw full-cream milk stored at 5°C, treated with KIO_3 for t_{24} h

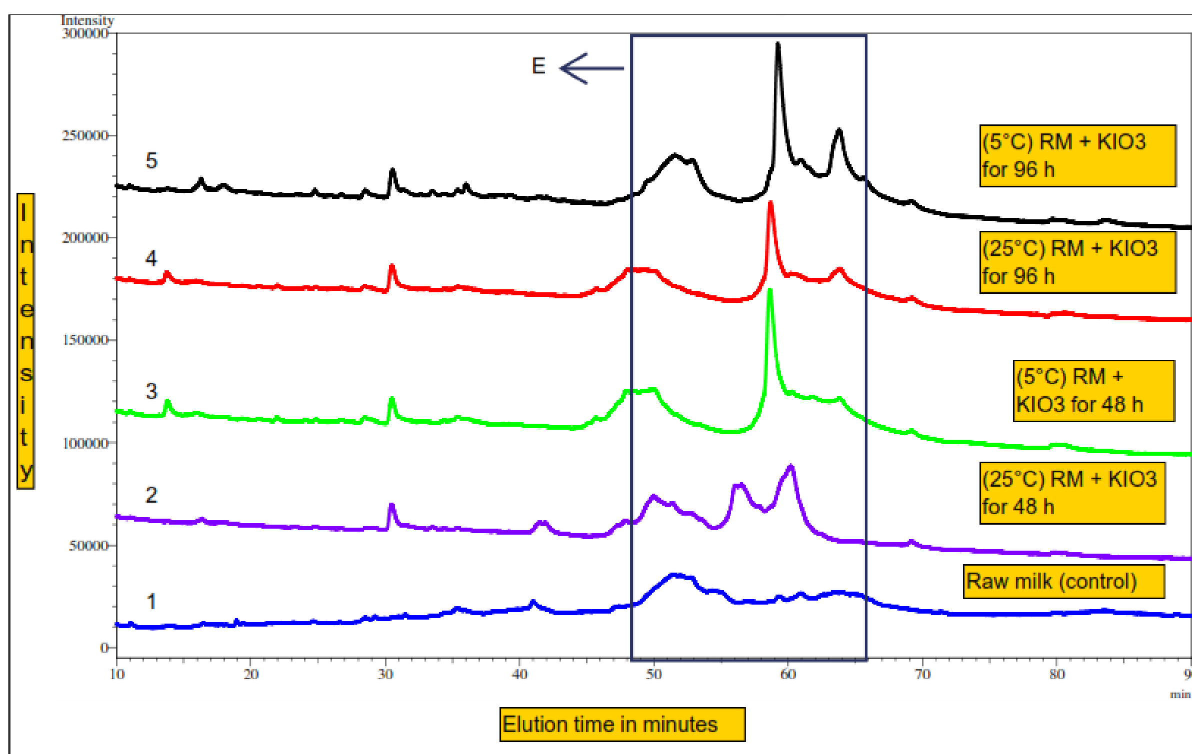

Figure 4.3b: RP-HPLC chromatogram peptide profiles in milk samples treated with KIO_3 (48-96h) and Region E includes the characteristic plasmin protease peaks. (1) Raw full-cream milk before any treatment; (2) Raw full-cream milk stored at 25°C, treated with KIO_3 for t_{48} h; (3) Raw full-cream milk stored at 5°C, treated with KIO_3 for t_{48} h; (4) Raw full-cream milk stored at 25°C, treated with KIO_3 for t_{96} h; and (5) Raw full-cream milk stored at 5°C, treated with KIO_3 for t_{96} h

Fig 4.3c exhibits the total area (retention time) characteristic for plasmin protease induced by PG activation (KIO_3). The extent of proteolytic activity was expressed in percentages (increase). **The following equation was used;**

Percentage (%) = [(TA) of UHT milk proteolyzed by PL protease / (TA) of UHT milk (control)] × 100
* TA= Total area

The difference in the enzyme activity is clearly shown between the two temperatures and more of plasmin activity (as a result of PG activation) was present in milk samples stored at refrigeration temperature to a greater extent than at room temperature.

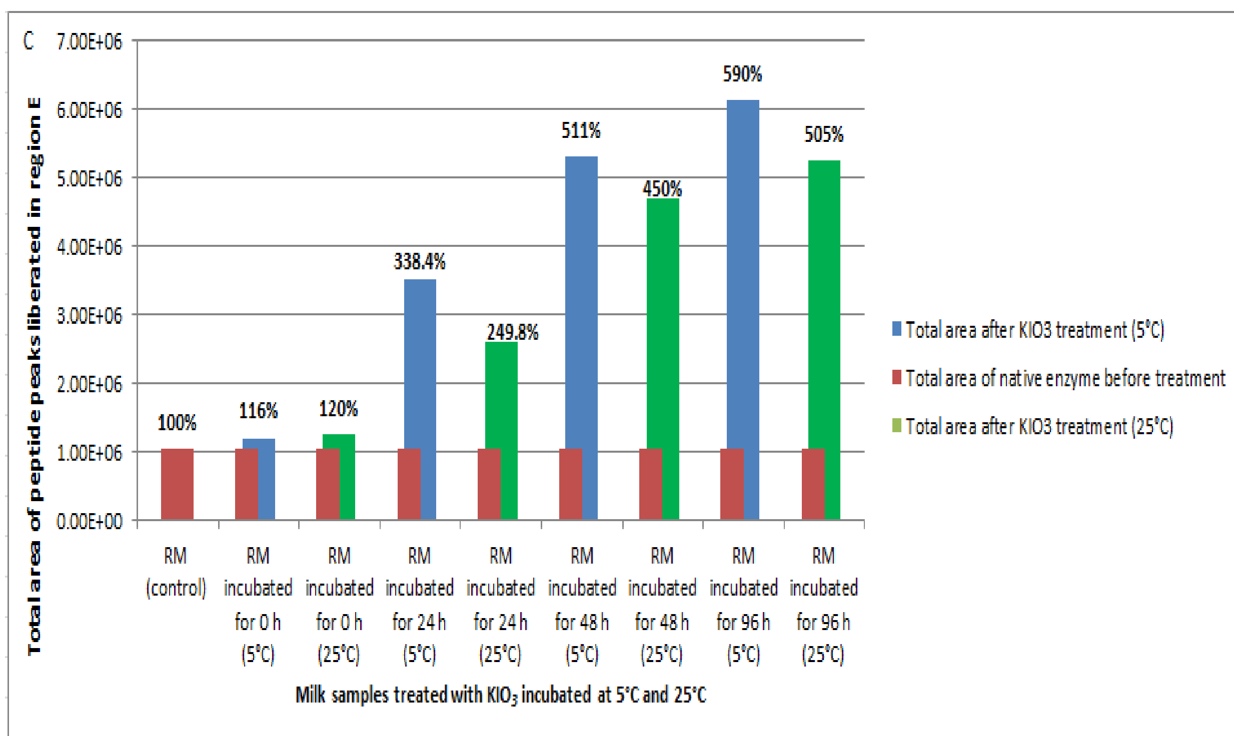

Figure 4.3c: Effect of refrigeration and room temperature conditions on plasmin (PL) activity (induced by PG activator KIO_3) in raw full-cream milk

4.5.3.2. Hydrolysis by commercial Plasmin protease

It was evident through RP-HPLC data that plasmin protease digested the casein to a greater extent during storage at room temperature (25°C) in comparison to refrigeration temperature (5°C). This was evident in the chromatographic profiles Fig 4.4a and 4.4b. At the elution times 50 min to 65 min (Region E), the characteristic plasmin protease peaks were eluted (Hattingh, 2017). The area of peptide peaks liberated at time 0, 24 h, 48 and 96 h for milk stored at 25°C was higher in comparison to peptide peaks liberated at 5°C.

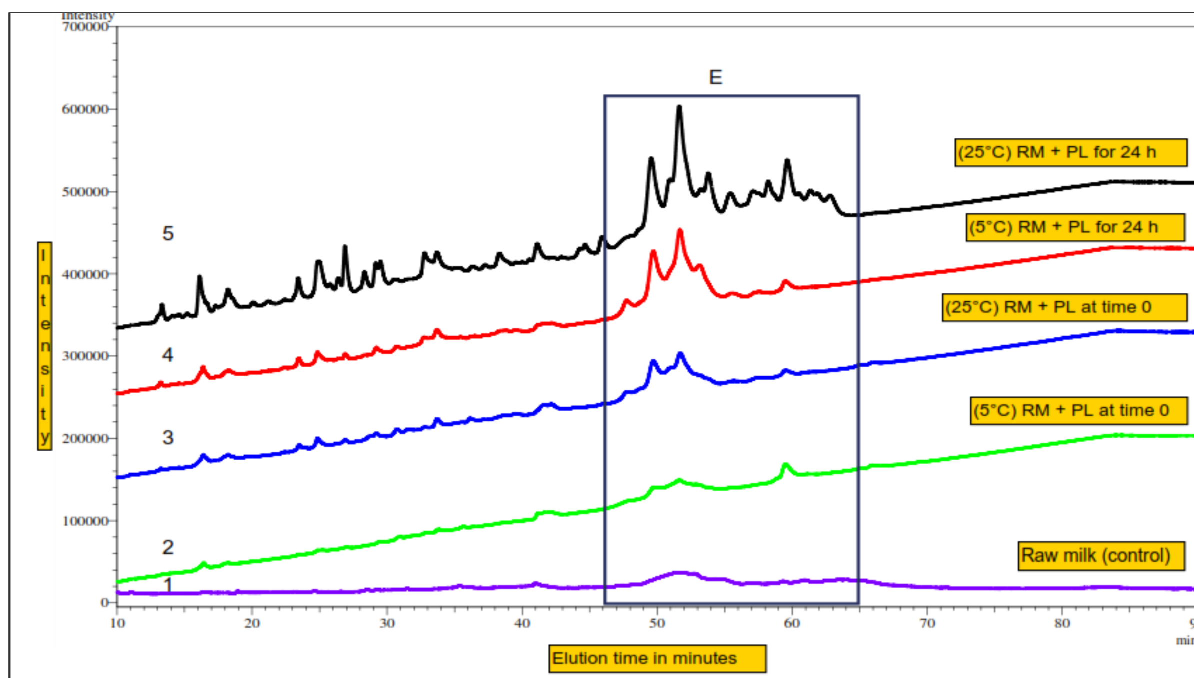

Figure 4.4a: RP-HPLC chromatogram peptide profiles in milk samples treated with PL (0-24h) and Region E includes the characteristic plasmin protease peaks. (1) Raw full-cream milk before any treatment; (2) Raw full-cream milk stored at 5°C, treated with PL at time 0; (3) Raw full-cream milk stored at 25°C, treated with PL at time 0; (4) Raw full-cream milk stored at 5°C, treated with PL for t_{24} h; and (5) Raw full-cream milk stored at 25°C, treated with PL for t_{24} h

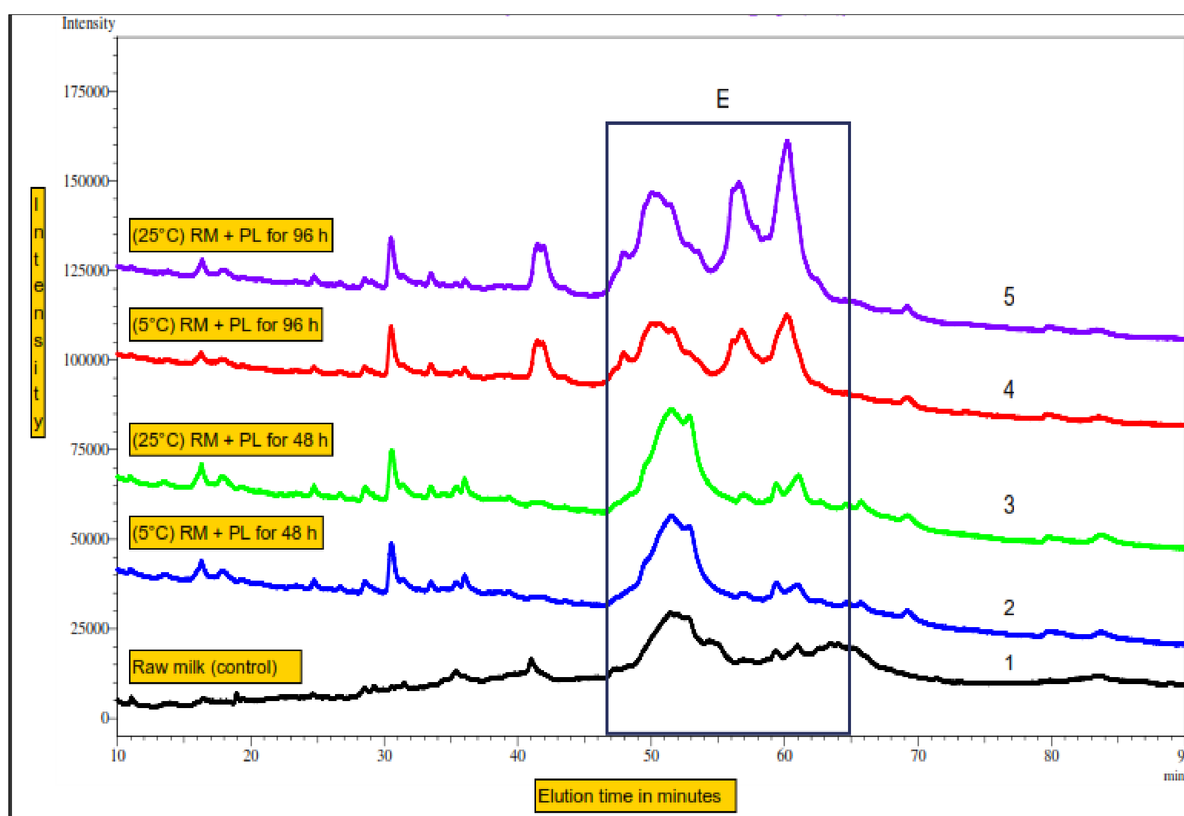

Figure 4.4b: RP-HPLC chromatogram peptide profiles in milk samples treated with PL (48-96h) and Region E includes the characteristic plasmin protease peaks. (1) Raw full-cream milk before any treatment; (2) Raw full-cream milk stored at 5°C, treated with PL for t_{48} h; (3) Raw full-cream milk stored at 25°C, treated with PL for t_{48} h; (4) Raw full-cream milk stored at 5°C, treated with PL for t_{96} h; and (5) Raw full-cream milk stored at 25°C, treated with PL for t_{96} h

Figure 4.3c exhibits the total area (retention time) characteristic for plasmin protease induced by commercial PL. The extent of proteolytic activity was quantified in percentages. From this figure, the differences in enzyme activity between two temperatures are shown and higher plasmin activity was present in milk samples stored at room temperature than at refrigeration temperature.

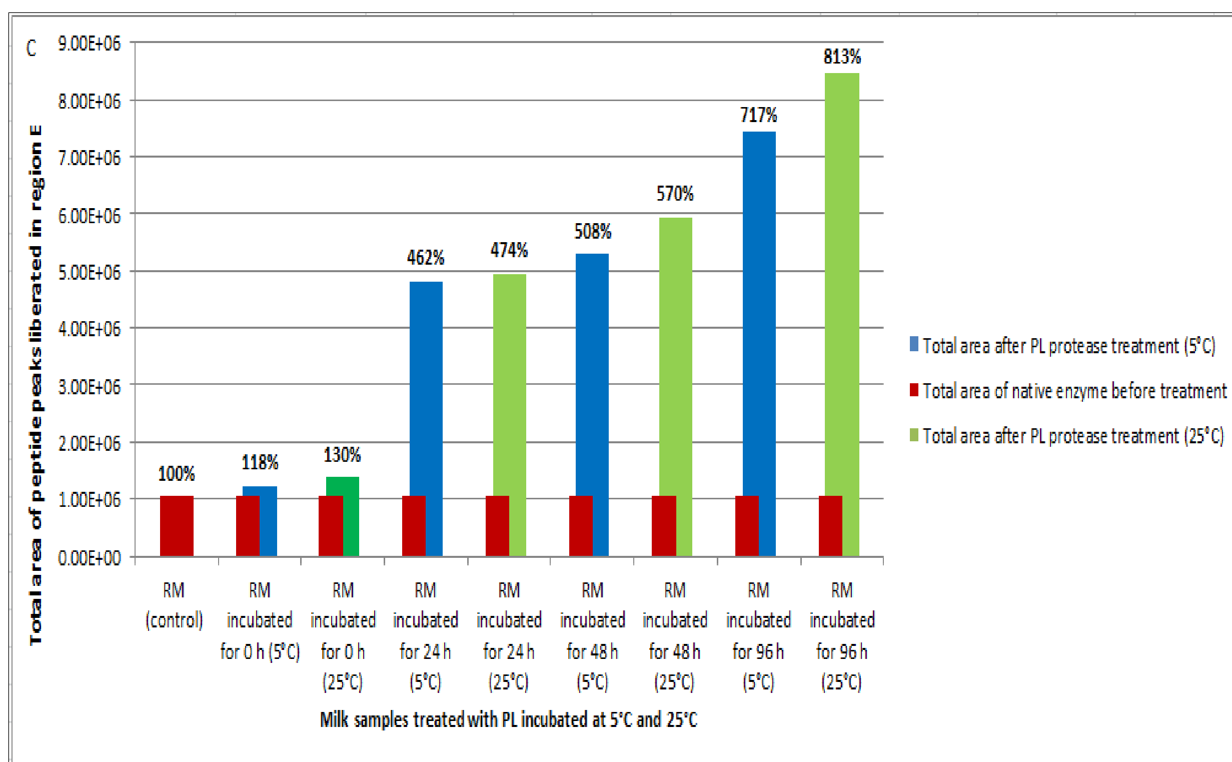

Figure 4.4c: Effect of refrigeration and room temperature conditions on PL activity (commercial PL) in raw full-cream milk

There is a lot of controversy on the occurrence of age gelation (destabilization of casein) at low temperatures and higher temperatures in milk. In the current work, it was found that milk samples treated with KIO_3 activated PG to PL protease and the induced PL by KIO_3 digested the casein extensively at 5°C in comparison with the samples incubated at 25°C. This is in contradiction with the findings reported by Al-Saadi *et al.*,(2015) that more proteolysis occurs at higher temperatures i.e. 20°C, 37°C and 45°C than 5°C. The occurrence of more hydrolysis at 5°C than 25°C can be ascribed to the dissociation of β-casein into the serum fraction during storage at 5°C, and because of the change in the location of β-casein, PL protease is enabled easier access to the casein (Crudden *et al.,* 2005, Ismail, *et al*., 2011). It can also be ascribed to the change in pH above that of normal milk pH (6.6). The pH of milk stored under refrigeration temperatures (5°C) after KIO_3 treatment increased as a function of time to 6.9 whilst that of milk stored at room temperature (25°C) after KIO_3 increased to 6.8. According to the findings reported by Sinaga, *et al*., (2017), the increase in the milk pH affects the casein micelle structure i.e. the micelles become looser; the total size of the micelles increases and the casein swells at pH above 6.6. Thus, this casein structural change caused the casein micelle structure to becomes more susceptible to plasmin attack (induced by KIO_3) during storage at 5°C in comparison with 25°C.

In the case of commercial (pure) plasmin treatment, it was found that the casein was hydrolysed more readily at 25°C as compared to 5°C. The pH of the milk samples increased from 6.6 to 6.9 under both temperature conditions. The current findings are in agreement with the findings reported by Al-Saadi *et al*., (2015) that at higher temperatures namely 20°C, 37°C and 45°C, more proteolysis of milk casein protein occurs during extended incubation periods than at lower temperatures (5°C). The destabilization mechanism of pure plasmin and plasmin-induced by plasminogen activation (KIO_3) on the casein protein is different and these differences warrant further study.

4.6. Conclusions

The presence of plasmin (induced by KIO_3) and commercial plasmin resulted in milk destabilization of the casein and as early as from time 48 h to more than 96 h of incubation at refrigeration temperatures (5°C) and at room temperatures (25°C) respectively (according to the results obtained in the alizarol test and milk agar test). According to the RP-HPLC data, it was evident that samples treated with KIO_3 had similar proteolysis peptide patterns to those treated with commercial plasmin protease, meaning that KIO_3 has the ability to mediate the conversion of plasminogen to plasmin under refrigeration temperatures and at room temperature. Furthermore, it was surprising to learn that higher plasmin proteolysis (induced by KIO_3) occurred in milk stored under refrigeration temperatures in comparison with milk stored under higher temperatures (25^oC). Thus this means that enormous damage could still be inflicted to the casein structure during milk storage at 5°C at the farm due to proteinase induced by plasminogen activators.

4.7. References

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Chapter 5: The Inhibition of Age Gelation in Milk

5.1. Introduction

Gelation of UHT milk during storage is a major factor that reduces its shelf life. Due to the high temperatures of processing (140–145°C for 10 sec), UHT milk is usually considered sterile and when it is aseptically packaged it is bacteriologically stable at ambient storage temperatures (Datta and Deeth, 2001). More so, milk is pasteurized to inhibit microbial growth and undergoes UHT treatment to destroy most of the undesired proteases. However, these thermal processes often lead to some denaturation of the milk fat globule membrane proteins (Ye *et al.,* 2017). Additionally, there are a lot of changes that take place during storage and ultimately result in the reduction of shelf-life. Amongst them is the change in the physical state which is exhibited by the rise in viscosity which is irreversible (age gelation) (Datta and Deeth, 2001).

The changes that occur in milk during storage are usually attributed to proteinase from psychrotolerant bacteria (*Bacillus* spp. and *Pseudomonas* spp.) that grow during refrigerated storage before the processing of milk and the native milk proteinase known as plasmin. UHT milk is even more sensitive to proteolytic attack than pasteurized milk due to the extended storage time at ambient temperatures of the former. Low residual or high proteinase levels present in the milk makes it susceptible to destabilization during storage and lead to serious defects during storage (Garcia-Risco *et al*., 2001).

However, there is a possibility that enzymes other than the bacterial proteinase and plasmin can contribute to biochemical changes during storage of UHT milk. The changes that occur in UHT milk not only depend on the enzyme activity but also depend on the composition of the milk, the processing conditions and temperatures (Garcia-Risco, *et al*., 2001).

To date, UHT milk has been introduced to consumers as the safest milk and milk with extended shelf life. However, the changes in physical properties create a big problem in a sense that consumers loose trust in the product and the dairy industry encounters considerable financial loses, hence the pressing need to lower or inhibit proteolytic systems responsible for age gelation in milk (Buthgamuwa, 2008).

5.2. Objectives:

1: To investigate the impact of additives and gases on the inhibition of proteolytic activity in the milk and to lower the susceptibility of the casein towards proteolytic attack.
2: To determine how long the inhibition effects of plasmin and *Bacillus* protease remains after N_2 pre-treatment.

5.3. Materials and Reagents

Objective 1 and 2:

(1) To investigate the impact of additives and gases on the inhibition of proteolytic activity in the milk and to lower the susceptibility of the casein towards proteolytic attack.

(2) To determine how long the inhibition of plasmin and *Bacillus* protease remain active after N_2 pre-treatment.

5.3.1. Milk

UHT milk was purchased from Checkers local supermarket in Brandwag, Bloemfontein, Free State, (RSA). The fat content was measured by a Foss MilkcoScan, and it was 0% for UHT fat-free and 2,9% for UHT full-cream.

Raw-full cream milk was collected from a local dairy plant, Dairy Cooperation, Bloemfontein, (RSA). The fat content was measured by a Foss MilkcoSca and it was 3,1%.

5.3.2. Enzymes/proteases

The commercial enzymes, Bovine plasmin (10602370001) and *Bacillus* (P5380) proteinases were purchased from Sigma-Aldrich Chemical South Africa. The two proteolytic enzymes had an activity of 5 U/mg for plasmin protease and 11 U/mg of *Bacillus* protease, respectively. Due to the high activity of the *Bacillus* protease, the enzyme was diluted 10 times in a buffer (1.1 U/ml).

5.3.3. Acidic precipitation of casein

Hydrochloric acid (batch no: 10108772) was purchased from Sigma-Aldrich Chemical (RSA) and an 0.1 N solution was used to lower the pH (acidification) in order to precipitate the casein of all the samples.

5.3.4. Additives (chemicals)

The additives namely, Sodium hexametaphosphate (SHMP), Calcium chloride ($CaCl_2$), and Sodium citrate ($Na_3C_6H_5O_7$) were all purchased from Merck (RSA). Ethylenediaminetetra-acetic acid (EDTA) was purchased from Protea Laboratory Services.

5.3.4. Gases

The gases namely, carbon dioxide (CO_2), helium (He) and nitrogen (N_2) were purchased from Afrox (RSA).

5.3.5. Oxygen meter and redox meter

An Oxygen meter and Redox meter were purchased from Labotec (RSA) and they were used to detect O_2 percentage (%) and redox in mV (Both instruments have a separate temperature probe to compensate for temperature). The instruments were calibrated with calibration liquids provided by the supplier.

5.3.6. Merck protease assay kit

The protease assay kit (catalog no: 539125-1KIT) was purchased from Sigma-Aldrich (RSA) and it was used to assay the protease activity as prescribed by the supplier.

5.4. Methods

Objective 1: To investigate the impact of additives and gases on the inhibition of proteolytic activity in milk and to lower the susceptibility of the casein to proteolytic attack.

5.4.1. Preparation of additives stock solution

The experiment was done using UHT fat-free milk with a fat content of 0% (confirmed by Milkoscan). The stock solution was prepared by adding 0.1 g of various additives (chemicals)

to 100 mL of UHT fat-free milk separately as indicated in Table 5.1. Sodium azide (0.01 g) was added to each 100 mL of milk to prevent microbial growth during the incubation period.

Table 5.1: The concentration of additives (chemicals) and their reaction volumes

Additives (Chemicals)	**Ethylendiaminetetra-acetic acid (EDTA)**	**Calcium Chloride ($CaCl_2$)**	**Sodium Hexametaphosphate (SHMP)**	**Sodium citrate** ($Na_3C_6H_5O_7$)
Concentration (g/100 mL)	0.1g/100 mL	0.1g/100 mL	0.1g/100 mL	0.1g/100 mL
1st Reaction Volume	995 µL	995 µL	995 µL	995 µL
2nd Reaction Volume	990 µL	990 µL	990 µL	990 µL

5.4.2. Treatment of milk with N_2, CO_2, and He gases respectively

The gases namely, nitrogen, carbon dioxide, and helium were bubbled through 250 mL milk (UHT fat-free, UHT full-cream and raw full-cream milk) in sterile Schott bottles separately using a sterilized Pasteur pipette connected to an Afrox nitrogen supply. The Schott bottles were tightly closed after the bubbling process in order to prevent any evaporation of gas. The gas flow controller was set at 1000 kPa. The measuring time intervals were set to 2 min during which O_2 and redox were measured. The gases were bubbled through the milk until the O_2 level stopped declining (levelled out).

5.4.3. Treatment of milk with commercial proteases (*Bacillus* protease and plasmin protease)

5.4.3.1. Milk samples treated with additives

The reaction volume (995 µL) was firstly removed from each stock solution as indicated in Table 5.1 and was added separately to 2 mL Eppendorf tubes and it was subjected to treatment with 5 µL of *Bacillus* protease. The final activity of *Bacillus* protease was 0.0055 U/mL. The reaction volume (990 µL) was secondly removed from each stock solution and was added separately to a 2 mL Eppendorf tube and it was subjected to treatment with 10 µL of plasmin protease. All the samples were incubated for 6 hours at 37°C.

After incubation, the samples were divided into two sets; the reaction in the first set of samples was stopped by heating (boiling) at 90°C for 10 min. The casein was precipitated

using 0.1 N HCl acid and centrifuged in a bench top Eppendorf Centrifuge (Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan), at the speed of 10 000 rpm for 20 min. The supernatant of the samples was analysed using RP-HPLC. The second set of samples were directly precipitated using 0.1 N HCl acid without stopping the reaction by heating and centrifuged at 10 000 rpm for 20 min. The supernatants obtained were assayed using a Merck protease assay kit.

5.4.3.2. Milk samples treated with gases

After treatment of milk samples with various gases as stipulated in section 5.4.2 the aliquots of 995 µL were first aseptically removed in a laminar flow hood from each Schott bottle with a different gas and was added separately to 2 mL Eppendorf tubes. The samples were then subjected to treatment with 5 µL of *Bacillus* protease. Similarly, the same methodology was followed for treatment with plasmin protease where aliquots of (990 µL) were subjected to treatment with 10 µL of plasmin.

The samples were incubated for 6 hours at 37°C and they were divided into two sets. The casein was precipitated using 0.1 N HCl acid and centrifuged in a bench top Eppendorf Centrifuge (Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan), at the speed of 10 000 rpm for 20 min. The supernatant of the samples was subjected to RP-HPLC analysis. The second set of samples was directly precipitated using 0.1 N HCl acid without stopping the reaction by heating and centrifuged at 10 000 rpm for 20 min. The supernatants obtained were analysed using a Merck protease assay kit and the reaction was read in a spectrophotometer (Cecil 2021, 2000 series supplied by Lasec RSA).

5.4.4. Analysis of peptide profiles using the Reverse-Phase High-Performance Liquid Chromatography

Aliquots of a 200 µL clear supernatant as collected in section 5.4.2.1 and 5.4.2.2 were stored in various Eppendorf tubes prior to RP-HPLC analysis at -20°C. Immediately before the RP-HPLC analysis, the samples were thawed and transferred to RP-HPLC vials. (Refer to Chapter 3, section 3.4.2 for the RP-HPLC running conditions).

5.4.5. Analysis of protease activity using the Merck protease assay kit

The same methodology for protease assay as mentioned in Chapter 3, objective 1 in section 3.4.6 was followed. In this case, the protease assay was performed to establish the level of protease activity (U/mL) after the addition of additives and after gas treatment.

5.4.5.1. Additives (chemicals):

The samples spiked with EDTA, $CaCl_2$, SHMP, and Sodium citrate were prepared according to the following protocol for the Merck protease assay: A volume of 25 µL of the incubation buffer was added followed by 50 µL of the samples supernatant (The clear supernatant obtained under section 5.4.2.1 and 25 µL of FTC-casein. The reagent blank was prepared by adding 25 µL of the incubation buffer followed by 50 µL of distilled water and 25 µL FTC-casein. The protease positive control was prepared by adding 10 µL of protease positive control (part of the assay kit) and 90 µL of distilled water.

5.4.5.2. Gas bubbling/treatment:

Similarly, the same methodology for Merck protease analysis as mentioned in section 5.4.4.1 was performed in samples treated with CO_2, N_2, and He gases, respectively.

Objective 2: To determine how long the inhibition of PL and *Bacillus* protease remain active after N_2 pre-treatment.

5.4.6. Preparation of a stock solution (gases)

Sodium azide (0.05 g) was added to 1 L of UHT fat-free milk. The milk sample was placed into six different 100 mL sterile Schott bottles.

5.4.7. Treatment of milk with nitrogen gas and incubation conditions

The treatment with nitrogen gas was performed in the same manner as previously mentioned in section 5.4.2. After the gas treatment process, the Schott bottles containing the treated milk samples were incubated (stored) at 5°C for time 0 h, 168 h and 744 h.

5.4.8. Incubation of nitrogen treated UHT fat-free milk with commercial proteases (*Bacillus* protease and plasmin protease)

After time 0 h, 168 h and 744 h, aliquots (995 µL) as indicated in section 5.4.6 were first aseptically removed in a laminar flow hood from each sample (at different times) bubbled with a nitrogen gas in the Schott bottles and was added separately into 2 mL Eppendorf tubes. The samples were then treated with 5 µL of *Bacillus* protease and at a final activity of 0.0055 U/mL. Similarly, using the same methodology mentioned above, plasmin protease with a final activity of 0.05 U/mL was added to aliquots of 990 µL. All the above-mentioned

samples with added protease were incubated in a water bath at 37°C for 6 h in order to investigate the effectiveness of N_2 gas on the digestion of the casein protein.

After the 6 h incubation, the milk samples were divided into two sets. In the first set of samples, the enzyme reaction was stopped by heating (boiling) at 90°C for 10 min. The casein was precipitated using 0.1 N HCl acid and centrifuged by a bench top Eppendorf Centrifuge (Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan), at a speed of 10 000 rpm for 20 min. The supernatant of the samples was subjected to RP-HPLC analysis. The second set of samples were directly precipitated using 0.1 N HCl acid without stopping the reaction by heating and centrifuged at 10 000 rpm for 20 min. The supernatants obtained were analysed using the Merck protease assay kit.

5.4.9. Analyses of peptide profiles by Reverse-Phase High Performance Liquid Chromatography

Aliquots of 200 µL clear supernatant as collected in section 5.4.8 were stored in various Eppendorf tubes prior to RP-HPLC analysis at -20°C. Immediately before analysis by RP-HPLC, the samples were thawed and transferred to RP-HPLC vials. (Refer to Chapter 3, section 3.4.2 for the RP-HPLC running conditions).

5.4.10. Analysis of protease activity using the Merck protease assay

The same methodology for protease assay as mentioned in Chapter 3, objective 2 in section 3.4.6 was followed. In this case, the protease assay was performed to establish the level of protease activity (U/mL) after gas treatment for 0 h, 168 h, and 744 h.

5.4.10.1. Gas bubbling/treatment:

The same methodology for Merck protease analysis as mentioned in section 5.4.4.1 was performed in samples treated with N_2 for 0 h, 168 h and 744 h respectively.

*NOTE: The experiments were done in triplicates.

5.4. Results and Discussion

Objective 1: To investigate the impact of additives and gases on the inhibition of proteolytic activity in milk and to lower the susceptibility of the casein to proteolytic attack.

5.5.1. The inhibitory effect of additives (chemicals) on milk casein as evaluated using the Merck protease assay kit

5.5.1.1. *Bacillus* protease in UHT fat-free milk

The inhibitory effect of various additives was evaluated on UHT fat-free milk hydrolyzed by *Bacillus* protease. Fig 5.1a below exhibits the average proteolytic activity level in each milk sample treated with various additives separately. The average proteolytic activity in milk samples treated with EDTA was 0.358 U/mL, SHMP was 0.392 U/mL and $CaCl_2$ was 0.398 U/mL in comparison to milk samples hydrolysed by *Bacillus* protease without any additives (0.461 U/mL). The latter indicated that EDTA, SHMP, and $CaCl_2$ were able to inhibit the proteolytic attack induced by *Bacillus* protease. Leviton, *et al*., (1962) reported in the literature that polyphosphates such as SHMP lengthen the shelf life of UHT milk and retard gelation but it was not clear if it retards gelation induced by bacterial protease or an indigenous plasmin protease. The ability of polyphosphates to lengthen the shelf life of milk is due to the inability of cyclic polyphosphates to form complexes with Ca^{2+} which is known to have anti-gelation traits (Ca^{2+} have anti-gelation traits) (Data and Deeth, 2001). Additionally, Chavan *et al*., (2011) reported that EDTA is a chelator, it binds metals and it inhibits *Pseudomonas* enzyme which is a metalloenzyme. *Bacillus* protease is also a metalloenzyme which requires divalent cations like Ca^{2+} and Zn^{2+} for stability and optimum functioning; therefore the addition of EDTA binds the metals and blocks the enzyme activity. However, milk samples treated with sodium citrate had an average proteolytic activity of 0.466 U/mL and it slightly increased/enhanced the proteolytic activity. The colloidal calcium phosphate linkages that include ions speed up the aggregation of the casein that has been destabilized (Datta and Deeth, 2001).

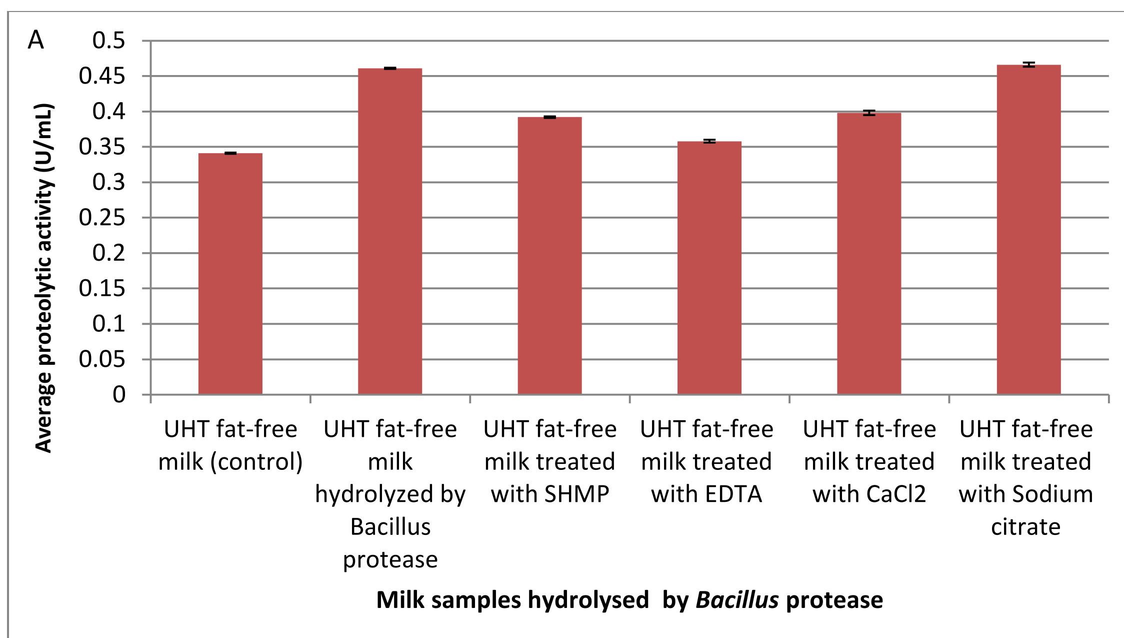

Figure 5.1a: Protease activity (U/mL) in milk after treatment with various additives (chemicals) and *Bacillus* protease using the Merck protease kit

5.5.1.2. Plasmin protease in UHT fat-free milk

The inhibitory effect of various additives was evaluated in UHT fat-free milk hydrolyzed by plasmin protease. Fig 5.1b exhibits the average proteolytic activity level in each milk sample treated with additives separately. The average proteolytic activity in milk samples treated with SHMP was 0.364 U/mL and $CaCl_2$ was 0.369 U/mL in comparison to milk samples hydrolyzed by plasmin protease without any additives 0.379 U/mL. The latter indicated that SHMP and $CaCl_2$ were able to retard proteolytic attack induced by plasmin protease. Milk samples treated with the additive EDTA had a proteolytic activity of 0.378 U/mL which showed no pronounced differences in comparison to the milk that was hydrolyzed by plasmin without any additives (0.379 U/mL). This is due to the fact that plasmin is a serine enzyme and EDTA is a chelator that binds metals, therefore EDTA had no influence on the enzyme. Milk samples treated with sodium citrate had an average proteolytic activity of 0.381 U/mL. Sodium citrate slightly increased/enhanced the proteolytic activity.

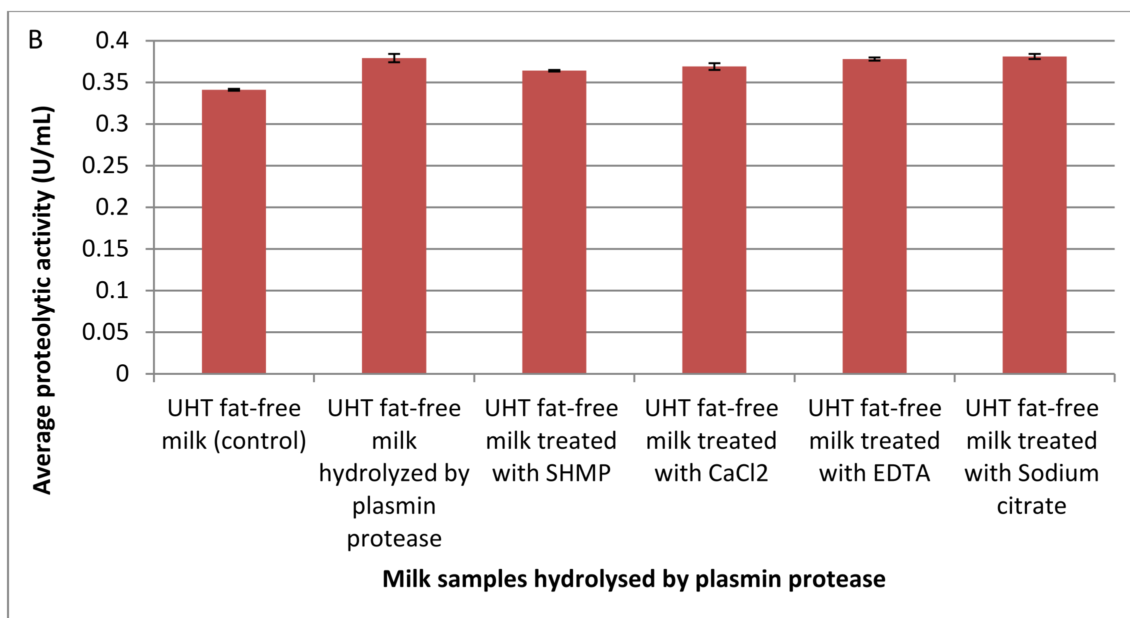

Figure 5.1b: Protease activity (U/mL) in milk after treatment with various additives (chemicals) and plasmin protease using the Merck protease kit

5.5.2. The inhibitory effect of additives (chemicals) on milk casein as analyzed by RP-HPLC

5.5.2.1. *Bacillus* protease in UHT fat-free milk

Additives (chemicals) were added to milk to evaluate their impact on the inhibition of proteolytic attack induced by bacterial (*Bacillus*) proteinase. Fig 5.2a shows the peptide peaks eluted between 30-35 min; 45-49 min, and 50-70 min which is characteristic for *Bacillus* protease. Profile 2 (UHT fat-free milk hydrolysed by *Bacillus* protease) shows high peptide peaks liberated as a result of *Bacillus* protease action and the treatment of UHT fat-free milk with EDTA, SHMP, as well as $CaCl_2$ suppressed the peptide peaks meaning that it retarded the bacterial hydrolysis shown by profile 3, 4 and 5. The area of peptide peaks liberated in milk sample treated with sodium citrate showed no differences when compared against the area of peptide peaks liberated in milk treated with only *Bacillus* protease. The results obtained using the RP-HPLC corresponds with the results obtained by Merck protease assay with the exception of sodium citrate.

The extent of proteolytic activity from the chromatographic profiles was not quantified in percentages. Thus, the calculations were based on the total integrated area (retention time)

characteristic for *Bacillus* protease as mentioned in Chapter 3, section (3.5.1). **The following equation was used;**

Percentage (%) = [(TA) of UHT milk proteolyzed by protease – (TA) of UHT milk (control)/ (TA) of UHT milk proteolyzed by protease] × 100

It was evident that EDTA retarded *Bacillus* protease activity by 21.2%, SHMP by 19.8%, and $CaCl_2$ by 5.5% whereas sodium citrate slightly increased the protease activity by 1.8% (Bar graph not shown).

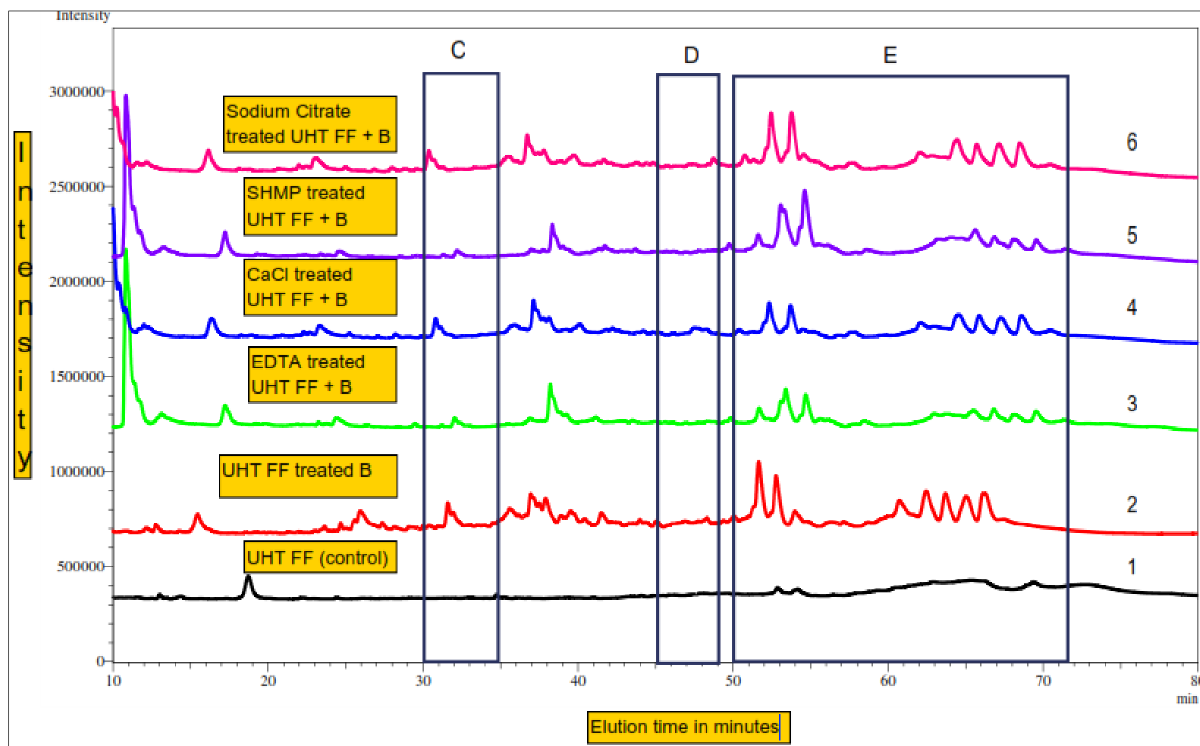

Figure 5.2a: Peptide profiles for (1) UHT fat-free milk (control); (2) UHT fat-free milk proteolysed by *Bacillus* protease; (3) EDTA treated UHT fat-free milk proteolysed by *Bacillus* protease; (4) $CaCl_2$ treated UHT fat-free milk proteolysed by *Bacillus* protease; (5) SHMP treated UHT fat-free milk proteolysed by *Bacillus* protease and (6) Sodium Citrate treated UHT fat-free milk proteolysed by *Bacillus* protease

5.5.2.2. Plasmin protease in UHT fat-free milk

Fig 5.2b shows the peptide peaks eluted between 18-20 min; 23-28 min and 50-69 min characteristic for plasmin hydrolysis. Profile 2 (UHT fat-free milk hydrolysed by plasmin protease) shows high peptide peaks liberated as a result of plasmin protease action and the

treatment of UHT fat-free milk with $CaCl_2$ and SHMP retarded plasmin hydrolysis shown by profile 3 and 4 respectively. The area of peptide peaks liberated in milk treated with EDTA (profile 6) showed no pronounced differences when compared against the area of peptide peaks liberated in milk treated with PL without any additives. Additionally, the area of peptide peaks liberated in milk samples treated with sodium citrate slightly increased (profile 5). These results correspond to the results obtained using Merck protease assay.

The extent of proteolytic activity from the chromatographic profiles was expressed as percentages. Thus, the calculations were based on the total integrated area (retention time) characteristic for plasmin protease as mentioned in Chapter 3, section 3.5.1.

It was established by RP-HPLC analysis that SHMP and $CaCl_2$ were able to retard proteolytic attack induced by plasmin protease by 60.9%, and 4.7%, respectively whilst EDTA played no significant role in the inhibition of the enzyme (1.5% reduction). Sodium citrate on the other hand slightly enhanced plasmin activity by 2.8% (Bar graph not shown).

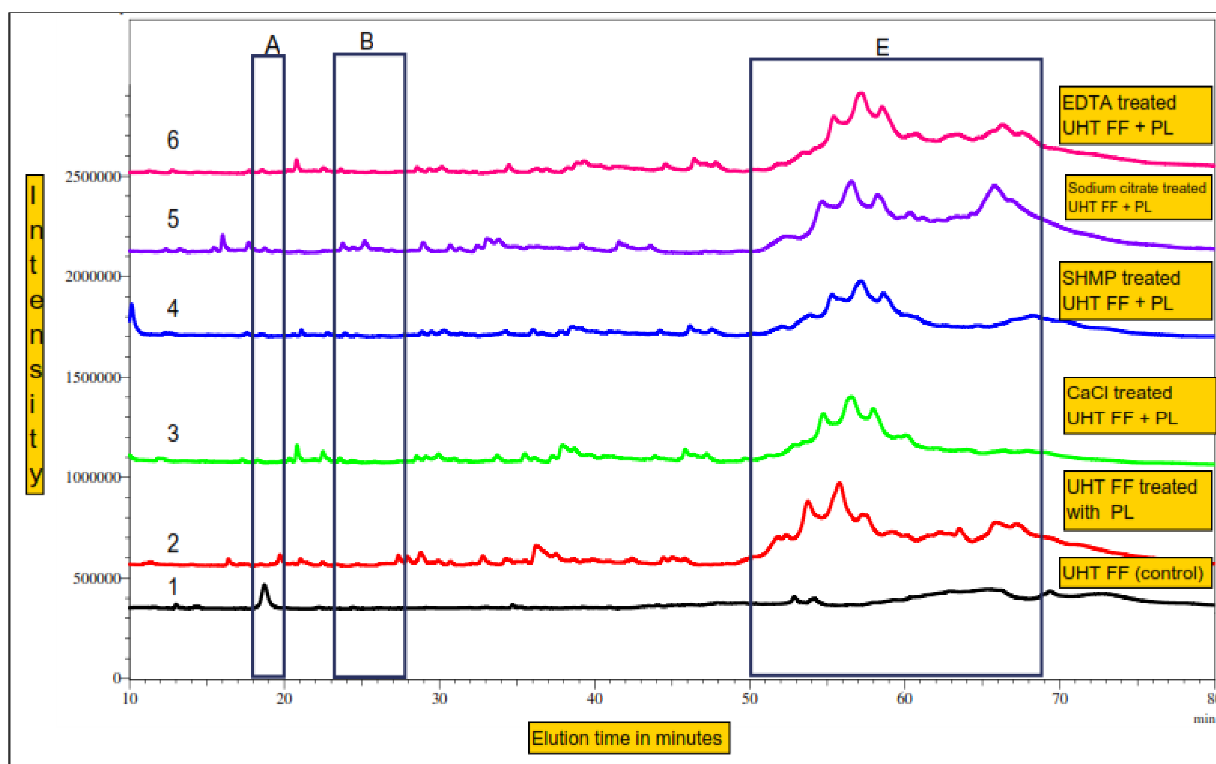

Figure 5.2b: Peptide profiles for (1) UHT fat-free milk (control); (2) UHT fat-free milk proteolysed by plasmin protease; (3) $CaCl_2$treated UHT fat-free milk proteolysed by plasmin protease; (4) SHMP treated UHT fat-free milk proteolysed by plasmin protease; (5) Sodium Citrate treated UHT fat-free milk proteolysed by plasmin protease and; (6) EDTA treated UHT fat-free milk proteolysed by plasmin protease

5.5.3. Gases:

5.5.3.1. Oxygen concentration (%) and redox potential (mV)

Oxygen in the milk was measured and monitored to evaluate what happens to the casein or how susceptible the casein becomes to proteolytic attack at lower levels of dissolved oxygen concentration. The initial O_2 concentration in raw full-cream milk was 75% and it was reduced to around 15% in milk bubbled with N_2 gas, around 5% in the case of CO_2, and around 22.2% in the case of He. The redox potential ranged between 60 and 66.5 mV in milk bubbled with gases already mentioned.

Exactly the same methodology mentioned above was adopted for UHT fat-free and UHT full-cream which was only bubbled by N_2 gas. The initial O_2 concentration in UHT fat-free milk was 85% and it was reduced to around 10.0 %. In the case of UHT full-cream milk, the initial

O_2 concentration was 78%; and it was reduced to around 14.1%. The redox potential in both types of UHT milk ranged between 104 and 106 mV.

5.5.3.2. The inhibitory effect of gases in milk on proteolytic activity as evaluated using the Merck protease assay

5.5.3.2.1. *Bacillus* protease in raw full-cream milk

The inhibitory effect of nitrogen, carbon dioxide, and helium gas was evaluated on raw full-cream milk hydrolyzed by *Bacillus* protease. Fig (5.3a) exhibits the average proteolytic activity level in each milk sample bubbled with the gases mentioned above. The average proteolytic activity in raw full-cream milk samples bubbled with nitrogen gas was (0.386 U/mL) in comparison to raw full-cream milk samples hydrolyzed by *Bacillus* protease without any gas (0.387 U/mL). Nitrogen gases showed no pronounced differences in comparison to raw full-cream milk samples hydrolyzed by *Bacillus* protease without any gas. The average proteolytic activity in raw full-cream milk samples bubbled with CO_2 was 0.359, and helium gas was 0.360 U/mL, in comparison to raw full-cream milk samples hydrolyzed by *Bacillus* protease without any gas (0.387 U/mL). Helium gas and CO_2 retarded proteolytic action induced by *Bacillus* protease. According to literature reported by (Sorhaung and Stepaniak, (1997), carbon dioxide and nitrogen reduce protease secretion by *Pseudomonas* spp. at low temperatures and it was not known if this was also the case for protease secreted by *Bacillus* protease and indigenous plasmin protease. Thus far, no information is reported in the literature regarding the effectiveness of He gas.

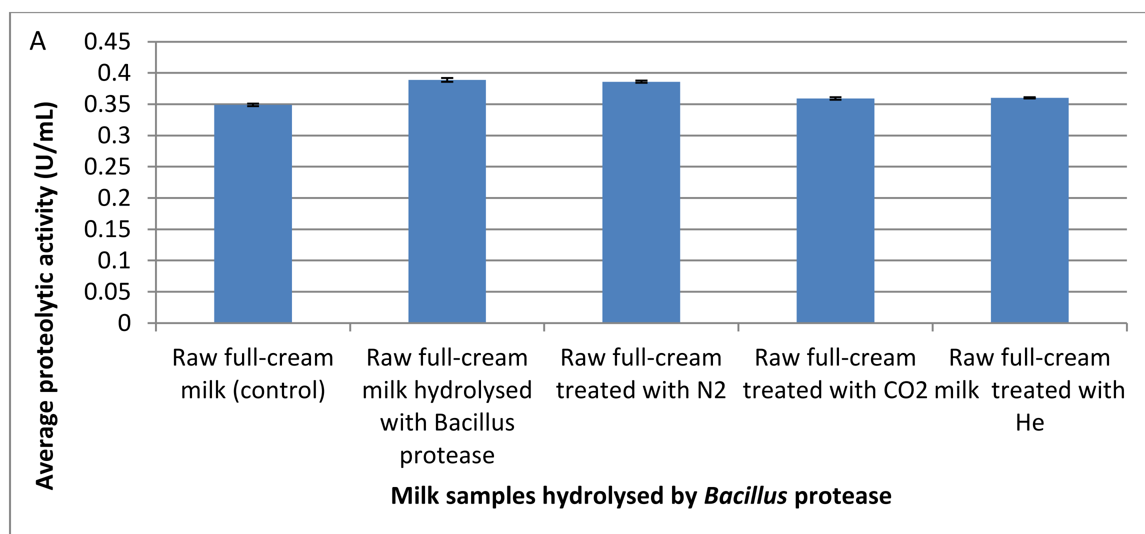

Figure 5.3a: The final protease activity (U/mL) in milk after bubbling with nitrogen, carbon dioxide, helium gas and treatment with *Bacillus* protease

5.5.3.2.2. Plasmin protease in raw full-cream milk

The inhibitory effect of nitrogen, helium and carbon dioxide gases were evaluated on raw full-cream milk hydrolyzed by plasmin protease. Fig 5.3b below exhibits the average proteolytic activity levels in each milk samples bubbled with gases mentioned above. The average proteolytic activity in raw full-cream milk samples bubbled with nitrogen was 0.350 U/mL; helium was 0.353 U/mL and carbon dioxide gas was 0.355 U/mL in comparison to raw full-cream milk samples hydrolyzed by plasmin protease without any gas (0.399 U/mL). Treatment of milk with all these gases showed pronounced differences in comparison to raw full-cream milk hydrolyzed by plasmin protease without any gases.

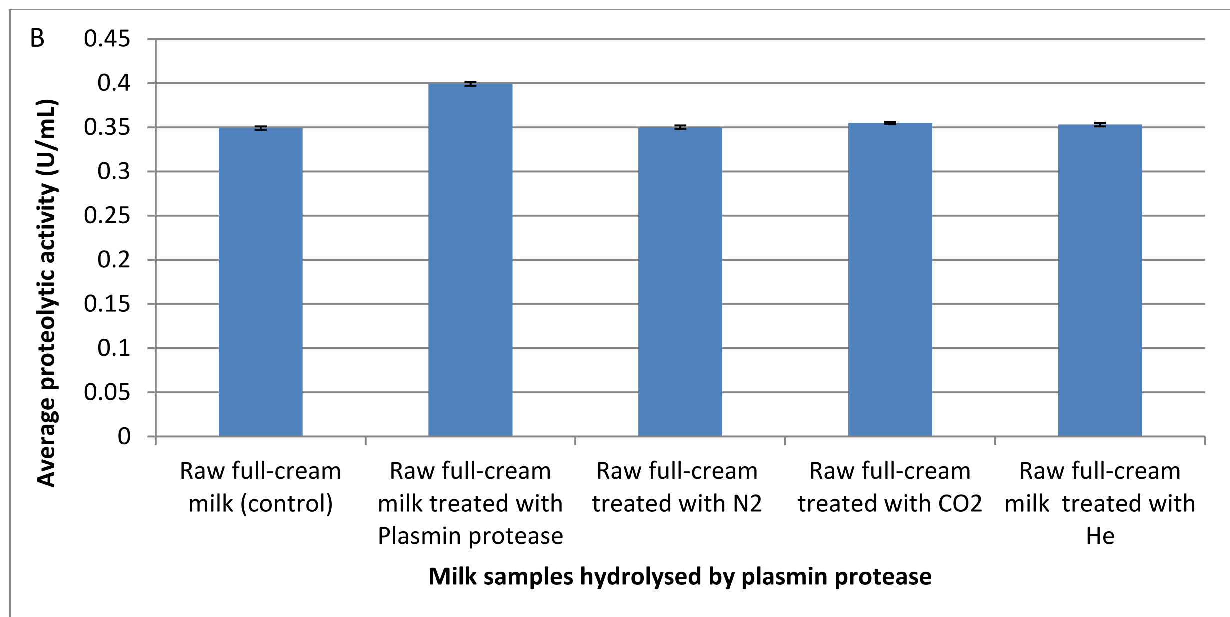

Figure 5.3b: Protease activity (U/mL) in milk after bubbling with the nitrogen, carbon dioxide, and helium gas and treatment with plasmin protease

5.5.3.2.3. Plasmin protease in UHT full-cream and UHT fat-free milk

The inhibitory effect of nitrogen gas was also evaluated on UHT fat-free milk and UHT full-cream milk hydrolyzed by plasmin protease. Fig 5.4a exhibits the average proteolytic activity level in each milk sample bubbled with nitrogen gas. The average proteolytic activity in UHT fat-free milk samples bubbled with nitrogen gas was 0.365 U/mL in comparison to UHT fat-free milk samples hydrolyzed by plasmin protease without any gas 0.398 U/mL. The average proteolytic activity in UHT full-cream milk samples bubbled with nitrogen gas was 0.344U/mL in comparison to UHT full-cream samples hydrolyzed by plasmin protease

without any gas 0.378 U/mL. Nitrogen gas was able to retard proteolytic activity in both UHT fat-free and UHT full cream milk.

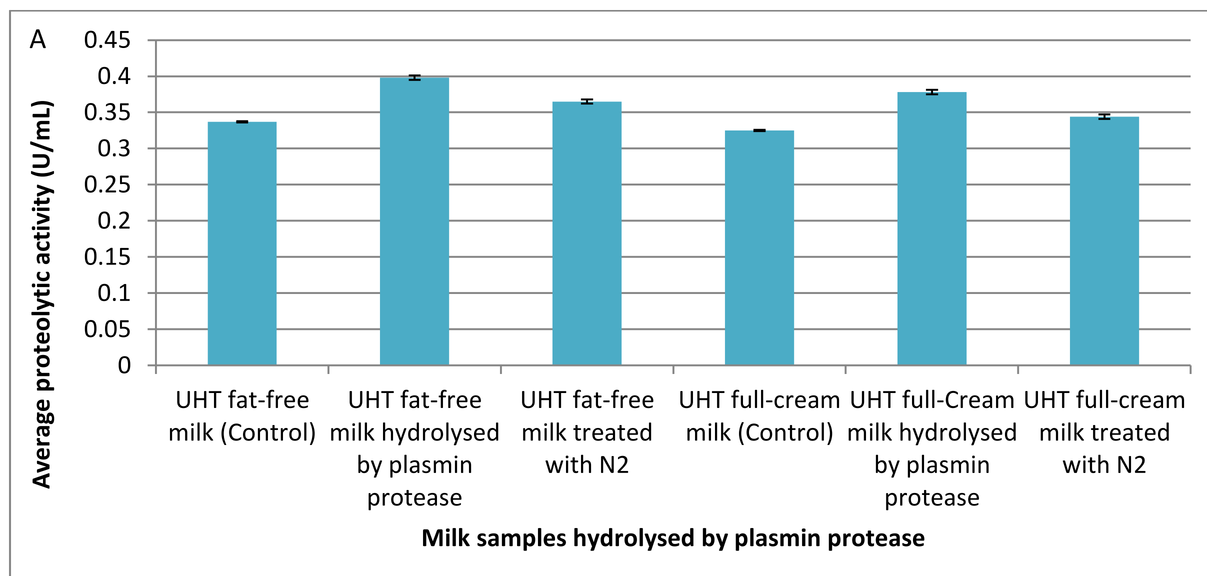

Figure 5.4a: Protease activity (U/mL) in milk after bubbling with nitrogen gas and treatment with plasmin protease

5.5.3.2.4. *Bacillus* protease in UHT full-cream and UHT fat-free milk

The inhibitory effect of nitrogen gas was evaluated on UHT fat-free milk and UHT full-cream milk hydrolyzed by *Bacillus* protease. Fig 5.4b exhibits the average proteolytic activity level in each milk sample bubbled with nitrogen gas. The average proteolytic activity in UHT fat-free milk samples bubbled with nitrogen gas was 0.389 U/mL in comparison to UHT fat-free milk samples hydrolyzed by *Bacillus* protease without any gases 0.461 U/mL. The average proteolytic activity in UHT full-cream milk samples bubbled with nitrogen gas was 0.382 U/mL in comparison to UHT full-cream samples hydrolyzed by *Bacillus* protease without any gas 0.432 U/mL.

It was established that bubbling N_2 gas through UHT fat-free milk and UHT full-cream milk retarded proteolytic attack induced by *Bacillus* protease in comparison to non-bubbled UHT fat-free milk.

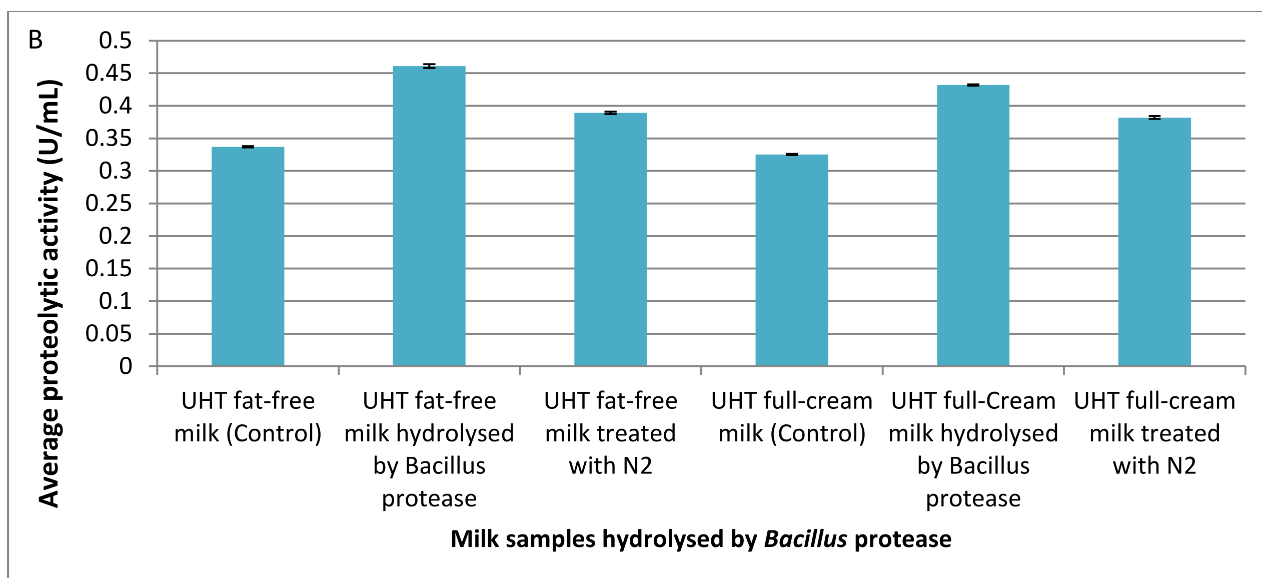

Figure 5.4b: Protease activity (U/mL) in milk bubbled with the nitrogen gas and treated with *Bacillus* protease

5.5.3.3. Inhibitory effect of gases in milk on proteolytic activity as analysed by RP-HPLC

5.5.3.3.1. *Bacillus* protease in raw full-cream milk

Raw full-cream milk was bubbled with nitrogen, carbon dioxide, and helium gases respectively as shown in Fig 5.5a below. Treatment of milk with CO_2 and He shown in profile 4, and 5, retarded the proteolytic action induced by bacterial hydrolysis as the peaks mainly in region D (45-49 min) were suppressed by 9.11%, and 6.9% respectively. Region D, elution time 45-49 min is characteristic for *Bacillus* protease hydrolysis (refer to section 3.5.1 in Chapter 3). Treatment of milk with N_2 did not play any pronounced role in retarding bacterial proteolytic digestion shown in profile 3 (the difference was only 1.6%). The size of peptides peaks as detected by RP-HPLC liberated from milk bubbled with N_2 gas is similar to the size of peptide peaks liberated when milk was hydrolyzed by *Bacillus* protease without the bubbling of the above-mentioned gases. These results correspond to the results obtained by the Merck protease assay.

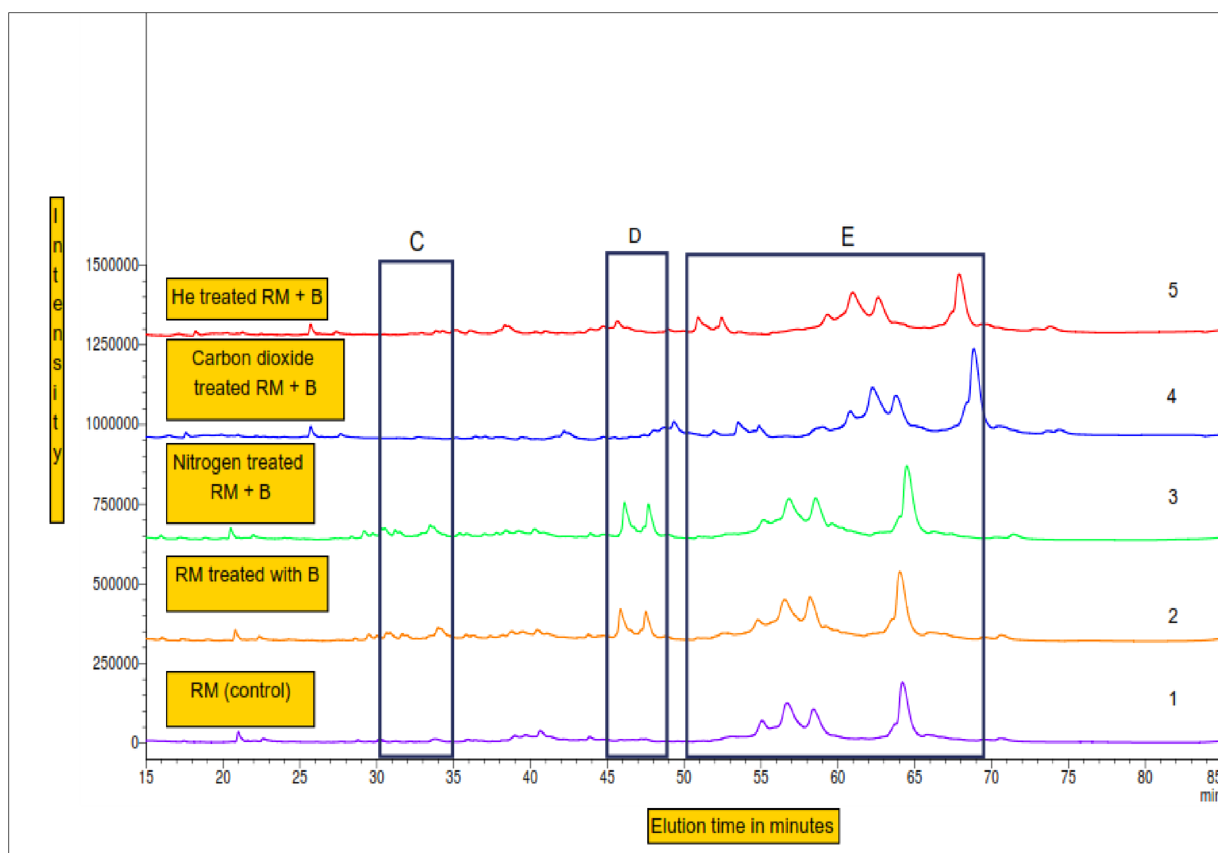

Figure 5.5a: RP-HPLC chromatogram peptide profiles for *Bacillus* proteolysed raw milk treated with gasses (1) Raw full-cream milk (control); (2) raw full-cream milk proteolysed by *Bacillus* protease; (3) N_2 treated raw full-cream milk proteolysed by *Bacillus* protease; (4) CO_2 treated raw full-cream milk proteolysed by *Bacillus* protease and (5) He treated raw full-cream milk proteolysed by *Bacillus* protease

***RM** = Raw full-cream milk
***B** = *Bacillus* protease

5.5.3.3.2. Plasmin protease in raw full-cream milk

There was little proteolysis in the control (profile 1) which may be attributed to the number of SCC and protease present in raw milk (Fernandes *et al.*, 2008). Profile 2 (raw full-cream milk) in Fig 5.5b below shows the peptide peaks liberated as a result of plasmin protease hydrolysis. These peptide peaks are mainly eluted at the time 50-69 minutes (refer to section 3.5.1 in chapter 3). The same gases as previously mentioned were introduced into the milk medium to evaluate their impact on the plasmin activity/action. It is evident that all these gases retarded the plasmin action, as the peptide peaks liberated mainly in region E (50-69 min), were suppressed in comparison to raw full-cream milk hydrolysed by plasmin protease

without any gases (profile 2). The effectiveness of various gases namely CO_2, He and N_2 was evaluated on raw full-cream milk. It was evident by means of total area integration that all the gases mentioned above had the ability to retard plasmin protease activity by 16.9%, 6.9%, and 6.8%, respectively in raw full-cream milk (Bar graph indicating percentages is not shown).

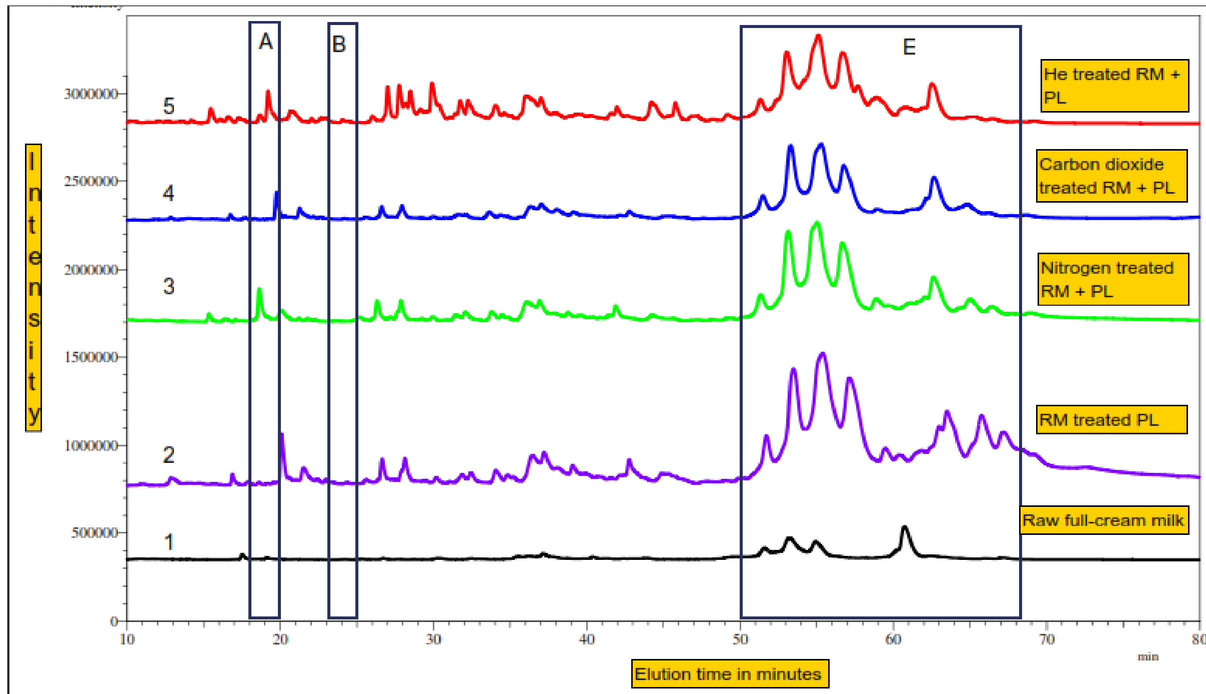

Figure 5.5b: RP-HPLC chromatogram peptide profiles for plasmin proteolysed raw full-cream milk treated with gasses (1) Raw full-cream milk (control); (2) raw full-cream milk proteolysed by plasmin protease; (3) N_2 treated raw full-cream milk proteolysed by plasmin protease; (4) CO_2 treated raw full-cream milk proteolysed by plasmin protease and (5) He treated raw full-cream milk proteolysed by plasmin protease

5.5.3.3.3. Plasmin protease in UHT full-cream and UHT fat-free milk

There was little proteolysis in the control (profile 1) which may be attributed to the number of SCC and protease present in raw milk (Fernandes *et al*., 2008). The chromatographic profiles in Fig 5.6a shows that treating milk with plasmin protease hydrolyzes the casein substrate, therefore, releasing peptides as the by-products of hydrolysis (profile 4 and profile 6). The peptide peaks were eluted mainly in region E (50-65 min). Introducing nitrogen gas into the milk medium retarded the proteolytic attack in UHT fat-free milk shown by sample 6 in comparison to milk hydrolysed by plasmin without any gas (profile 5). The peptide peaks

were also suppressed in UHT full cream milk treated with nitrogen gas (profile 4) in comparison to milk hydrolysed by plasmin without any gas (profile 3). This meant that the treatment of milk with inert nitrogen gas has a positive impact on the inhibition of proteolytic action induced by plasmin protease. Thus, it was established by means of total area integration that N_2 gas retarded proteolytic attack induced by plasmin protease in both UHT fat-free milk and UHT full-cream milk by 35.8% and 9.3% respectively. (Bar graph indicating percentages is not shown).

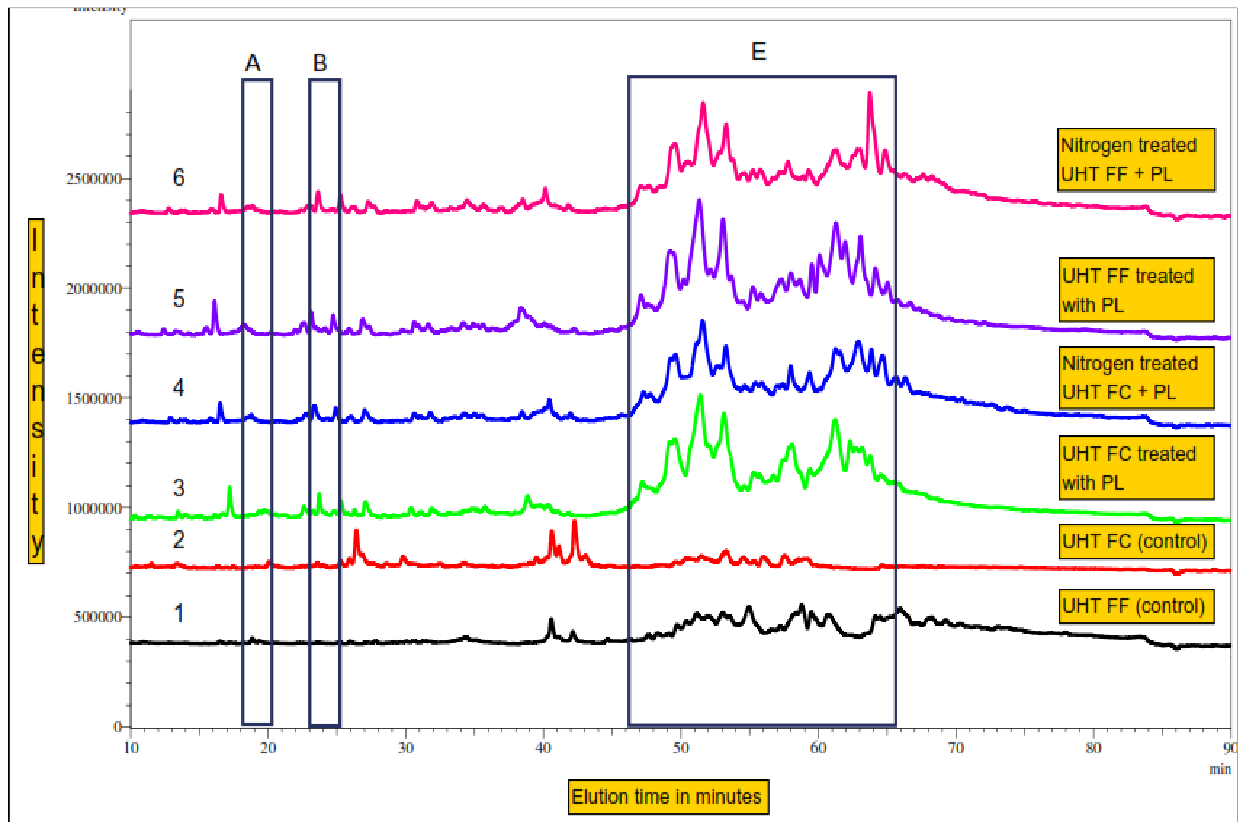

Figure 5.6a: RP-HPLC chromatogram peptide profiles for (1) UHT fat-free milk (control); (2) UHT full- cream milk (control); (3) UHT full-cream milk proteolysed by plasmin protease; (4) N_2 treated UHT full-cream milk proteolysed by plasmin protease; (5) UHT fat-free milk proteolysed by plasmin protease; and (6) N_2 treated UHT fat-free milk proteolysed by plasmin protease

5.5.3.3.4. *Bacillus* protease in UHT full-cream and UHT fat-free milk

The chromatographic profile in Fig 5.6b shows that *Bacillus* protease does hydrolyse the casein and release the peptides as by-products (profile 6 and profile 4). It is evident that there were high peptide peaks liberated in UHT fat-free milk hydrolysed by *Bacillus* protease

shown in sample 6 and introducing nitrogen gas into the milk medium (profile 5) retarded the proteolytic attack induced by bacterial hydrolysis. Additionally, there were small differences observed in terms of the peak sizes eluted in profile 3 (N_2 treated UHT full-cream milk hydrolyzed by *Bacillus* protease) in comparison to sample 4 (UHT full-cream milk hydrolyzed by *Bacillus* protease). The peptide peaks were slightly more suppressed in profile 3 than in profile 4. The peptide peaks were slightly more suppressed in profile 3 than in profile 4. This meant that the passing, sparging or bubbling of inert nitrogen gas through milk has a positive impact on the inhibition of proteolytic action induced by a bacterial protease.

Thus, it was established by means of total area integration that bubbling N_2 gas retarded proteolytic attack induced by *Bacillus* protease in UHT fat-free milk and in UHT full-cream milk by 30.7% and 9.1%, respectively. (Bar graph indicating percentages is not shown).

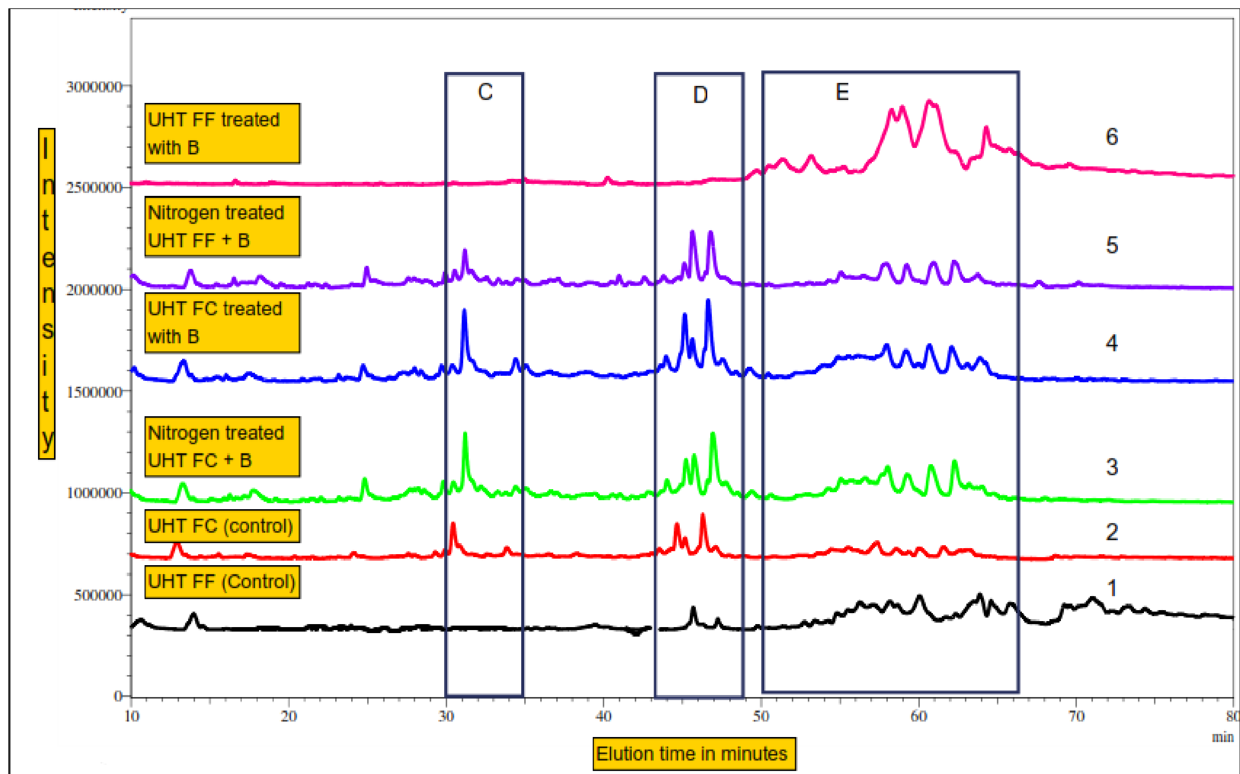

Figure 5.6b: RP-HPLC chromatogram peptide profiles for (1) UHT fat-free milk (control); (2) UHT full- cream milk (control); (3) N_2 bubbled UHT full-cream milk proteolysed by *Bacillus* protease and (4) UHT full-cream milk proteolysed by *Bacillus* protease; (5) N_2 bubbled UHT fat-free milk proteolysed by *Bacillus* protease; and (6) UHT fat-free milk proteolysed by *Bacillus* protease

After all the gases were evaluated, irrespective of their ability to retard proteases, CO_2 had a detrimental effect on the composition of the milk (lower pH), He gas was not economically feasible (too expensive) and nitrogen gas was cheap and readily available. Therefore, for this reason, further evaluation on the impact of gases was performed only on N_2 gas.

Objective 2: To determine how long the inhibition effects of PL and *Bacillus* protease remain after N_2 pre-treatment.

Due to the fact that modern consumers are unsettled by addition of additives in their food, bubbling gases through milk might be helpful in terms of delaying gelation processes and may be accepted by the consumers and no legislation limitation is set on the use of gases (Regulations relating to milk and dairy products: R1555 of 21 November 1997). In this study, in-depth investigations were done to evaluate the extent and the duration of inhibition induced by nitrogen gas.

5.5.3.4. The inhibitory effect of nitrogen gas in UHT milk on proteolytic activity as analysed using the Merck protease assay

Table 5.2: Proteolytic activity measured by Merck protease assay

Treatment nr.	Treatment	Proteolytic activity (U/mL)
1	UHT fat-free milk (control)	$0.33^{a} \pm 0.01$
2	UHT N_2 bubbled fat-free milk (control)	$0.34^{ab} \pm 0.01$
3	UHT fat-free milk + PL (main control)	$0.43^{d} \pm 0.01$
4	N_2 bubbled UHT fat-free milk @ t_0 + PL	$0.36^{abc} \pm 0.01$
5	N_2 bubbled UHT fat-free milk @ t_{168} + PL @ 5°C	$0.38^{bc} \pm 0.06$
6	N_2 bubbled UHT fat-free milk @ t_{744} + PL @ 5°C	$0.43^{cd} \pm 0.01$
7	UHT fat-free milk + *Bacillus* protease(main control)	$0.51^{e} \pm 0.02$
8	N_2 bubbled UHT fat-free milk @ t_0 + *Bacillus* protease	$0.34^{ab} \pm 0.02$
9	N_2 bubbled UHT fat-free milk @ t_{168} + *Bacillus* protease @ 5°C	$0.33^{a} \pm 0.03$
10	N_2 bubbled UHT fat-free milk @ t_{744} + *Bacillus* protease @ 5°C	$0.54^{f} \pm 0.01$
Significance level		$p < 0.001$

* Means with different superscripts in the same column differ significantly
* t = time in hours

Treating UHT fat-free milk with the N_2 gas showed significant differences in the inhibition of PL between the proteolysis in the main and the reading at t_0 h, t_{168} h. No significant difference was found at t_{744} h when milk was stored at 5°C in comparison to milk proteolyzed by PL

without N_2 as an inhibitor. The difference was 0.07 U/mL at t_0 h, and 0.05 U/mL at t_{168} h in comparison to milk that was not bubbled with nitrogen gas.

Treating UHT fat-free milk with N_2 gas showed significant differences in the inhibition of *Bacillus* protease at t_0 h, t_{168} h when milk was stored at 5°C in comparison to milk proteolyzed by *Bacillus* without N_2 as an inhibitor. The difference was 0.17 U/mL at t_0 h and 0.18 U/mL at t_{168} h in comparison to milk that was not bubbled with nitrogen gas. However, treating UHT fat-free milk with N_2 gas for t_{744} h showed an increase in *Bacillus* protease activity in comparison to milk proteolyzed by *Bacillus* without N_2 as an inhibitor and the increase was 0.03 U/mL. According to literature reported by Sorhaung and Stepaniak, (1997), nitrogen gas reduces protease secretion by *Pseudomonas* spp. at low temperatures but it was not specified how long the inhibition by nitrogen gas lasted.

5.5.3.5. The Inhibitory effect of nitrogen gas in UHT fat-free milk on proteolytic activity as analysed by Reverse-Phase High-Performance Liquid Chromatography

In Fig 5.7a, profile 1 and 2 shows that there were almost no peptide peaks liberated from the control samples before the enzyme treatment. There were high peptide peaks liberated in profile 3 as a result of casein hydrolysis by plasmin protease and the peptide peaks were eluted mainly between 50-65 min. The area of peptide peaks liberated in profile 4 (0 h), and profile 5 (168 h) were suppressed due to the presence of nitrogen gas in comparison to profile 3 (UHT fat-free milk hydrolysed by plasmin protease) which was not treated with N_2 gas. This indicated that up to 168 h, nitrogen gas still effectively retarded proteolysis induced by plasmin protease during storage at 5 °C. However, no inhibition of plasmin protease activity was observed at 744 h.

The extent of proteolytic activity in the chromatographic profiles was expressed as percentages. Thus, the calculations were based on the total integrated area (retention time) characteristic for plasmin protease as mentioned in Chapter 3, section 3.5.1. **The following equation was used:**

Percentage (%) = [(TA) of UHT milk proteolyzed by protease – (TA) of UHT milk (control)/ (TA) of UHT milk proteolyzed by protease] × 100

It was then evident that protease activity induced by plasmin protease was partially reduced using inert N_2 gas. The reduction was 28% at t_0 h, 26% at t_{168} h, and 0.9% at t_{744} h (Bar graph not shown).

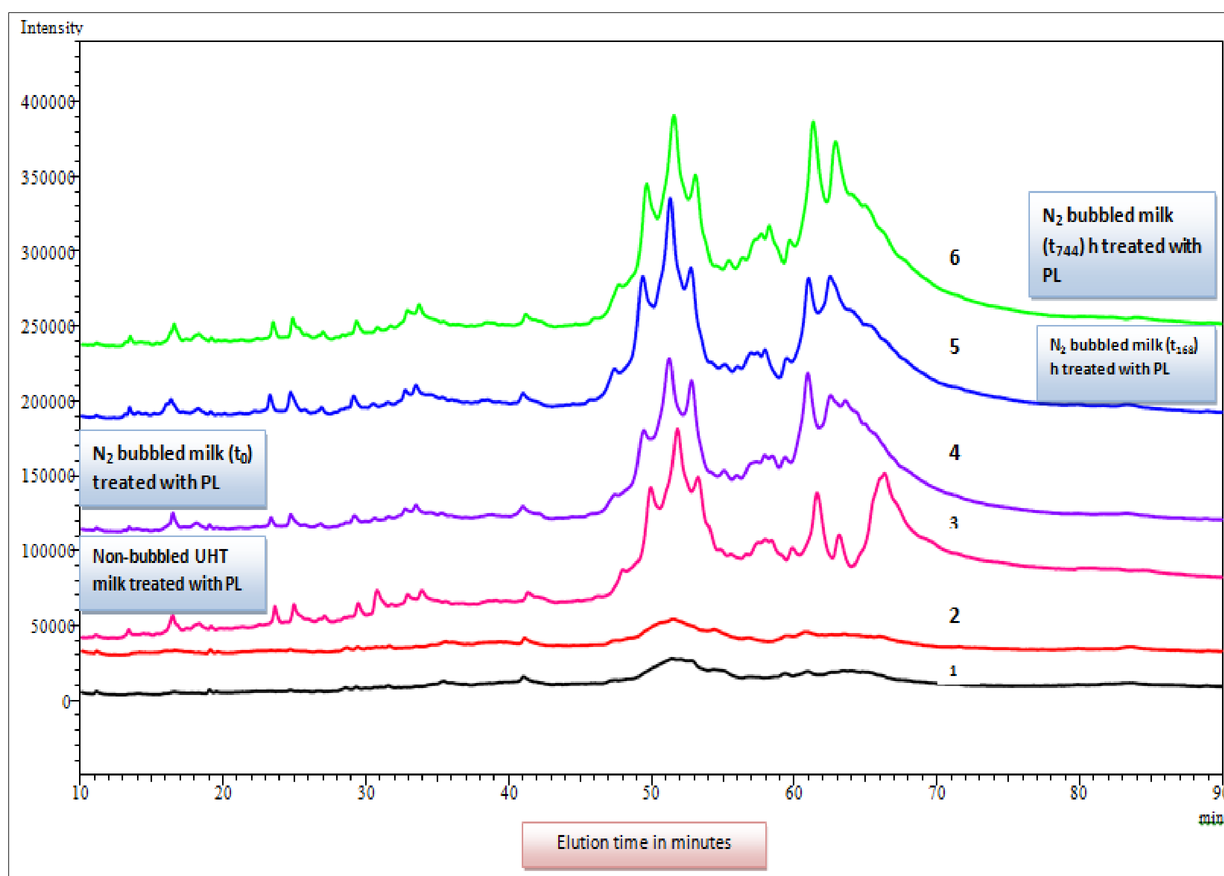

Figure 5.7a: RP-HPLC chromatogram peptide profiles for (1) UHT fat-free milk (control); (2) N_2 bubbled UHT fat-free milk (control); (3) UHT fat-free milk hydrolysed by plasmin protease; (4) N_2 bubbled UHT fat-free milk @ time 0 hydrolysed by plasmin protease; (5) N_2 bubbled UHT fat-free milk @ time 168 h hydrolysed by plasmin protease and (6) N_2 bubbled UHT fat-free milk @ time 744 h hydrolysed by plasmin protease

Fig 5.7b the chromatographic peptide profiles of milk hydrolysed by *Bacillus* protease after nitrogen gas treatment are shown. Profile 1 and 2 were the control samples before enzyme treatment and no peptide peaks were liberated. Profile 5 is the UHT fat-free milk hydrolyzed by *Bacillus* protease and the peptide peaks were eluted mainly between 50-65 min. The peptide peaks liberated were high which is indicative of casein hydrolysis by *Bacillus* protease. The area of peptide peaks after enzyme treatment in profile 3 (0 h), and sample 4 (168 h) were greatly suppressed due to the presence of nitrogen gas in comparison to profile 5 (UHT fat-free milk hydrolyzed by *Bacillus* protease) which was not treated with nitrogen gas. However, the area of peptide peaks in profile 6 (744 h) was again high in comparison to

plasmin protease. This indicated that the gas was no longer effective and the casein protein was digested unhindered.

Furthermore, the extent of proteolytic activity in the chromatographic profiles was expressed as percentages. It was evident that protease activity induced by *Bacillus* protease was partially reduced using inert N_2 gas. The reduction was 48% at t_0 h, and 51% at t_{168} h. There was an increase in *Bacillus* protease activity of 96% at t_{744} h (Bar graph not shown). This tendency of *Bacillus* protease inhibition by N_2 gas is the same as that of plasmin protease.

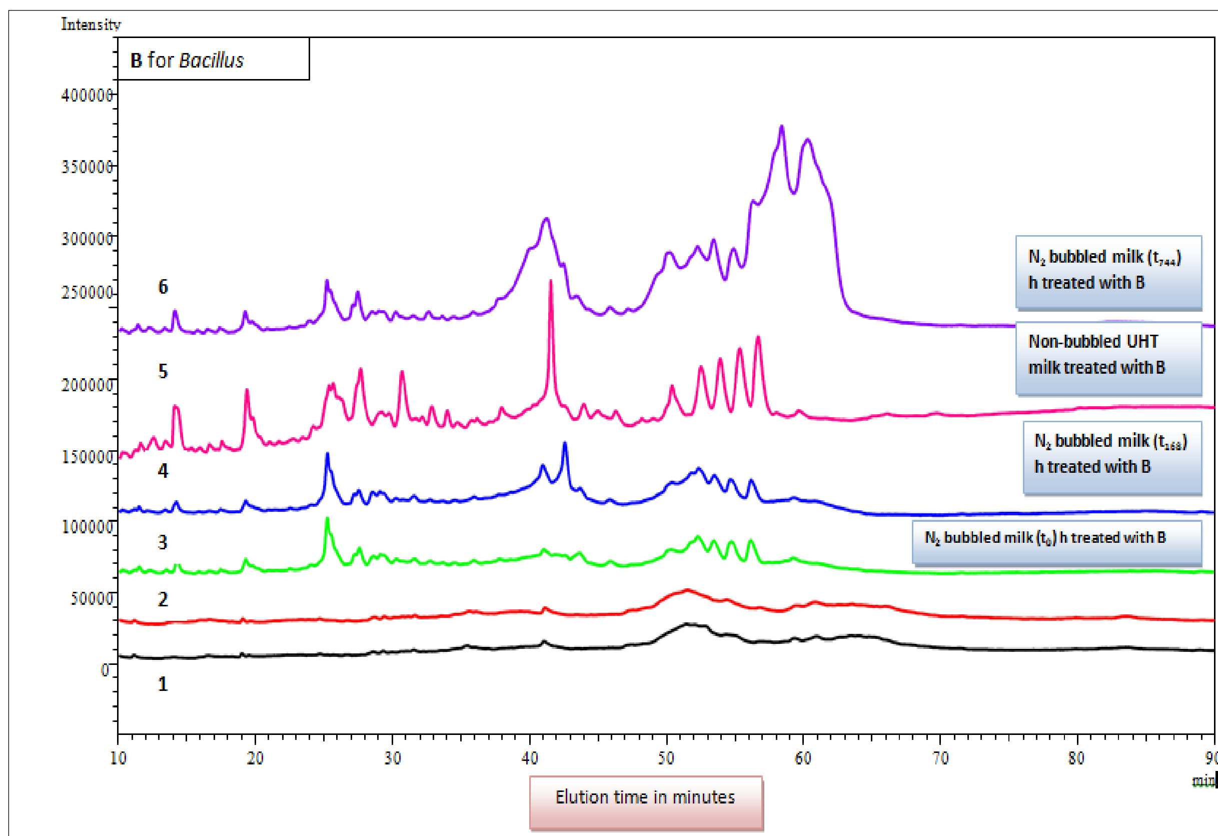

Figure 5.7b: RP-HPLC chromatogram peptide profiles for (1) UHT fat-free milk (control); (2) N_2 bubbled UHT fat-free milk (control); (3) N_2 bubbled UHT fat-free milk @ time 0 hydrolyzed by *Bacillus* protease; (4) N_2 bubbled UHT fat-free milk @ time 168 h hydrolyzed by *Bacillus* protease; (5) UHT fat-free milk hydrolyzed by *Bacillus* protease; and (6) N_2 bubbled UHT fat-free milk @ time 744 h hydrolyzed by *Bacillus* protease

5.5. Conclusions

Various additives (chemicals) were evaluated in UHT fat-free milk to assess their inhibitory effect towards proteolytic attack. Two main techniques were used for analysis namely, RP-HPLC and the Merck protease assay. From the data obtained, it was evident that SHMP and $CaCl_2$ inhibited *Bacillus* and plasmin protease attack. It was also evident that EDTA only inhibited the proteolytic action induced by *Bacillus* protease and had no inhibitory impact on plasmin protease activity. The additive sodium citrate on the other hand slightly increased protease action induced by both *Bacillus* and plasmin protease.

Unfortunately, the addition of chemicals/additives in milk might result in legal complications, therefore the impact of bubbling gases through milk was investigated. The effectiveness of various gases namely, CO_2, He, and N_2 were evaluated on raw full-cream milk. It was evident that all the gases mentioned above had the ability to retard plasmin protease activity whereas only CO_2 and He retarded bacterial proteolysis (*Bacillus*) in raw full-cream milk.

Additionally, due to the fact that age gelation occurs predominantly in UHT milk and N_2 gas is by far the cheapest and readily available gas; its effectiveness was further evaluated on UHT fat-free milk and UHT full-cream milk. It was evident that N_2 gas retarded proteolytic attack induced by *Bacillus* protease and plasmin protease in both UHT fat-free milk and UHT full-cream milk. It was also evident that the inhibition by N_2 is more effective in UHT fat-free milk than it is in either UHT full-cream milk or raw full-cream milk.

Finally, the extent of N_2 inhibition in UHT fat-free milk over time (up to 744 h$\approx$ 1 month) was evaluated. It was evident that N_2 was effective against proteolytic attack induced by plasmin protease and by *Bacillus* protease up to 168 h of storage at 5°C. Thus, it is concluded that N_2 gas was able to retard protease activity induced by bacterial (*Bacillus*) protease better than it does to protease action induced by the native plasmin enzyme.

5.6. References

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Chapter 6: General conclusions

Age gelation in milk is a major factor that limits the shelf life of UHT milk; and this defect consequently costs the dairy industry enormous financial losses (millions of rand per annum). It is reported in the literature that age gelation is caused by a proteolytic enzyme secreted predominantly by psychrotolerant bacteria (*Bacillus* spp. and *Pseudomonas* spp.) as well as plasmin protease which is indigenous in milk. Microbial protease production is often associated with lipase production.

Throughout the study, it was clear that milk-fat plays an important role in the susceptibility of casein towards proteolytic attack. It was evident that UHT fat-free milk is more susceptible to bacterial protease than UHT low-fat and full-cream milk. In contrast, plasmin protease hydrolysed UHT fat-free, low-fat and full-cream milk equally.

During the treatment of milk fat with *Pseudomonas* lipase enzyme, it was evident that the free-fatty acids (FFA'S) released during fat hydrolysis activates plasminogen (PG) to plasmin (PL) protease. From these findings, further investigations were performed to study the individual and combined roles of FFA's on the activation of PG to PL protease. Surprisingly, it was found that most of the FFA's between C4-C18:1 had the ability to activate PG to PL. This activation of PG to PL by FFA's is astonishing, and it will bring in a totally new approach towards the understanding of age the gelation phenomenon.

Often, milk is stored in the cold chain at temperatures in the vicinity of 5°C. According to enzyme kinetics, low levels of casein hydrolysis are supposed to occur under these conditions in comparison to room temperature. In literature, controversy exists on this matter. In this study, it was reported that proteolysis by plasmin (activated by KIO_3) protease occurs to a greater extent at refrigeration temperatures than at room temperatures. Thus, it is clear that not only psychrotolerant bacterial protease is causing problems at lower temperatures, but that plasminogen can be activated during storage under refrigeration temperatures and as a result, plasmin can effectively contribute to this problem.

It is clear that protease from psychrotolerant bacteria predominantly *Bacillus* and *Pseudomonas* protease and plasmin causes age gelation. Therefore, means to inhibit proteolytic attack using chemicals and gases were evaluated. It was established that EDTA has an inhibitory effect on *Bacillus* protease in milk due to its ability to bind metals (*Bacillus*

protease is a metalloenzyme) and therefore inhibit enzyme activity. In the case of plasmin which is a serine enzyme, EDTA had no impact. However, SHMP and $CaCl_2$ inhibited both *Bacillus* protease and plasmin protease. Sodium citrate, on the other hand, enhanced the activity of both PL and *Bacillus* protease.

Unfortunately, the addition of additives/chemicals might result in legal complications; therefore, the next option of bubbling gases through milk was investigated. The actions of *Bacillus* protease or a plasmin protease were retarded mainly by CO_2 and He gases in raw full-cream milk. Nitrogen gas, on the other hand, was mainly effective against proteolytic attack induced by bacterial protease (*Bacillus*) and plasmin protease more in UHT fat-free milk than in UHT full-cream milk. Finally, it can be concluded that the effectiveness of N_2 gas is short-lived (only effective up to 168 h $\approx$ 1 week), thus the use of N_2 gas is not ideal and cannot be recommended as a definitive practical solution to the problem of age gelation in UHT milk.

Chapter 7: Summary

Dairy industries lose millions of rand per annum due to the age gelation of milk that reduces its shelf life. This can be induced by anything that has an impact on the suspension of the negative charge on the casein micelle during storage of the UHT milk. This includes the enzymatic or chemical processes that occur during storage of milk. The enzymatic processes involve the action of the plasmin (native enzyme) or the heat stable microbial protease.

During storage at the processing plant or on the farms, psychrotolerant bacteria predominantly the *Pseudomonas spp.* or *Bacillus spp*. start to grow in the milk and produce enzymes called protease. Additionally, during storage of milk at the processing plant, inactive plasminogen in milk can be activated to plasmin protease during storage at 5°C. The heat stable proteases are not destroyed during UHT treatment and remain active in UHT milk. This can result in gelation of milk being observed within few weeks after storage.

In order to implement means to combat the age gelation problems, knowledge of plasminogen activators that mediate the conversion of plasminogen to plasmin after heat treatment is required. In this study, it was found that free-fatty acids were able to act as activators in this conversion. Furthermore, once the mechanisms are in place, stopping the age gelation of milk is nearly impossible. Retarding the flocculation processes however may be possible by making use of chemicals such as EDTA, SHMP, $CaCl_2$ and Sodium citrate, or by bubbling gases such as CO_2, He and N_2 through milk.