A BIOCHEMICAL STUDY OF TISSUE TYPE PLASMINOGEN ACTIVATOR IN BOVINE MILK

by

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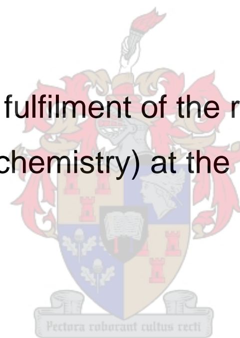
Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (Biochemistry) at the University of Stellenbosch



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SUMMARY

This study describes:

1. The isolation and the purification of tissue type plasminogen activator and urokinase plasminogen activator in bovine milk.

2. The biochemical characterisation of tissue type plasminogen activator in bovine milk.

3. An investigation of the influence of the addition of purified tissue type plasminogen activator to ultra high temperature milk, Gouda cheese and yoghurt.

OPSOMMING

Hierdie studie beskryf:

1. Die isolering en suiwering van weefseltipe-plasminogeenaktiveerder en urokinase-plasminogeenaktiveerder in beesmelk.

2. Die biochemiese karakterisering van weefseltipe-plasmingeenaktiveerder in beesmelk.

3. `n Ondersoek na die invloed van die byvoeging van gesuiwerde weefseltipe-plasminogeenaktiveerder by ultra hoë temperatuur melk, Gouda kaas en joghurt.

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been subjected to any university for a degree.

1st March 2007

F.P. Cilliers

Date

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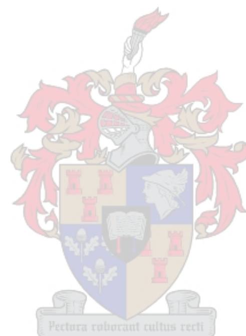

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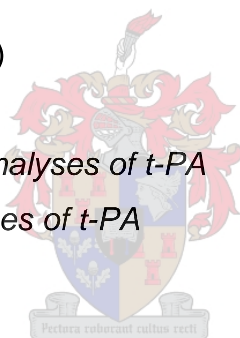

ABBREVIATIONS

ADSA	American Dairy Science Association
BSA	Bovine serum albumin
CdN	Cd-ninhydrin
CCP	Colloidal calcium phosphate
cfu	Colony forming units
CN	Casein nitrogen
CP1	Casein pellet 1
CP2	Casein pellet 2
cpi	Centipoise
Da	Daltons
DAN	Diazoacetylnorleucine methyl ester
DFP	Di-isopropylfluorophosphate
DMF	Dimethylformamide
D-value	Decimal reduction time
EDTA	ethylenediaminetetraacetic acid
EPNP	1,2 epoxy-3(p-nitrophenoxy) propane
FAO/WHO	World Health Organisation
FDM	% Fat in dry matter
Fp	Freezing point
FTIR	Fourier transform infrared
GDP	Gross domestic product
HIV-AIDS	Human Immunodeficiency Virus, Acquired Immunodeficiency Syndrome
HPLC	High performance liquid chromatography
HPGPC	High performance gel permeation chromatography
IDF	International Dairy Federation
IEF	Iso-electric focusing

Ig	Immunoglobulin
IL	Interleukin
kDA	Kilo Daltons
MAP	Milk alkaline protease or plasmin
MCP	Microbial protein
MFGM	Milk fat globule membrane
Mr	Molecular mass
NCN	Non-casein nitrogen
NDA	National Department of Agriculture
NEM	N-ethylmaleimide
NPN	Non-protein nitrogen
OPA	*o*-phthaldialdehyde
PA	Plasminogen activator
PAI	Plasminogen activator inhibitor
PCMB	Para-chloromercuribenzoate
PG	Plasminogen
PI	Plasmin inhibitor
pI	Isoelectric point
PL	Plasmin
PMN	Poly-morphonuclear leukocytes / Neutrophils
PMSF	Phenylmethylsulfonyl fluoride
PP	Proteose peptones
RCP1	Reconstituted casein pellet 1
RSP1	Reconstituted somatic cell pellet 1
RSP2	Reconstituted somatic cell pellet 2
RM	Raw milk (fresh) bovine
SA	Specific activity
SCC	Somatic cell count
SDS	Sodium dodecyl sulphate gel electrophoresis
SP	Somatic cell pellet
SPC	Standard plate count

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SUP1	Supernatant 1: Defatted milk
SUP2	Supernatant 2: Milk serum
SUP3	Supernatant 3: t-PA fraction
TA	Titratable acidity
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TN	Total nitrogen
TNF-α	Tumour necrosis factor alpha
t-PA	Tissue plasminogen activator
TPCK	Soybean trypsin inhibitors
TPN	Total protein nitrogen
TS	% Total solids
UDP	Undegraded dietary protein
UHT	Ultra-high temperature
u-PA	Urokinase plasminogen activator
V_0	Void volume (gel filtration)
V_e	Total volume (gel filtration)
WPN	Whey protein nitrogen
WSN	Water soluble nitrogen
ZNF	Zinc chelate fraction (t-PA)

ab asino lanam

“blood from a stone”

Chapter 1

INTRODUCTION

In 1905 "*Herr Doktor*" Albert Einstein, the brilliant physicist who's legendary formula changed the world of physics, published four papers that were later described as *"…the papers which, after half a century, still had a major influence on ongoing research"* [1]. Milk was the subject of study for the scientific icon of the 20th century, when he formulated his theory on Brownian movement. In Einstein's publication on Brownian movement in dairy research, he investigated the behaviour of casein particles in milk during the manufacturing of cheese, which is still being researched today [2].

Milk and milk constituents have always provided a model system for scientific investigation for numerous reasons. Milk is a nutrient dense product which is secreted by the female of all mammalian species, primarily to meet the nutritional requirements of the neonate. Milk contains lipids (which include essential fatty acids), proteins and peptides, essential amino acids, immunoglobulins, enzymes, enzyme inhibitors, growth factors, hormones and anti-bacterial agents, lactose, vitamins and inorganic elements such as calcium and water.

Milk is the most nutritional and complete single food available in nature which, as a result, is very important in a socio-economic context in the world today. Milk and dairy products are important components in the human diet in many parts of the world, especially in developing countries such as South Africa, as it provides a rather inexpensive, valuable source of nutrition. It is stated by The National Department of Agriculture (NDA) that as much as 30% of the dietary protein in developed countries, such as the United States and Australia, is supplied by milk and dairy products. Since milk can be converted into a wide range of products containing important dietary nutrients it is not only beneficial to the consumer, but

also to the industry. Therefore the food industry in South Africa is faced with various challenges, such as providing better quality raw products and final product solutions to address important factors such as child nutrition, malnutrition, HIV-AIDS and obesity.

According to the NDA primary agriculture contributes about 2.6% to the gross domestic product (GDP) and almost 9% to formal employment. However, there are strong backward and forward linkages into the economy, so that the agro-industrial sector is estimated to comprise 15% of the GDP. In the past five years agricultural exports have contributed approximately 8% towards South African exports. The South African dairy industry is an important employer, with 4,300 milk producers that employ about 60,000 farm workers, and indirectly providing employment to some 40,000 people. Milk production for 2004/2005 was estimated at 2.90 million tonnes. Therefore, scientific research and ultimately technology transfer to the primary and secondary dairy industries are key drivers in the expansion of a developing market within the agro-industrial sector.

Milk contains approximately 30 indigenous enzymes, some of which are associated with the casein micelles and others that can be found in the serum phase of the milk [3-5]. These enzymes originate from the blood, the secretory cell cytoplasm or the fat globule membrane [4]. Bovine milk contains several endogenous proteases; these include plasmin, plasminogen, plasminogen activators (PA), plasmin inhibitors (PI), plasminogen activator inhibitors (PAI), thrombin, cathepsin D, acid milk proteases and aminopeptidases [5,6]. Several proteases also derive from leukocytes (somatic cells) and bacteria in milk [3].

One of the major enzymes in milk is milk alkaline protease (MAP), commonly referred to as plasmin. Under certain physiological conditions, such as bacterial infection, the inactive precursor form of the enzyme is converted into the active form by self-activation (autolysis) or by limited proteolysis by another protease [7-10]. The physiological mechanism by which the inactive precursor zymogen,

plasminogen, is converted to plasmin, is the result of a cascade of reactions producing plasmin or fibrinolysin. In the case of plasminogen the conversion to the active enzyme plasmin occurs as a result of the specific action of PA [7,8]. Plasmin is an anti-coagulant that lyses fibrin, therefore preventing coagulation or clotting of blood [6,11].

Protease-catalysed hydrolysis of micellular and casein dispersions in milk by the fibrinolytic system, of which plasmin and PA are key elements, causes multiple changes in the functional properties of milk and dairy products and directly influences the quality of milk as a base for all dairy products [12-16]. Enhanced proteolysis in dairy products directly influences the quality of the final product. Controlled proteolysis is important for the flavour development in cheese, on the contrary uncontrolled proteolysis can cause detrimental effects in dairy products, such as: the gelation of Ultra High Temperature (UHT) milk, the manufacte of poor quality cheeses, poor ripening of cheese, declining cheese yield, degradation in stored casein products (decrease in viscosity) and decreased heat and ethanol stability of raw and processed milks, to name but a few.

The aim of the study was to purify and characterise PA from bovine milk and to investigate and quantify the effect of the addition of the purified PA to bovine milk prior to secondary processing.

The mechanism of activation of the serine proteinase plasmin in the fibrinolytic system of human and bovine milk is described in Chapter 2. Thrombin, through thrombomodulin, activates protein kinase C. Activated protein kinase C increases the concentration of PA [17,18]. Although the physiological mechanism of PA function and release is still unclear, it stimulates and activates the conversion of plasminogen to plasmin [19]. Research also indicates that during mastitis (infection in the udder of the cow) there is a 10 to 20-fold increase in PA activity, but the mechanism causing this rapid increase in PA concentration is still unknown [20]. It is believed to be as a result of a second messenger system, but

this still remains to be proven. The increase in PA supposedly also influences the ratio of the different somatic cells in the milk (macrophages, neutrophils and lymphocytes) which is an indicator of general herd health [20,21].

Chapter 3 gives an overview of the protein composition in bovine milk. The activation of the plasmin results in the breakdown of several milk proteins, especially β- and α_s- casein, yielding a number of peptides referred to as the γ-caseins and the proteose peptones [19]. The κ-casein component is relatively resistant against cleavage by the enzyme [15,16,22,23]. The breakdown of the milk proteins will influence the textural, physical and chemical properties of milk and dairy products, as discussed in Chapter 4.

Chapter 5 describes the isolation and characterization of plasminogen activators. Plasmin, plasminogen and PA are associated with the casein miscelles and the fat globule membranes in milk [22,23]. The PI and PAI that occur in the serum phase of the milk that further control the activity of PA [22,23]. There are at least two PAs present in bovine milk, two of which have been identified as urokinase plasminogen activator (u-PA) and tissue type plasminogen activator (t-PA) [22]. Both activators have already been partially isolated and characterised and according to these preliminary studies also exhibit different structural, physiological, kinetic and immuno-chemical properties [22,24]. While it is well established that plasmin in milk and other dairy products occurs mainly in precursor form (plasminogen - activated during storage), the origin, characteristics and the factors affecting the activity of plasminogen activators are still unknown [22-28].

Chapter 6 describes the results of adding the purified PA fraction to milk and incubating before using the milk for the manufacturing of ultra high temperature (UHT) milk, Gouda cheese and yoghurt. The hypothesis was that protease hydrolysis of the micellular and caseinate dispersions can cause changes in the functional properties and characteristics of the final products.

The results of this study are discussed in Chapter 7, and the experimental protocols in Chapter 8. PA was isolated and purified from bovine milk, using a combination of fractionation, solubilisation and chromatography steps. Partial purification was done using size exclusion and metal chelate chromatography. Colorimetric assays were used to detect the purified PA and their activity. The amino acid sequence of PA was determined by high-pressure liquid chromatography (HPLC) analysis with *o*-phthaldialdehyde (OPA) detection.

In summary the elucidation of the PA system is arguably the most crucial part of interest to the biochemical study of milk proteases. The elucidation can lead to practical applications that will be of great benefit to the primary and the secondary dairy industries.

Chapter 2

A PHYSIOLOGICAL REVIEW OF THE FIBRINOLYTIC SYSTEM IN HUMAN AND BOVINE MILK

2.1. Introduction

Milk can be defined as the unadulterated, fresh liquid that is expressed from the udder of cows when milked. This, however, excludes the liquid expressed 15 days before and 5 days after calving, this is known as colostrum.

Good quality raw milk is required to make good quality dairy products. Once raw milk is defective and of inferior quality, it cannot be improved during processing, and defects often become more pronounced. Mastitis, an infection of the udder of the cow, is one of the most common herd health concerns. Mastitis in dairy cows, which is most often the result of a bacterial infection or inferior milking practices, causes an increase in the herd Somatic Cell Count (SCC) of the milk.

The SCC is the number of white blood cells, epithelial and tissue cells counted in 1 ml of milk. When inflammation (haemostasis and thrombosis) in the udder of the cow occurs, the natural defense mechanisms of the cow are activated to combat the infection, which leads to an increased blood supply and concentration of white blood cells, especially neutrophils, to the infected areas, this causing and increase in SCC. SCC is, therefore, an important indicator of mastitis and an invaluable tool for herd management and indicative of the “udder-health” of the cow.

In South Africa the legal specification for the acceptance of raw bulk milk for further processing according to the ”Regulations Relating to Milk and Dairy

products" is 500,000 or fewer somatic cells per 1,0 ml of bovine milk (Table 2.1). As indicated in Table 2.2 counts exceeding 200,000 SCC.ml^{-1} generally indicate some level of mastitis (clinical or sub-clinical) in the herd and thus the potential for quality defects in raw milk and in processed dairy products. Quality defects are generally the result of enzymes associated with infection and somatic cells that degrade protein, milk fats and other components resulting in reduced cheese yields and flavour defects (such as bitterness, rancidity) in cheese, pasteurised milk and other dairy products. Table 2.3 summarises the effect of mastitis on milk components [29].

Table 2.1: South African Regulations Relating to Milk and Dairy products: Regulations regarding sale of raw milk for further processing[i]

Parameter	**Specification**
Antibiotics	Absent
Pathogenic organisms, extraneous matter or inflammatory products	Absent
Clot-Boil test	Negative
Standard Plate Count (cfu.ml^{-1})	< 200,000
Coliform bacteria (cfu.ml^{-1})	< 10
Escherichia coli (cfu.ml^{-1})[ii]	Absent
Escherichia coli (cfu.0.01ml^{-1})[iii]	Absent
Somatic Cell Count.ml^{-1} [iv]	< 500,000
Alcohol Test (68% ethanol v/v)	Negative

[i] *Published under Government Notice No. R. 1555 of 21 November 1997, As corrected by: Government Notice No. R.1278 of 29 October 1999, Government Notice No. R. 488 of 8 June 2001, As amended by: Government Notice No. R.9 of 7 January 2000, Government Notice No. R.53 of 28 January 2000, Government Notice No. R.755 of 28 July 2000, Government Notice No. R.837 of 25 August 2000, Government Notice No. R.1052 of 27 October 2000, Government Notice No. R.489 of 8 June 2001*

[ii] *Modified Eijkmann Test*

[iii] *VRB Mug Agar Method and dry hydrated film method*

[iv] *The Standard Method for Counting Somatic Cells in Bovine Milk is set forth in International Dairy Federation (IDF) Bulletin No. 114 of 1979.*

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Table 2.2: Relationship between herd Somatic Cell Count and mastitis in bovine milk

Herd SCC / ml	Mastitis	Projected % infected quarters in herd
Less than 200,000	Excellent	None
250,000 - 500,000	Good	Less than 20 %
500,000 - 750,000	Danger zone	20 - 40 %
More than 750,000	Definite problem	More than 40 %

Table 2.3: Relationship between mastitis and milk constituents in bovine milk [29]

Component	Effect	% Composition	
		Normal	Mastitis
Fat	Decrease	3.8	3.6
Protein	Slight decrease	3.6	3.5
Casein	Decrease	2.8	2.3
Whey Proteins	Increase	0.8	1.2
Lactose	Decrease	4.9	4.4
Sodium (Na^+)	Increase	0.05	0.08
Chlorine (Cl^-)	Increase	0.10	0.18
Calcium (Ca^{2+})	Decrease	0.13	0.09

Factors causing mastitis and an increase in SCC, such as overmilking, injury to the udder of the cow and infection in the teat canal resulting in inflammation, are discussed in more detail in Chapter 4.

2.2. The physiology of bovine milk synthesis

In order to understand the effect of milk proteases on milk and milk composition it is important to have a basic understanding of the physiological process of milk synthesis.

The udder of a full-grown cow can weigh up to 50 kilograms (kg), just prior to milking, and is divided in individual milk glands or quarters. Each quarter has one teat, with no connection between individual quarters. The ideal udder should shrink after complete milking, but still be elastic. Generally, the rear quarters are slightly more developed and produce 60% of the milk, whereas the front quarters only produce 40%. The major components of the udder are as follows (Fig. 2.1) [30,31]:

Ligaments: Together with the skin, ligaments and connective tissue support and maintain the udder close to the body of the cow. The ligaments are divided into the median suspensory ligaments, these divide the left and the right halves of the udder of the cow, and the lateral suspensory ligaments which give support to the outside and underside of the udder.

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Secretory and duct system: The udder is known as an exocrine gland because milk is synthesized in a specialized cell known as an alveolus (grouped in alveoli), and is then excreted outside the body through a duct system. The alveoli are made up of a single layer of myoepithelial cells, which surround the lumen on the inside of each alveolus, into which the milk is secreted. A dense layer of myoepithelial cells (muscle tissue) and capillary blood vessels surround the alveoli. The function of the alveolus is to (i) remove nutrients from the blood, (ii) to transform these nutrients into milk and (iii) to discharge the milk into the lumen. Each lumen drains to smaller milk collection ducts which are connected to the gland cistern directly above the teat of the gland. The milk can be withdrawn from the gland (or quarter) via the teat and teat canal.

Blood supply and capillary structures: Milk production demands a lot of nutrients; these are brought to the udder via the bloodstream. To produce 1 kg of milk, 400 to 500 kg of blood must pass through the udder. In addition, the blood carries hormones that control udder development, milk synthesis, and the regeneration of the secretory cells between lactations, also known as the dry period.

Lymph system: Lymph is a clear fluid that comes from tissues highly irrigated by blood. The lymph helps to balance the fluid flowing in and out of the udder and helps to combat infections. Sometimes the increased blood flow at the onset of lactation leads to an accumulation of fluid in the udder until the lymph system is able to remove this extra fluid.

Innervation of the udder: Nerve receptors on the surface of the udder are sensitive to touch and temperature. During the preparation of the udder for milking, these nerves are triggered which initiates the "milk let down" reflex that allows the release of milk. The hormone, oxytocin, and the nervous system are also involved in the regulation of blood flow to the udder. When a cow is startled or feels physical pain, the concerted action of adrenaline and the nervous system decreases blood flow to the udder, thus inhibiting the "milk let down" reflex and as a result milk production is lowered.

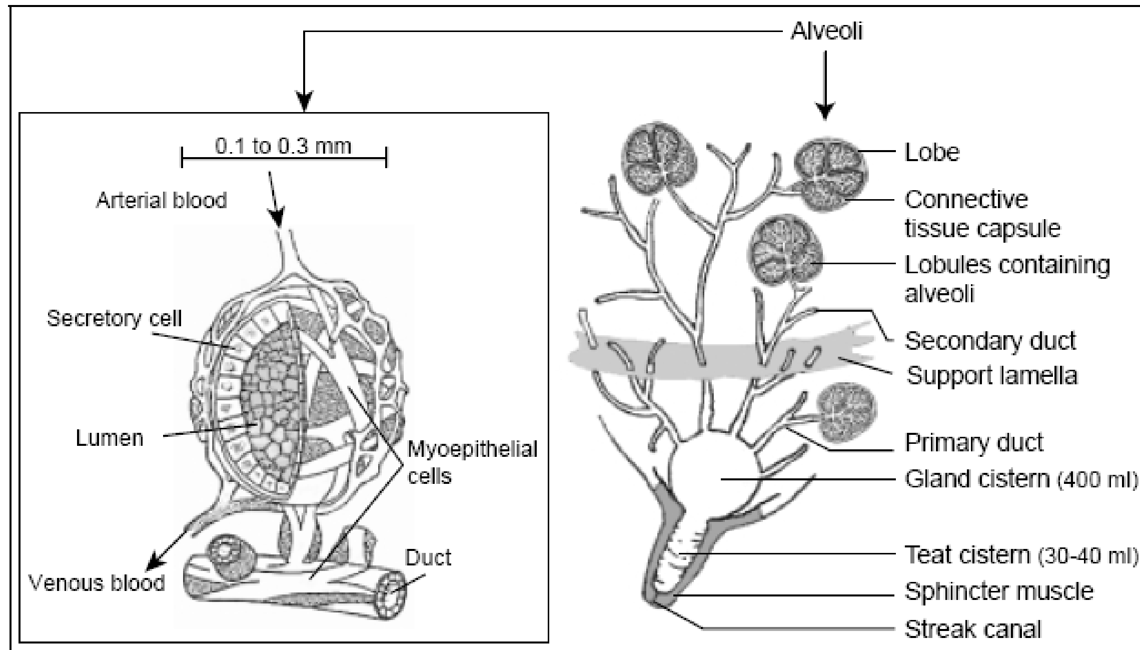

Figure 2.1: Alveoli and ducts of the milk secretory system [31].

Milk secretion by the secretory cells is a continuous process that involves intricate biochemical reactions as diagrammatically summarised in Fig. 2.2. Nutritional factors which influence rumen fermentation and the end products of fermentation such as volatile fatty acids, ammonia and peptides, influence milk composition since microbial protein and volatile fatty acids are precursors for milk component synthesis. The principle nutrients available to the udder for synthesis of milk solids are glucose, acetate, β-hydroxy butyrate, long chain fatty acids and amino acids. As molecules of lactose are produced, water moves into the cell to equalise osmotic pressure, the same is true for the ash or minerals component. The rate of lactose synthesis and secretion therefore regulates milk yield. Lactose is produced from glucose, most of which is synthesised in the liver from propionate, a ruminally derived volatile fatty acid. Under some circumstances glucose can also be synthesised from amino acids and starch.

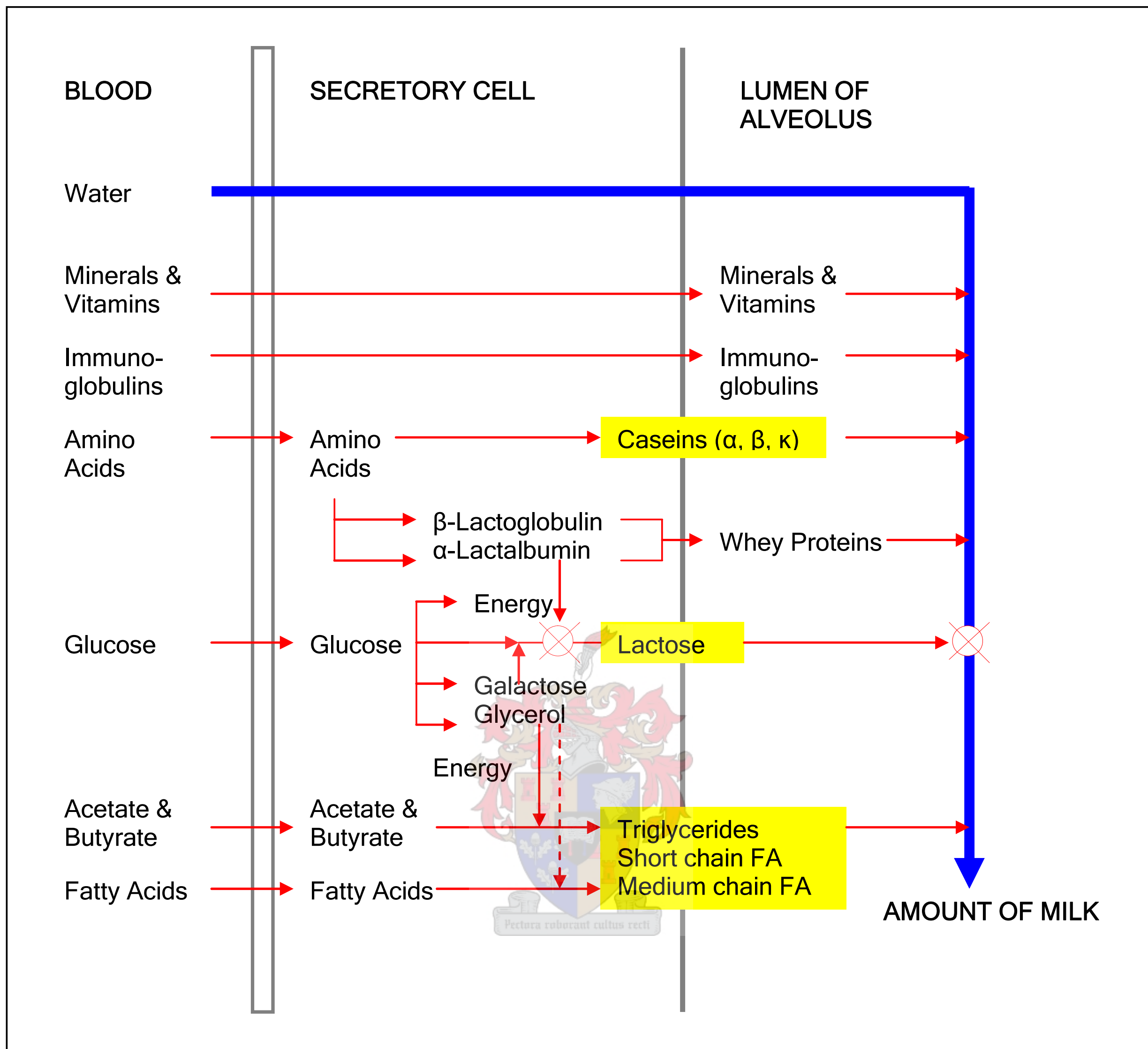

Figure 2.2: Milk secretion in the secretory cells (crossed circles indicate key regulatory steps) [31].

Milk protein is synthesised in the mammary gland from amino acids extracted from blood as it flows through the mammary gland. The amino acids are supplied by ruminally synthesised microbial protein (MCP) and ruminally undegraded dietary protein (UDP). Colostrum milk contains extensive amounts of globulin proteins which move directly from blood to milk.

Milk fat is synthesised in the mammary gland from two basic types of precursors. Short chain fatty acids are synthesised primarily from acetate and β-hydroxy-butyrate which are derived from the rumen as a result of carbohydrate digestion by microbes. The longer chain fatty acids are extracted from the circulating blood by the mammary gland. These fatty acids are primarily of dietary origin but may also be derived from body fat mobilisation or by liver metabolism. About 50% of total fatty acids are synthesised in the mammary gland while the remaining 50% are derived directly from the blood.

The energy required by the udder for synthesis of milk solids is derived largely from the oxidation of acetate, the volatile fatty acids produced in the largest quantity in the rumen. Smaller amounts of energy are derived from the oxidation of β-hydroxy-butyrate (synthesised in the liver from butyrate, the volatile fatty acid produced in the third largest quantity in the rumen), amino acids and glucose [31,32].

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2.3. The fibrinolytic system

The fibrinolytic system acts as an anti-coagulant that solubilises fibrin clots in the blood. The proteolytic system contains five different enzymes, namely: the inactive precursor zymogen plasminogen, the active protease plasmin, plasminogen activators (PA), plasmin inhibitors (PI) and plasminogen activator inhibitors (PAI) [20,22].

The fibrinolytic system is in a state of dynamic equilibrium in which fibrin clots in the blood are constantly being formed and dissolved [6,9,10,11].

2.3.1. Haemostasis and thrombosis

Haemostasis and thrombosis are processes that encompass the coagulation or the clotting of blood. Haemostasis is the cessation of bleeding from a cut or severed vessel. Initially there is vasoconstriction of the injured vessel that reduces the flow of blood to the area of injury. Thrombosis occurs when the endothelium lining in the blood vessels is injured or removed (Fig. 2.3).

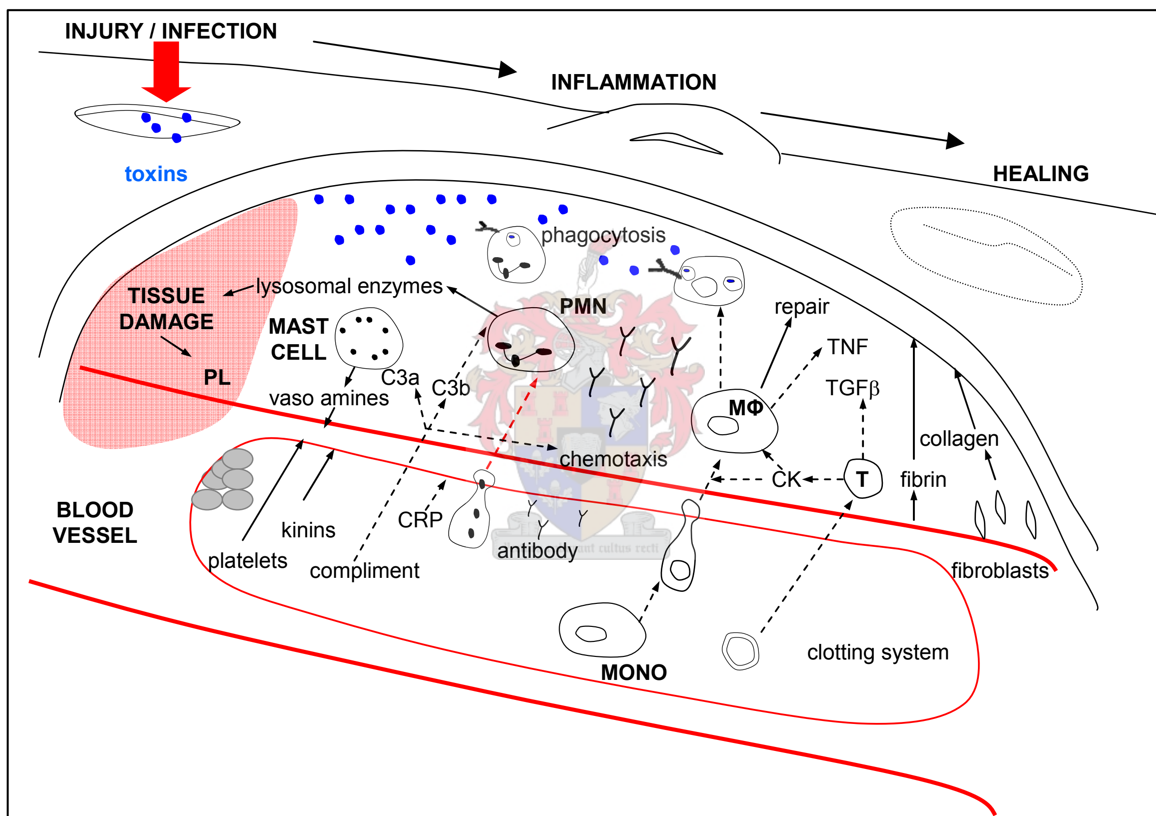


Figure 2.3: Diagrammatic representation of haemostasis during acute inflammation [33].

Both haemostasis and thrombosis can be divided into three phases: (i) the formation of loose and temporary platelet aggregates at the site of the injury, (ii) the formation of a fibrin network that binds to the platelet aggregate forming

hemostatic plug or thrombus, and (iii) the partial or complete dissolution of the thrombus, due to the function of the anti-coagulant plasmin, takes place.

As shown in Fig. 2.4, thrombus formation begins with the release of the pro-inflammatory cytokine interleukin-5 (IL-5) and the tumour necrosis factor alpha (TNF-α). The tissue factor (TF) stimulated by interleukin-6 (IL-6) and other cytokines, are expressed on the surface as activated mononuclear cells and endothelial cells.

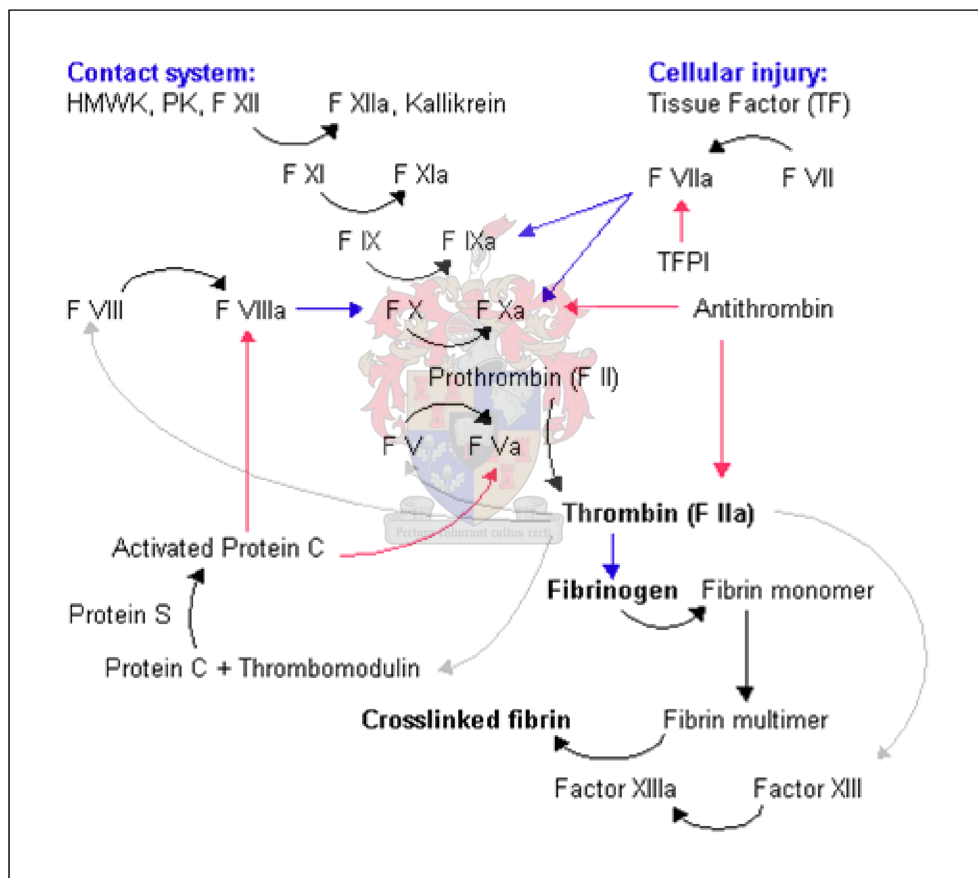
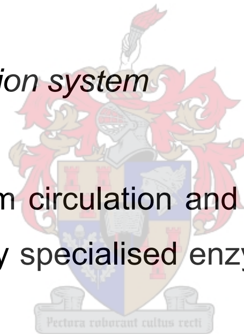


Figure 2.4: Diagrammatic representation of fibrinolysis during acute inflammation with special mention to the activation systems is place [34].

TF binds to factor VII to form the activated TF-factor VIIa complex. TF-factor VIIa directly or indirectly (via activated factor IX and factor VII) activates factor X. Activated factor X and activated factor V converts prothrombin (factor II) to thrombin (factor IIa).

Impairment of the three coagulation systems involving anti-thrombin III, protein C and tissue factor pathway inhibitor (TFPI) is initiated primarily by the release of TNF-α. The resulting intra-vascular formation of fibrin is not balanced by the adequate removal of fibrin, because fibrinolysis is inhibited by the high levels of PAI 1, mainly because of the influence of TNF-α. Increased PAI 1 inhibits PA activity, therefore reducing the rate of plasmin formation. The end result is the deposition of fibrin in the microvasculature and compromised perfusion [34].

2.3.2. The plasminogen activation system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The removal of blood clots from circulation and the turnover of the extracellular matrix proteins are facilitated by specialised enzymes, one of the most important being plasmin [6,7].

Plasmin performs many functions, but its primary role is to degrade fibrin that can be seen as the "structural matrix" of a blood clot. Plasminogen is activated to plasmin by serine plasminogen activator (u-PA and t-PA) enzymes [20]. The proteolytic activity of plasmin is regulated by PAI 1&2 (Figs. 2.5 and 2.6).

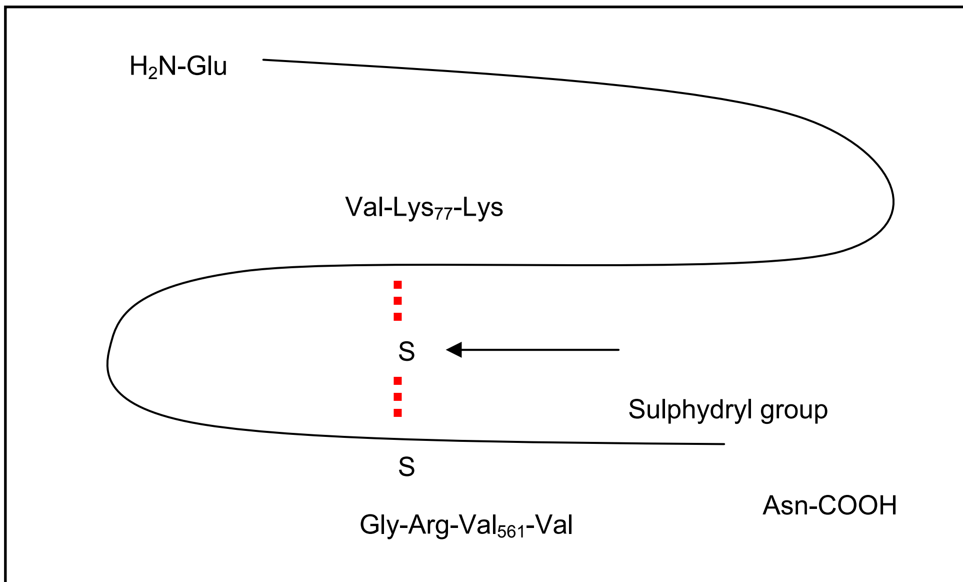

Figure 2.5: Inactive precursor zymogen plasminogen before activation to the two-chained active plasmin molecule [6].

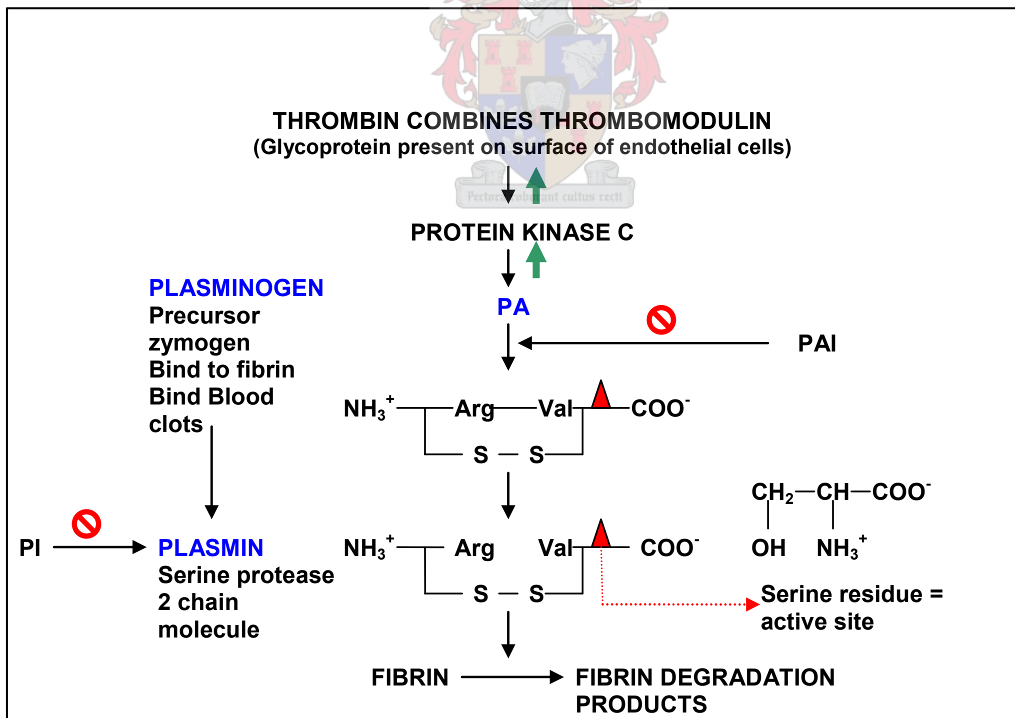

Figure 2.6: Activation of plasminogen activators during fibrinolysis (adapted from Ganong, W.F.) [34].

The plasminogen activating system actively participates in cell movement (cellular migration), wound healing (blood clot dissolution or fibrinolysis), metastatic spread of cancer and contributes to the turnover of the extracellular matrix in the central nervous system [35,36].

2.4. The fibrinolytic system in human milk

Although there is almost a 75% homology between bovine and human PA, extrapolation of results from human to bovine milk is not justified due to the difference in the composition of the milk between the species. Therefore results from research on human milk can be utilised as a guideline but should not be accepted for different models.

2.4.1. Human plasminogen and plasmin

During the first few days after birth high levels of PA and plasmin activity occur in human milk. After a few weeks the PA activity gradually decreases [37]. Comparison of the molecular weights of different PA's in human milk correlates well with the PA in blood, suggesting that the PA in milk originates from the blood, rather than being produced by the tissue of the mammary gland itself [38-41].

Human plasminogen is a single peptide chain with the N-terminal glutamine and the C-terminal asparagine [42,43]. Native plasminogen can be divided into two subgroups, namely Glu-plasminogen 1 (Mr 91,500 Da) and Glu-plasminogen 2 (Mr 89,300 Da) [40]. Glu-plasminogen 1 has four subforms with different pI values, namely 6.2, 6.3, 6.4 and 6.6. Glu-plasminogen 2 has 2 different subforms with different pI values, namely 6.4 and 6.6 [44]. Activation of

plasminogen to plasmin by t-PA or u-PA is as a result of the proteolyitic cleavage of a single peptide bond at Arg_{561}-Val_{562} in human plasminogen [46]. The N-terminal peptide is released, forming Lys-plasminogen 1 and Lys-plasminogen 2. Lys-plasminogen 1 has three subforms with different isoelectric (pI) points, namely 6.7, 7.2 and 7.5. Lys-plasminogen 2 has three different subforms with different pI values, namely 7.5, 7.8 and 8.1 [40,41]. A three dimensional model of plasminogen reveals triple disulfide loops or the so-called kringles, whereas plasmin contains five kringle domains on the N-terminal A chain [44].

Human plasmin (Mr approximately 90,000 Da) is capable of hydrolysing a broader spectrum of proteins than any other blood protease, but is highly specific for the peptide bonds containing lysine residues [38,39]. Plasmin catalyses the hydrolysis of the peptide bonds on the C-terminal side of the Lys and Arg residues [40]. The active site on the plasmin molecule is located at the serine residue on the C-terminal B chain and is connected to the N-terminal A chain two by a disulfide bridge [41,42].

Plasmin digests fibrin to form soluble degradation products, thus solubilising the fibrin-clot. Both plasmin and PA is then released into the fluid phase of the blood; subsequent inactivation by their natural inhibitors, PAI and PI, renders these molecules inactive [38,43].

2.4.2. Human plasminogen activators

Plasminogen activator activity is present in human milk and human mammary gland tissue [37]. The sources of PA are the mammary epithelial cells, endothelial cells or the leakage of PA from the mammary epithelial tissue via the blood into the milk. PA is native to human milk and can be divided into two distinct groups, namely: u-PA and t-PA [38-46]. Plasmin and plasminogen is functionally the same in milk and blood. PA activates plasminogen to plasmin;

PA is inhibited by PAI. The activation of plasmin results in an increase in proteolysis in blood and milk, in the latter instance casein is converted into smaller peptides. Plasmin inhibitors (PI) also inhibit the conversion of plasmin activity [21,23].

2.4.2.1. Urokinase plasminogen activators

Urokinase-PA is a serine proteinase and is probably the best-characterised PA with a molecular weight of 54,000 Da that can be divided into two subunits, a C-terminal B chain (containing the serine proteinase domain) and a N-terminal A chain, with molecular weights of 27,000 Da each. These two subunits are linked with a disulfide bond. The N-terminal A-chain contains a growth factor domain (amino acids 1-49), a kringle domain (amino acids 50-131), and an inter-domain linker region (amino acids 132-158) [40]. The one chain zymogen precursor of u-PA, pro-u-PA, has an activity of 250-fold less than that of the two chained u-PA. The activation of the pro-u-PA occurs by proteolytic cleavage of Lys_{158}-Ile_{159} in human u-PA [47]. U-PAs with molecular weight of 93, 57, 42, 35 and 27 kDa have been isolated from bovine and human milk and are associated with the somatic cells (leukocytes) in milk. The u-PA is synthesised by all cell types such as macrophages, fibroblasts and epithelial cells. The main function of u-PA is the degradation of extracellular matrix.

Glu-plasminogen 1 and 2 are converted to plasmin due to activation by urokinase. There are two proteolytic cleavages that firstly release a pre-activation peptide from the N-terminal with a molecular weight of 7,000. The cleavage takes place between Lys_{76} and Lys_{77} to yield either Lys-plasminogen 1 or 2 [40,41,43]. The second takes place between Arg_{560} and Val_{561} to yield Glu-plasminogen [45]. If the pre-activation peptide has already been released Glu-plasminogen will be converted to Lys-plasminogen. The release of the pre-activation peptide induces conformational changes in the Glu-plasminogen that enhance the activation of Lys-plasminogen. The dissociation of non-covalent

bonds at residues 45-51 and unidentified residues elsewhere on the molecule result in the Arg_{561}-Val_{562} bond being more accessible for proteolytic cleavage [40,44,45].

2.4.2.2. Tissue type plasminogen activator

Tissue type PA is a serine-protease (Mr approximately 90,000 Da) which is released from the vascular endothelium tissue in a single-chain form. The single chain t-PA can be proteolytically converted to a two-chained form, by a cleavage of a single polypeptide bond at Arg_{275}-Ile_{276} [45]. The two chains are held together by a disulfide bond, The N-terminal A chain (starting from the N-terminal) contains a fibronectin type II domain, a growth factor domain, and two kringle domains. The C-terminal B chains contains the serine proteinase domain [48,49]. T-PA is released into the blood stream where it binds to fibrin, and is biologically inactive when it is not bound to fibrin. Upon binding with fibrin, the t-PA cleaves the inactive precursor zymogen, plasminogen, between the Arg_{561}-Val_{562} bond to render the two-chain active protease plasmin [45,48,49]. The plasminogen activity of the single-chain t-PA is 10 to 50-fold lower than that of the two-chained form [48,49].

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2.5. The fibrinolytic system in bovine milk

In fresh bovine milk plasmin is present in low concentration, but during storage the plasmin concentration increases significantly [9,10,20,22]. Any factor or combination of factors that activates the inactive plasminogen to the active protease plasmin will have a negative influence in the overall quality of the milk, due to the effect on protein functionality. In dairy products, proteolysis due to an increase in plasmin negatively influences the body, texture, consistency and flavour of dairy products. Plasmin is a heat stable enzyme that survives pasteurisation and UHT treatment. The proteolytic cleavage of casein by

proteases also influences a number of dairy processing factors, such as the yield of cheese, gelation of UHT milk, milk protein functionality and overall dairy product quality [44,50-54].

In bovine milk plasmin, plasminogen and the plasminogen activators are associated with the casein micelles and plasminogen activator inhibitors and plasminogen inhibitors with the serum phase of the milk [55,56].

2.5.1. Bovine plasminogen and plasmin

Inflammation is the host defence mechanism against bacterial infection. During sub-clinical and clinical mastitis the invasion of antigens evokes an inflammatory response which includes the plasminogen activation system. T-cells sensitized toward antigens, release lymphokines which activate monocytes. The monocytes and macrophages migrate to the infected area and secrete mediators of the inflammatory response including proteinases [57].

In bovine milk the plasminogen acts as a proteolytic reservoir with concentrations between 0.8 and 1.6 $mg.l^{-1}$ [50,51,56]. The molecular mass of plasminogen and plasmin is 88 kDa and 58 kDa respectively [56-58]. The optimum pH for plasmin hydrolysis is pH 8. Plasmin can hydrolyse α_{s}-casein and β-casein in bovine milk yielding a number of peptides referred to as the γ-caseins and the proteose peptones [56,59].

Plasmin is associated with the iso-electric casein in milk (at a pH value of 4.60) or the casein micelles [3,6,23,56]. Plasmin is also the main protease associated with the milk fat globule membrane, although not the only protease present. Plasmin is an alkaline serine protease with trypsin-like activity, also inhibited by trypsin inhibitors (soybean trypsin inhibitors), but is not inhibited by chymotrypsin inhibitors [60-62]. Plasmin cleaves Lys-X and Arg-X at the C-terminal and the

rate of hydrolysis is faster at the Lys residues [60,63,64]. Maximum enzyme activity is exhibited at a pH of 7.5 and a temperature of 35°C. It is a two chain molecule with a heavy chain (Mr 60,000 Da) and a light chain (Mr 25,000 Da). These two chains are connected via a disulfide bond, the active site of the molecule is located in the light chain [20,23,25,28,56].

In bovine milk plasmin can be dissociated from casein and casein micelles *in vitro* by the addition of lysine or 6-aminohexanoic acid, in concentrations up to 1 M in milk or blood. In blood, plasmin is coupled to fibrin via lysine-binding sites on the plasmin. These lysine-binding sites interact with the lysine or 6-aminohexanoic acid, followed by the subsequent dissociation of fibrin from the fibrin–plasmin complex. Thus the interaction of plasmin in bovine milk with casein and the casein micelles is likely to be as a result of the interaction of lysine residues [23,65,66]. Blood only contains plasminogen, thus the presence of plasmin in freshly secreted milk suggests that the activation of plasminogen occurs while the milk is stored in the lumen of the udder prior to milking or perhaps earlier in the process of milk synthesis [50,51]. Both blood and milk plasminogen are inactive precursor zymogens that function as anti-clotting enzymes. The activated plasmin solubilises fibrin clots to form soluble products. Plasminogen concentration is higher in the colostrum than in late lactation milk [56,57,65].

In milk the major native proteolytic enzyme is plasmin, which functions optimally at a pH of 8.0. The plasmin readily hydrolyses the α_{s2}- and β-casein at the same rate to form shorter peptides referred to as γ-casein and the proteose peptones [52,67,68]. In β-casein there are 15 plasmin susceptible bonds, depending on the genetic variant. The first cleavage in β-casein of the peptide bonds by plasmin occurs at Lys_{28}-Lys_{29}, Lys_{105}-Hys_{106} and Lys_{107}-Glu_{108}. The three large C fragments released by the hydrolysis of β-casein is the $\gamma^{1\text{-}3}$-casein [69]. The $\gamma^{1\text{-}3}$-casein is considered to consist of fragmentation products rather than minor casein components. The position of the hydrolysis of the other caseins via

plasmin is not yet established, although the α_{s2}-casein yields three γ-caseins or fractions with molecular masses of 20.5, 12.3 and 10.3 kDa respectively. κ-Casein is resistant to proteolytic hydrolysis by plasmin [64, 70].

Plasmin has no effect on α_{s2}-lactalbumin and β-lactoglobulin, although these whey proteins inhibit plasmin activity to a certain extent [60].

2.5.2. Proteolysis of milk proteins by plasminogen

In the fibrinolytic system of bovine milk the proenzyme plasminogen is activated by PA to form the proteolytic enzyme plasmin as diagrammatically indicated in Fig. 2.7.

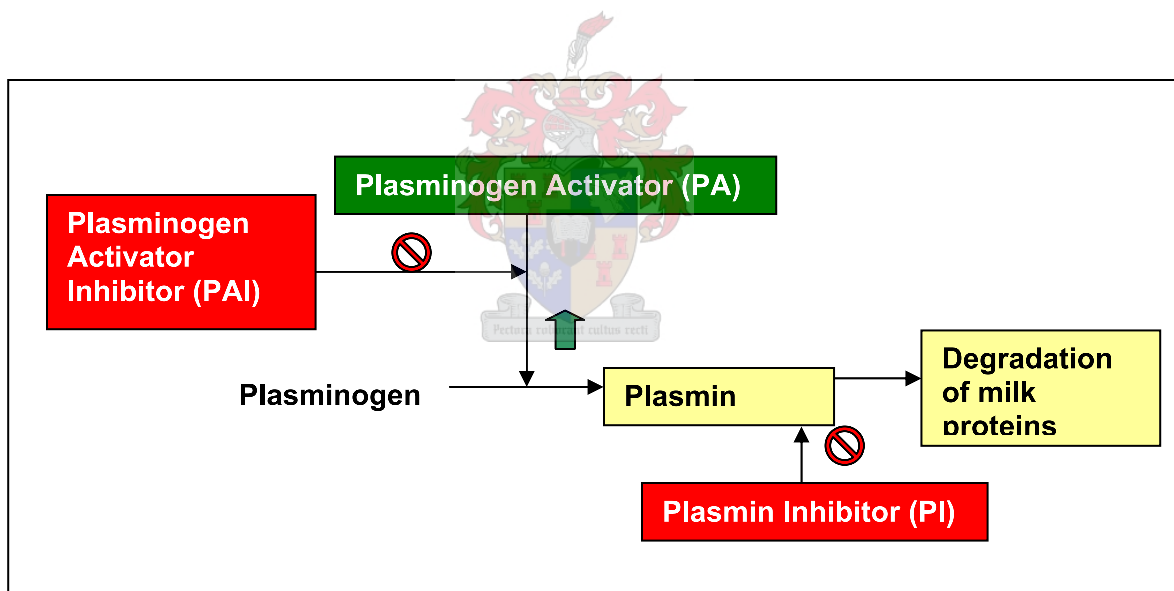


Figure 2.7: Native enzyme activator-inhibitor system in bovine milk with the activation of plasminogen to plasmin.

Alpha$_{s2}$- and β-casein are the preferred substrates for hydrolyses by plasmin. In trials with stored UHT milk the rates of hydrolysis of both α_{s2}- and β-casein were similar [6,14]. However, the whey proteins are resistant to the action of plasmin. Denatured β-lactoglobulin inhibits plasmin activity as the free sulfhydryl group of

the denatured β-lactoglobulin forms a thiol-disulphide interchange with the disulphide groups of the plasminogen. The allosteric hindrance renders the plasminogen molecule inactive for activation by PA [71,72,73].

2.5.3. Bovine plasminogen activators

Deharveng and Nielsen [22] reported the presence of at least two native PA in bovine milk. Plasminogen activator activity is associated with the casein micelles and the SCC in milk, whereas PAI and plasmin activity is localised in the milk serum. The serine proteases, t-PA and u-PA, activate plasminogen by the cleavage of its Arg_{561} – Val_{562} peptide bond. The newly formed amino group Val_{562} forms a salt bridge to Asp_{740}, resulting in conformational changes (specificity pockets) in the active domain of plasminogen to form the active form plasmin [22,45].

The cellular source of t-PA is still unknown, although the endothelial cells are rich in t-PA and is therefore probably the main source of t-PA extracts [20,56]. The t-PA activity in bovine milk is predominantly localised in the milk casein fraction. The molecular mass of the molecule is 75 kDa, and the activity of the t-PA is increase four-fold in the presence of fibrin, but remains unaffected by the presence of amiloride and urokinase [56].

The u-PA activity is localised in the somatic cell fraction of milk and has a molecular mass of 30 kDa and 50 kDa. Activity of the u-PA is enhanced by amiloride and urokinase, but remains unaffected in the presence of fibrin [57].

In bovine milk all the individual caseins (α_{s1}-, α_{s2}-, β- and κ-casein) enhance the activity of the PA. Optimal casein concentrations (α-casein 5 $\mu g.ml^{-1}$, β-casein 25 $\mu g.ml^{-1}$ and κ-casein 0 - 200 $\mu g.ml^{-1}$) that are needed to enhance PA activity were determined by Politus *et al.* [75]. It was also determined that when the

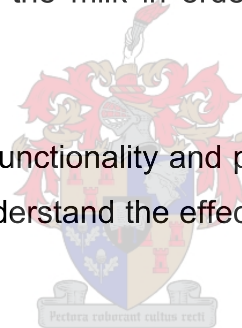
concentration of these caseins exceeded the optimum concentration the activity of the PA decreased. On a percentage weight for weight basis α-casein is the most effective enhancer of PA activity [65,74,75]. Bastian *et al.* [73] demonstrated that casein also acts as a competitive inhibitor of plasmin because casein is the natural substrate for plasmin in milk. When determining the activity under assay conditions casein will also compete with the chromaphore (D-Val-Leu-Lys-ρ-nitroanalide V0882) for the active site of the plasmin molecule. Once plasminogen is converted to plasmin, the plasmin promotes further activation of the plasminogen molecule, and can therefore be described as being autocatalytic (positive feedback mechanism) [72-73].

In bovine milk the u-PA and t-PA proteins contain 413 and 566 amino acids, respectively [57]. The t-PA and u-PA are believed to have different functions due to their different localization in milk fractions [9,45].

In addition to the native physiological PA in bovine milk, several pathogenic microorganisms also have developed PA (streptokinase produced by a variety of streptococci and staphylokinase produced by *Staphylococcus aureus*) [23,51,52]. Streptokinase, an extracellular protein of *Streptococcus uberis*, binds to plasminogen to form a plasminogen-complex in a 1:1 ratio [76]. The conformational changes to the plasminogen-complex render the plasminogen active and in cheese production will increase soluble nitrogen and increase the breakdown of β-casein [67]. In mastitis the function of these PAs produced by pathogenic microorganisms is to assist the proteolytic breakdown of fibrin and other extracellular matrix proteins, which facilitates bacterial penetration of normal tissue barriers and enables bacterial colonisation in deep tissue sites. The mechanism of function in cancer cells can be described as analogous to the invasion process of bacteria as they both immobilise plasmin on their surfaces in order to enhance proteolytic activity.

2.5.4. Serine Proteinase Inhibitors

Various plasmin inhibitors (PI) and plasmin activator inhibitors (PAI) inhibit the conversion of plasminogen to the active form plasmin [77]. Precetti *et al.* [78] isolated two major serine proteinase inhibitors in bovine milk, namely plasminogen activator inhibitor 1 (55,000 Da) and α_2-antiplasmin (60,000 Da). The principle plasmin inhibitor in bovine milk is α_2-antiplasmin that directly inhibits plasmin activity *in vivo* [79]. In humans the α_2-antiplasmin is a single chain glycoprotein with a molecular weight of 70,000 Da [80]. There is homology between human and bovine α_2-antiplasmin, particularly at the reactive site and at the N-terminal [81]. Bovine PAI–1 is also a 55,000 Da single chain glycoprotein. The low molecular mass of both α_2-antiplasmin and PAI–1 allows these inhibitors to migrate from the blood into the milk in order to participate in the control of plasmin activity.



In Chapter 3 the composition, functionality and protein structure of bovine milk is discussed in detail to better understand the effect of fibrinolytic milk proteases on milk proteins.

Chapter 3

COMPOSITION AND FUNCTIONALITY OF MILK PROTEIN

3.1. Introduction

In 1890 Stephen Babcock suggested the occurrence of an indigenous protease in milk and since the 1900's there have been numerous publications confirming the presence of proteases in milk [27]. There are also well documented examples of the effect that such protease activity induces on milk proteins, the character of milk and milk products [44,50-54]. At first it was believed that all protease activity occurs as a result of bacterial contamination. The presence of native milk proteases in milk has since been confirmed and as a result the native milk proteases have been studied intensely for the past three decades. In Table 3.1 a general classification of proteases in bovine milk, according to Hartley, is given [82].

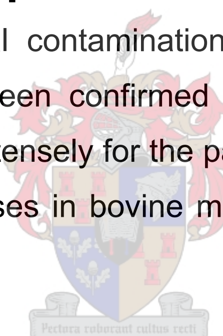


Table 3.1: General classification of proteases [82]

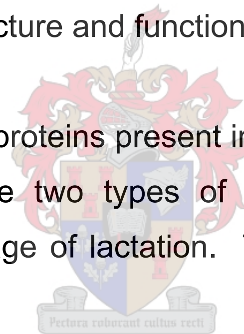
General classification of proteases		
Class	**Examples**	**Inhibitors**[i]
Serine proteases	trypsin, chymotrypsin, elstase, thrombin, plasmin	DFP, PMSF, natural trypsin inhibitor (soybean, pancreas)
Sulphydryl proteases	papain, bromelain, ficin	SH reagents (e.g. NEM, PCMB)
Metallo proteases	aminopeptidase, carboxypeptidases A and B, thermolysin	metallo complexing agents (e.g. EDTA)
Acid (carboxyl) proteases	pepsin, chymosin, gastricsin	DAN, EPNP, pepstatin

[i] DFP, di-isopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; DAN, diazoacetylnorleucine methyl ester; EPNP, 1,2 epoxy-3(p-nitrophenoxy) propane; PCMB, para-chloromercuribenzoate

The main indigenous protease identified in milk is plasmin, which occurs in its inactive precursor form plasminogen in milk [3-6,12,23,27,28]. Plasmin has been isolated and characterised, but the exact mechanism of its activation still remains the main focus of studies conducted on the fibrinolytic system in milk. The cascade of reactions resulting in the activation of plasminogen consists of various activator and inhibitor enzymes [8-10,19,20]. The fibrinolytic system acts as an anti-coagulant that solubilises fibrin clots in the blood [56,57,65].

3.2. Protein composition of bovine milk

In order to elucidate the functionality of proteases, especially with regard to the fibrinolytic system in bovine milk, it is important to understand the composition of milk in terms of the protein structure and functionality of its proteins.



There are two distinct types of proteins present in milk, namely caseins and whey proteins. The ratios of these two types of proteins can differ significantly throughout the season and stage of lactation. Typical values (mid-season) are indicated in Table 3.2 [59]:

Table 3.2: Typical composition of mid-lactation bulk milk [59]

The average composition of raw bulk milk		
Constituent	**Concentration ($g.l^{-1}$)**	**Percentage (m/m)**
Fat	37.00	3.58
Protein	34.00	3.29
Casein	27.60	2.67
α_{s1}-*casein*	10.54	1.02
α_{s2}-*casein*	2.86	0.27
β-casein[1]	10.62	1.03
κ -casein	3.58	0.35
Whey protein	6.40	0.62
β-lactoglobulin	0.61	0.06
α-lactoglobulin	0.24	0.02
Bovine serum albumin	0.09	0.01
Minor components[2]	0.20	0.02
Non-protein Nitrogen	1.90	0.18
Lactose	48.00	4.64
Ash (minerals)	7.00	0.68
Total Solids	127.90	12.37

[1]Including γ -casein

[2]Including immunoglobulins

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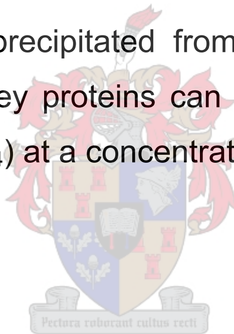
Due to the unique properties of the protein components in milk, the casein and the whey protein fractions can be separated for further classification using at least one of the following four techniques [83-86]:

<u>*Iso-electric precipitation*</u>: The caseins are insoluble at their iso-electric point (pI 4.6) at temperatures greater than 8 °C. Whey proteins are also insoluble at their iso-electric point (pI 5.0), but at a low ionic strength. However, in the ionic environment of milk they are solubilised at a pH of 5.0.

Rennet coagulation: The induction of coagulation of casein is caused by limited proteolysis, which is induced by crude protease preparations, known as rennets. Commercially this is the basic principle of the manufacturing of cheese.

Centrifugation/filtration: In milk, casein occurs in its natural form as large aggregates or micelles. Thus these casein micelles can be separated from molecularly dispersed whey proteins by ultracentrifugation techniques. Although not of significance in the dairy industry, it is commonly used in laboratory environments when casein micelle preparations are prepared (100,000 X g for 60 minutes). Micelles can also be separated from whey proteins by using ultrafiltration techniques. Ultrafiltration has been used with success in the dairy industry for the manufacture of cheese and fermented products.

Salting out: Casein can be precipitated from solution by a variety of salts. Casein together with some whey proteins can be salted out by the addition of ammonium sulphate ($(NH_4)_2SO_4$) at a concentration of 260 $g.l^{-1}$ of milk.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3.2.1. Casein

Iso-electric casein, which constitutes over 80 percent of the total protein in milk, can be divided into four primary caseins, namely: alpha$_{s1}$- (α_{s1}-), alpha$_{s2}$- (α_{s2}-), beta- (β-) and kappa (κ-) casein. The approximate ratio of these four caseins in bulk milk are 40 (α_{s1}):10 (α_{s2}): 35 (β): 12 (κ). The minor protein fraction in milk originates as a result of post-secretion proteolysis of the primary casein fractions due to the action of plasmin. These polypeptides include gamma-caseins (γ^1, γ^2, γ^3) and proteose peptones [84,85]. The four primary caseins exhibit "micro-heterogeneity" due to the variations in the degree of glycosylation, disulphide-linked polymerization and genetic polymorphism (genetically controlled amino-acid distribution) [84-88].

Of the 80 % of casein in milk, 94 % of the dry weight is the physical protein and the remaining 6 % consists of citrate and calcium, phosphate and magnesium ions [89-92]. The calcium and phosphate are referred to collectively as the colloidal calcium phosphate fraction in the milk.

The casein micelle has a porous structure and is composed of submicelles, 10-15 nm in diameter [93-95]. The micelles are highly hydrated, approximately 2 g $H_2O.g^{-1}$ protein [96]. Caseins are relatively small molecules as indicated by their respective molecular weights in Table 3.3. The casein micelles are composed of spherical submicelles, 10-15 nm in diameter. The hydrophobic core of the submicelles is considered to consist of the Ca-sensitive α_{s1}-, α_{s2}- and β-caseins, with variable amounts of κ-casein located principally on the surface. The hydrophobic N-terminal of the κ-caseins interact hydrophobically with the α_{s1}-, α_{s2}- and β-caseins, while the hydrophilic C-terminal protruding from the surface of the casein submicelles forms the "hairy" region [55].

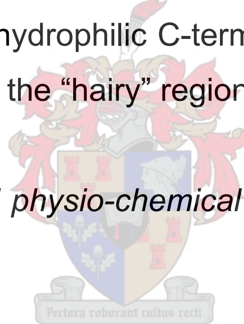

Table 3.3: Molecular mass and physio-chemical characteristics of bovine casein [59]

Casein	Moles P per mole	Molecular mass (Mr)	Charge at pH 6.6	Isoionic pH	$H\phi_{average}$ KJ per residue	Absorptivity (cm^2/g) at 280 nm
alpha$_{s1}$- (α_{s1}-)	8	23,600	- 20.9	4.96	4.87	1.05
alpha$_{s2}$- (α_{s2}-)	13	25,388	-18.0	5.19	-	1.10
alpha$_{s2}$- (α_{s2}-)	12	25,308	- 16.4	5.25	-	1.10
alpha$_{s2}$- (α_{s2}-)	11	25,228	- 14.8	5.32	-	1.10
alpha$_{s2}$- (α_{s2}-)	10	25,148	- 13.2	5.39	4.64	1.11
beta- (β-)	5	23,980	- 12.3	5.19	5.58	0.46
gamma- (γ-)	-	19,550	-	-	-	-
kappa (κ -)	1	19,037	- 3.9	5.43	5.12	0.95

The casein micelle structural network consists of α_s-casein molecules interacting with calcium phosphate by virtue of the serine phosphate esters in the α_s casein [86-88]. Kappa- and β-casein are also accommodated within this structural framework of the α_s-casein and the casein phosphate. Migration of the κ- and β-casein can occur in milk from the casein micelle itself into the serum phase of the milk. This normally occurs during prolonged storage of the milk at low temperatures [89-93,97]. During the heating of milk the β-and κ-casein re-associates with the casein micelle, but at this stage it is unclear whether the same native structure of the β- and κ-casein is attained. The κ-casein is mainly localised near the surface of the casein micelle, where it provides stability to the entire structure by virtue of its charge [94-96].

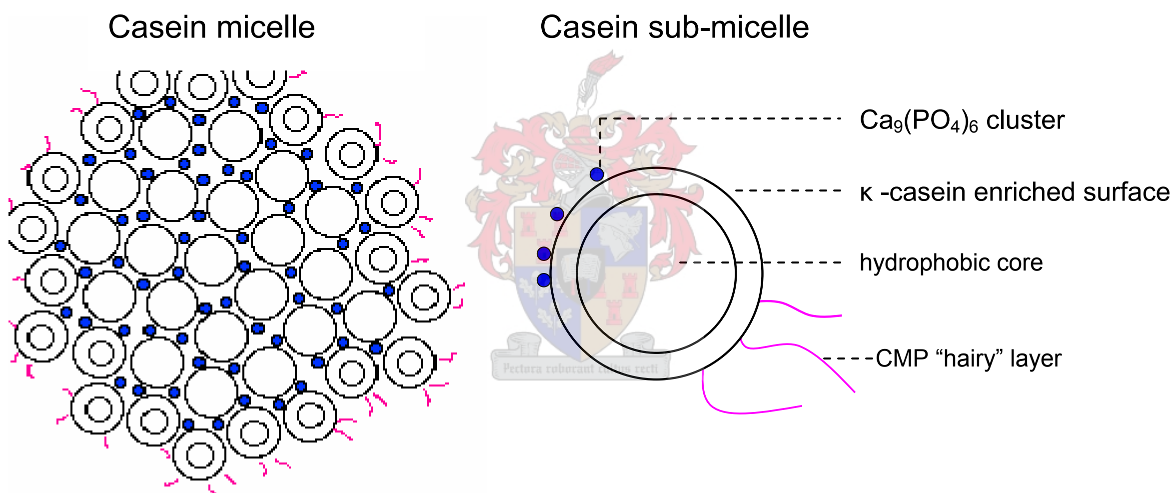

Figure 3.1: Diagrammatic representation of casein micelles and sub-micelles [98].

Natural casein is remarkably stable, and this can be attributed to a zeta potential of approximately -20 mV at 20°C and the steric stabilisation provided by the "hairy" layer [98]. All casein molecules are insoluble around their iso-electric points (pI 4.5 – 4.9). Caseins are strongly hydrophobic in the order β > κ > α_{s1} > α_{s2} and are small, random, unstructured, open molecules [95]. These features markedly influence their functional properties.

The α_{s1}-casein is a highly charged molecule (net charge at pH 6.6 = 20.6) whereas α_{s2}-casein is the most hydrophilic of all the casein species (net charge at pH 6.6 = 9.5). The α_{s2}-casein molecule can be divided into definite hydrophobic and hydrophilic domains. Beta-casein is also a highly charged molecule (net charge at pH 6.6 = 12) while κ-casein is an amphipathic molecule (net charge at pH 6.6 = 10). The α- and β-caseins are phosphoproteins with between 5 and 13 phosphoserine groups [91-94]. As a result of the high content of the phosphoserine residues, α_{s1}-, α_{s2}- and β-casein binds the polyvalent calcium (Ca^{++}) and zinc cations (Zn^{++}) strongly.

Kappa-casein has one serine phosphate ester and contains a charged carbohydrate moiety. Therefore, in contrast to the other casein species is insensitive to the addition of calcium [89,92].

Gamma casein is formed by hydrolyses of the β-casein molecules after secretion of the β-casein in the mammary glands into the milk. Gamma-casein constitutes only a small portion of the casein in good quality milk from cows in mid-lactation (less than 5% of the total casein fraction), but can be as high as 10% of the total casein fraction in cows in late lactation [90]. When the γ-casein exceeds this level, it is due to protease activity within the milk [99]. The source of the milk protease can either be of bacteriological origin, or could be as a result of native proteases present in the milk. Due to proteolysis it can be difficult to process the milk into certain types of products. To limit the bacteriological protease activity, better hygienic practices can be instigated in order to improve the bacteriological quality of the milk. However, in the case of native protease being present in the milk, no prompt remedial action can be taken [90].

Table 3.4: Average Characteristics of Casein Micelles [55]

Characteristic	**Value**
Diameter	120 nm (range: 50 – 500 nm)
Surface Area	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 $g.cm^{-3}$
Mass	2.2×10^{-15} g
Water content	63%
Hydration	3.7 g H_2O. g^{-1} protein
Voluminosity	4.4 cm^3. g^{-1}
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5×10^8 Da
Number of peptide chains	10^4
Number of particles / ml milk	10^{14} - 10^{16}
Surface area of micelles / ml milk	5×10^4 cm^2
Mean free distance	240 nm

The calcium concentration in bovine milk is of technological importance to the dairy industry as it influences a number of properties in the milk, such as heat stability, alcohol stability, rennet coagulability and the strength and syneresis properties of rennet gels [84]. Temperature, pH and ionic strength of the solution influences calcium binding to casein micelles. Calcium binding to the casein molecules takes place exclusively at the phosphoseryl residues, but at higher concentrations it can also bind to the Asp or Glu residues [67]. Binding of the casein to the calcium at the phosphoseryl groups reduces the net charge of the casein molecules, resulting in the association of the protein molecules [67,86-90]. Calcium and α_{s1}-casein form octamers, which aggregate and precipitate at higher calcium concentrations. Calcium mediated association with the hydrophobic β-casein is temperature dependant and β-casein is soluble in the presence of calcium at temperatures less than 20°C. Kappa-casein contains only one phosphoserine residue and, therefore, does not bind calcium strongly and

remains soluble at high calcium concentrations. Kappa-casein also binds with α_{s1}- and β-casein at high calcium concentrations to form colloidal particles. These colloidal particles, however, are less stable than the native casein micelles [83,84].

The phosphoserine resides are responsible for the unique properties of casein, such as the calcium sensitivity of milk protein [82-86]. With the addition of calcium in casein solutions such as milk, the casein coagulates due to inter-molecular calcium bridges that are formed. Eighty-five percent of the iso-electric α_{s1}-, α_{s2}- and β-casein are insoluble at calcium concentrations of above 6 mM [67,89]. Destabilisation of the casein micelles will result in coagulation, precipitation or gelation. The destabilisation of the casein micelle structure can be introduced by: (i) acid treatment (usage of lactic acid bacteria), (ii) organic solvents (such as hydrochloric and phosphoric acid), (iii) heat treatment, (iv) limited proteolysis (α_{s2}- and β-casein being the preferred substrates for native proteases and κ-casein being the substrate for rennet coagulation), (v) addition of ethanol, (vi) addition of calcium or a combination of the treatments mentioned above [82-96]. The knowledge of such behaviour and the application thereof in the food industry, directly relates to the manufacturing of cheese and the formation of acidic gels in yoghurt manufacture.

The clusters of the phosphoserine residues in the casein molecule are responsible for the highly charged hydrophilic areas in the molecule. When the separation of the hydrophobic and hydrophilic regions within the casein molecule takes place, it acts as an effective surface-active agent. As a result casein molecules are excellent stabilisers of foams and emulsions and therefore have widespread applications in the food industry.

In Fig. 3.2 a conceptual model by Carl Holt and co-workers at Hannah Research Institute, Scotland, is presented for the casein molecule, indicating the equilibrium between the micelle and the milk serum with acidification and with

heating [100]. The casein particle is demonstrated as a highly hydrated, spherical, open sized micelle with the polypeptide chains in the core partly cross-linked by nanometer sized clusters of calcium phosphate. The internal structure gives rise to an external region of lower segment density known as the hairy layer, which confers steric and/or charge stability to native casein particles.

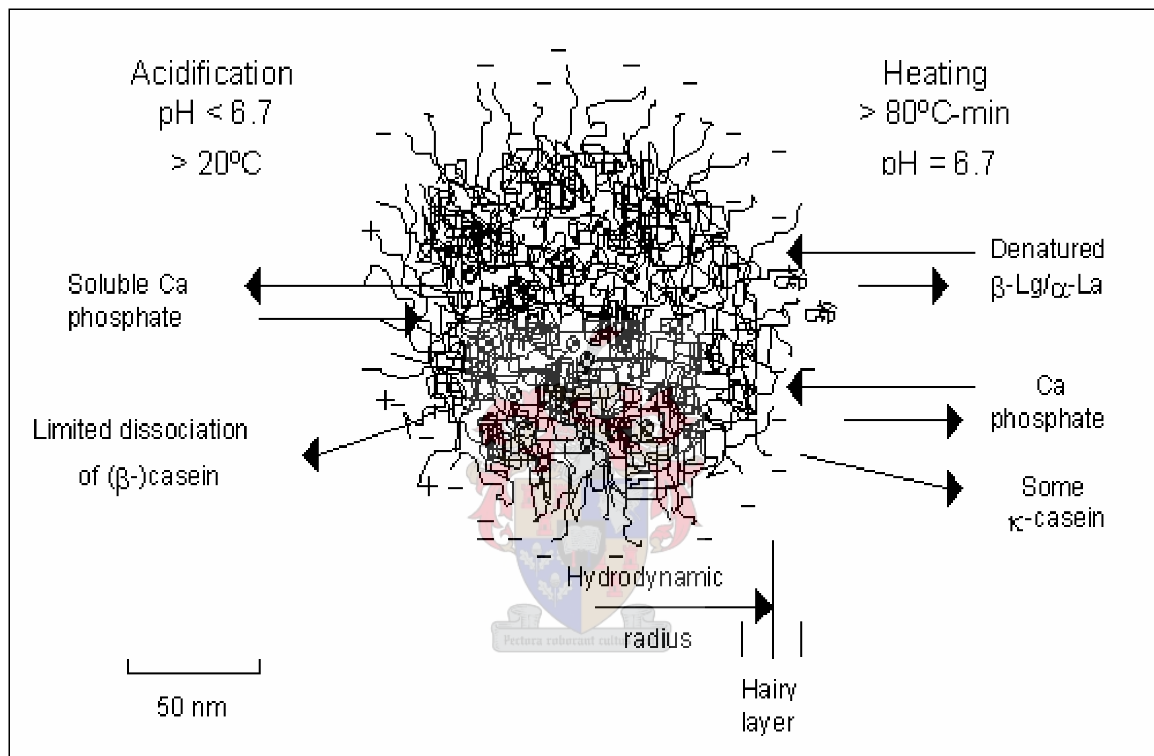

Figure 3.2: Casein model as suggested by Carl Holt and co-workers at Hannah Research Institute, Scotland [100].

Table 3.5 lists the amino acid composition of the principle proteins of bovine milk. In Figs. 3.3 to 3.5 the amino acid composition of α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein are indicated [55]. The plasmin cleavage on β-casein yields γ^1-, γ^2-, γ^3-caseins, which are indicated in Fig. 3.5.

Table 3.5: Amino Acid Composition of the Principle Proteins in Bovine Milk

Amino Acid	**α_{s1}-casein B**	**α_{s2}-casein A**	**β-casein A^2**	**κ-casein B**	**γ^1-casein A^2**	**γ^2-casein A^2**	**γ^3-casein A**	**β-LG A**	**α-LA B**
Asp	7	4	4	4	4	2	2	11	9
Asn	8	14	5	7	3	1	1	5	12
Thr	5	15	9	14	8	4	4	8	7
Ser	8	6	11	12	10	7	7	7	7
SerP	8	11	5	1	1	0	0	0	0
Glu	24	25	18	12	11	4	4	16	8
Gln	15	15	21	14	21	11	11	9	5
Pro	17	10	35	30	34	21	21	8	2
Gly	9	2	5	2	4	2	2	3	6
Ala	9	8	5	15	5	2	2	14	3
½ Cys	0	2	0	2	0	0	0	5	8
Val	11	14	19	11	17	10	10	10	6
Met	5	5	6	2	6	4	4	4	1
Ile	11	11	10	13	7	3	3	10	8
Leu	17	13	22	8	19	14	14	22	13
Tyr	10	12	4	9	4	3	3	4	4
Phe	8	6	9	4	9	5	5	4	4
Trp	2	2	1	1	1	1	1	2	4
Lys	14	24	11	9	10	$4	3	15	12
His	5	3	5	3	5	4	3	2	3
Arg	6	6	4	5	2	2	2	3	1
PyroGlu	0	0	0	1	0	0	0	0	0
Total residues	199	207	169	209	181	104	102	162	123
Mr	23612	25228	19005	23980	20520	11822	11557	18362	14174
$H\Phi_{ave}$ (kJ/residue)	4.89	4.64	5.12	5.58	5.85	6.23	6.29	5.03	4.68

1 Glu-Val-Leu-Asn-Glu-Asn-Leu-
H. Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-……………………………………
21
Leu-Arg-Phe-Phe-Val-Ala-(Variants B, C, D, E)
-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Gln-Leu
…………………………………………………… …(Variant A)
41 Ala (Variants A, B, C, E) Gln (Variants A, B, C, D)
Ser-Lys-Asp-Ile-Gly-*SerP*-Glu-*SerP*-Thr-Glu-Asp-Gln- -Met-Glu-Asp-Ile- -Met
ThrP (Variant D) Glu (Variant E)
61
Glu-Ala-Glu-*SerP*-Ile-*SerP*-*SerP*-*SerP*-Glu-Glu-Ile-Val-Pro-Asn-*SerP*-Val-Glu-Gln-Lys-His-
81
Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg
101
Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-*SerP*-Ala-Glu-Glu-Arg-Leu
121
His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-
141
Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-
161
Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-
181 Glu (Variants A, B, D) 199
Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser--Lys-Thr-Thr-Met-Pro-Leu-Trp. OH
Gly (Variants C, E)

Figure 3.3: Amino acid sequence information for α_{s1}*-casein; amino acid substitutions or deletions in principle genetic variants.*

1
H. Lys-Asn-Thr-Met-Glu-His-Val-*SerP*-*SerP*-*SerP*-Glu-Glu-Ser-Ile-Ile-*SerP*-Gln-Glu-Thr-Tyr-
21
Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro-Ser-Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-
41
Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Se-Ile-Gly-*SerP*-*SerP*-*SerP*-Glu-Glu-
61
SerP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-
81
Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe-Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-
101
Gln-Gly-Pro-Ile-Val-Leu-Asp-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-
121
Pre-Thr-Leu-Asn-Arg-Glu-Gln-Leu-*SerP*-Thr-*SerP*-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-
141
Met-Glu-*SerP*-Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-
161
Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-
181
Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-
201 207
Ile-Pro-Tyr-Val-Arg-Tyr-Leu. OH

Figure 3.4: Amino acid sequence information for α_{s2}*-casein, indicating 11 of the possible 13 phosphorylation sites.*

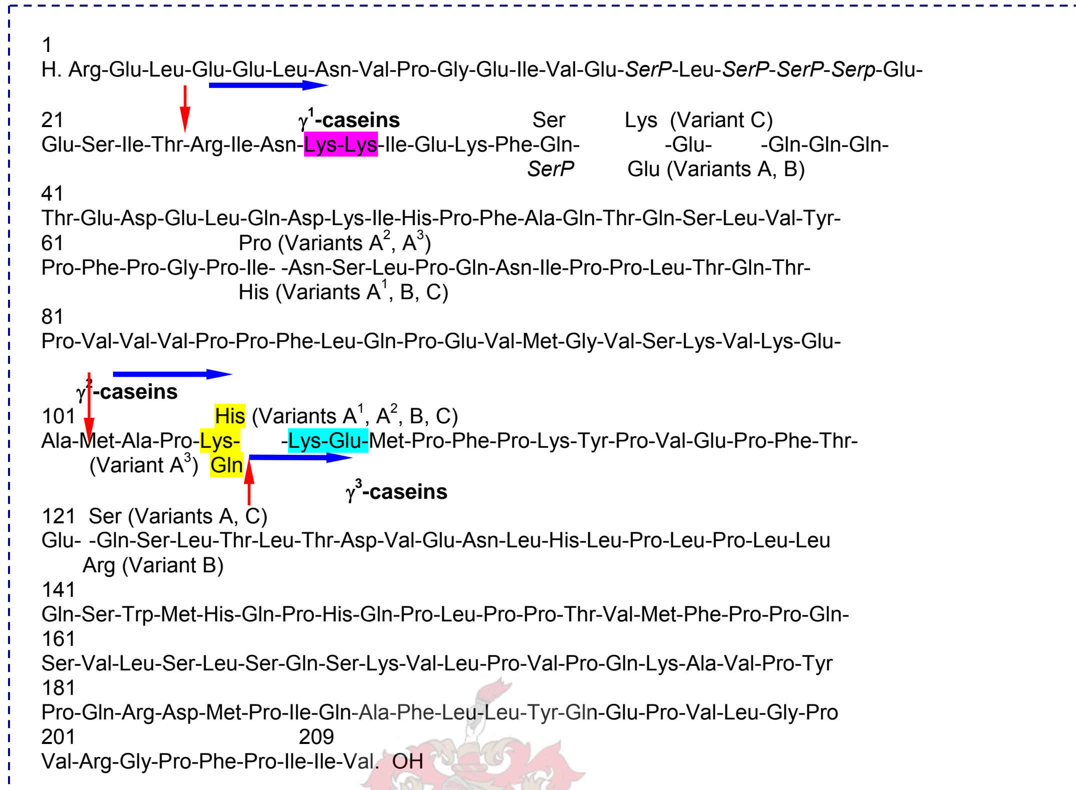

Figure 3.5: Amino acid sequence information for β-casein; amino acid substitutions in principle genetic variants and plasmin cleavage sites are indicated.

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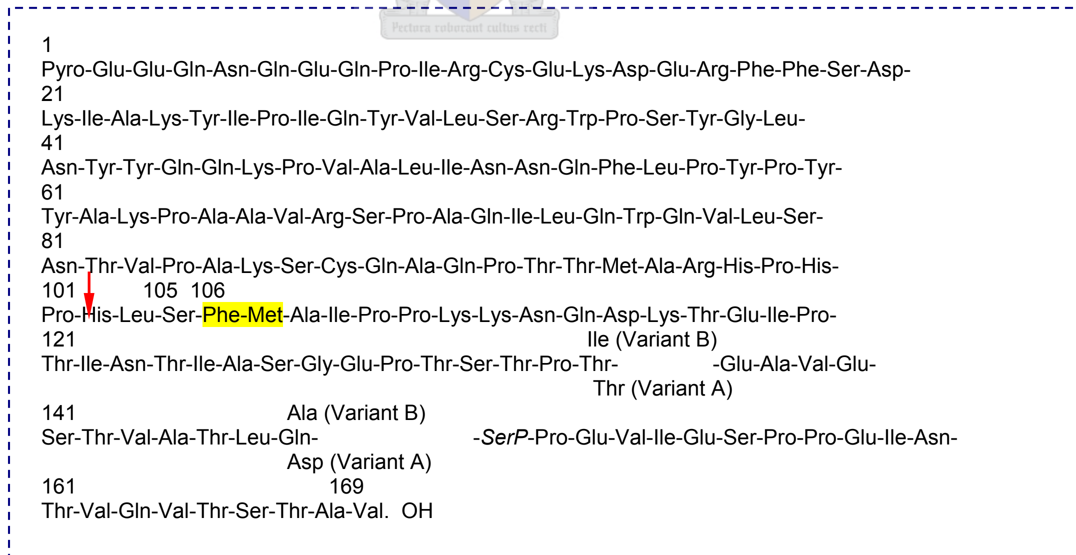


Figure 3.6: Amino acid sequence information for κ-casein; amino acid substitutions in principle genetic variants and chymosin cleavage sites are indicated.

3.2.2. Whey proteins

Whey proteins are typical globular proteins with high levels of secondary, tertiary and quarternary structures that can be denatured when heating takes place at temperatures above 65°C [67]. The four major constituents of the whey protein fractions in milk, from healthy mid-lactation cows, are: beta-lactoglobulin (β-lactoglobulin, 50%), alpha-lactalbumin (α-lactalbumin, 20%), bovine serum albumin (BSA, 10%) and immunoglobulins (Ig, 10%) - mainly IgG_1 with lesser amounts of IgG_2, IgM and IgA present [67]. During late lactation milk or milk with mastitis (with high somatic cell counts) increased levels of the blood-derived proteins such as bovine serum albumin and immunoglobulin may be present. Human milk contains no β-lactoglobulin and the principle Ig is IgA. The whey proteins in bovine milk are not phosphorylated and are insensitive to calcium; they contain intra-molecular disulfide bonds that stabilise their structure [67,86].

Beta-lactoglobulin is the most abundant protein in whey (about 6 $g.l^{-1}$) and is responsible for functional properties such as the ability of whey proteins to form a gel upon heating. Beta-lactoglobulin, contains the sulphur amino acid, cysteine, which upon heating may participate in sulfhydryl exchange reactions leading to inter-and intra-molecular cross-linking with other proteins [55,67]. One such reaction is the interaction of κ-casein and β-lactoglobulin which upon heating at 75°C for 15 seconds forms a complex [67]. The κ-casein and β-lactoglobulin complex can impair rennet coagulation properties of milk and alter the gel structure and rheological properties of acid-based gel products, such as yoghurt and fresh cheeses.

Beta-lactoglobulin is a dimer of identical units, each unit contains a number of β-sheet structures as well as some α-helix [101]. A special feature is that the β-sheets are arranged in such a way as to give rise to a central cavity (a β-barrel) where small hydrophobic molecules may be bound.

3.2.3. Proteose peptones

Proteose peptones are short peptides that are formed as a result of the breakdown of casein by proteases. The proteose peptone fraction in milk consist of approximately 38 components of which at least 65% can be attributed to hydrolysis of casein by the native enzyme plasmin [5,56,57,65]. Quantification of these results on gel electrophoresis indicated that 52% was as a result of the hydrolysis of β-casein, 29% from α_{s1}-casein, 9% from α_{s2}-casein and 4% from κ-casein [102]. The remaining 6% of the proteose peptone fractions can be attributed to the function of other proteases [56,102]. It has been shown that there is an almost linear relationship between plasmin activity and the proteose peptone fraction. Seventy seven percent of the fraction can be attributed to proteose peptones being produced within the secretory cell cytoplasm and the remaining 23% can be attributed to the function of post secretory plasmin activity [44,51,52].

3.2.4. Milk proteases

Milk proteases present in bovine milk originate from endogenous proteases or from proteases produced by microorganisms. Endogenous proteases are transferred from the blood to the milk through the blood-milk barrier, and proteases produced from microorganisms are secreted in the milk after contamination [4,55].

Endogenous enzyme originates from three different sources; namely the blood or other organs via the blood, the secretory cell cytoplasm and the fat globule itself (the outer layer of the fat globule membrane is made up of the apical membrane of the secretory cells, which is formed by the membranes of the Golgi-apparatus) [6].

Bacterial proteases can originate from multiple sources and do not necessarily occur after the secretion of milk. The remaining 45% of the proteose peptone fraction is attributed to the proteolytic activity of other proteases present in the milk. Infection can take place within the udder of the cow. Bacteria such as *Staphylococcus aureus, Streptococcus uberis, Escherichia coli, Streptococcus agalactia* and *Streptococcus dysagalactiae* can infect the cow's udder via the teat channel. The infection can take place due to a number of reasons, such as: over-milking, contaminated feed, contamination of the milk with soil and dust, contaminated silage, unhygienic milking utensils, contaminated water and unhygienic milking parlours [55]. Bacterial contamination of the milk can also take place after milk production, which normally relates to unhygienic practices such as incorrect cooling of the milk. The presence of psychrotrophic bacteria is the main contributing factor to extra cellular bacterial proteases in milk. Psychrotrophic bacteria are not part of the secreted udder-flora, and therefore their presence can normally be attributed to: (i) poor cleaning practices and hygiene on farms and in factories, (ii) extended storage of raw milk (more than 24 hours) at temperatures less than 4°C and (iii) post-pasteurization contamination [103,104]. Gram-negative *Pseudomonas* psychrotrophic bacteria, such as *Pseudomonas putida, Pseudomonas fragi and Pseudomonas fluorescens,* produce thermo-stable proteases, which are killed at normal pasteurisation temperature (72.5°C for 15 seconds). However, normal pasteurisation temperature do not inactivate psychrotrophic spore forming bacteria, such as *Bacillus*, which are more heat resistant [7,84,105,106]. Studies have indicated that contamination with psychrotrophic bacteria at levels of between 1.8 million and 9.5 million bacteria.ml^{-1} can hydrolyse between 61-91% of the κ-casein and 19-76% of the α-casein in 9 days [7,103,104].

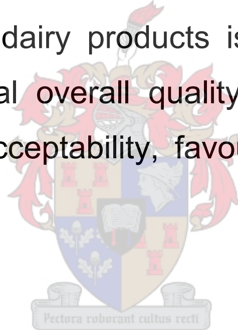
Acid milk protease has a pH optimum of 4.0 and a Mr of 36,000 Da. The properties of acid milk protease are similar to that of cathepsin D, with α_{s1}-casein being the preferred substrate for hydrolysis over β-casein. The enzyme is

associated with the casein micelles in the milk and due to its activity and plays a significant role during the ripening of cheese [61].

Thrombin is a serine protease that hydrolyses the peptide bonds containing arginine. Thrombin exhibits high substrate specificity and, therefore, the proteolysis effect in milk is strongly reduced in contrast to other proteases, such as plasmin and acid milk protease [6].

Aminopeptidases originate in the blood and have similar hydrolysis properties to plasmin. They are concentrated in the milk serum and can hydrolyse peptide bonds containing lysine, alanine and leucine [6].

In Chapter 4 the influence of the activity of plasmin and plasminogen activators on milk and some secondary dairy products is discussed. Plasmin activity in bovine milk influences the final overall quality of the product including such parameters as, organoleptic acceptability, favourable product composition and shelf-life.

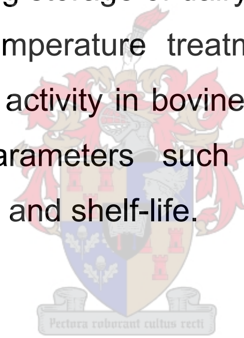


Chapter 4

THE INFLUENCE OF THE FIBRINOLYTIC SYSTEM IN BOVINE MILK ON THE DAIRY INDUSTRY

4.1. Introduction

The fibrinolytic system in bovine milk affects both the primary and secondary dairy industries. Two features of the plasmin protease system in milk makes it of particular interest to the manufacturers of dairy products. Firstly, the enzyme remains active indefinitely during storage of dairy products and secondly, plasmin activity survives high heat temperature treatments [107-111]. Therefore the reduction or control of plasmin activity in bovine milk will be of benefit to overall product quality; including parameters such as organoleptic acceptability, favourable product composition and shelf-life.



4.2. Raw, Pasteurised, UHT and Sterilised Milk

Milk proteases can induce thickening, gelation and coagulation upon storage of raw bulk milk, pasteurised milk, extended shelf-life milk, UHT milk and sterilised milk [7,13,14,28,55,106]. These proteases can be native to the milk (plasmin) or can be of bacteriological origin (contamination of milk with spoilage bacteria) such as the psychrotrophic bacteria *Pseudomonas fluorescens* and *Pseudomonas fragi* K122 [5,55]. Plasmin is a heat stable enzyme that survives normal pasteurisation and UHT processes. Proteolytic activity in the milk can also result in the breakdown of casein to form short peptides and free amino acids causing a bitter flavour.

Blood only contains plasminogen, thus the activation of plasminogen to plasmin occurs during milk synthesis, storage of the milk in the lumen of the udder (prior to milking), storage of milk post secretion and during the processing of milk [112-115].

In fresh milk the plasminogen is activated resulting in an increase in plasmin concentration and activity that will lead to the proteolysis of casein substrates. The ratio of plasminogen to plasmin can vary from 5:1 to 40:1 in milk of individual cows. The average ratio in good quality bulk milk with a low SCC and low bacterial count is approximately 8:1 [19,58].

Due to the increase of PA released from the leukocytes, plasminogen is activated to form plasmin. Plasmin catalyses the breakdown of casein with subsequent disruption of the casein micellular structure and liberation of the associated PA. The resulting effect is more activation of the pro-enzyme and more plasmin being formed as well as an increase in protease activity [6]. It is reported that there is an increase of the plasmin and plasminogen concentrations in milk of 82% and 21%, respectively, during sub-clinical mastitis [8].

In bovine milk the concentration and activity of the native protease plasmin may vary due to numerous factors.

4.2.1. The stage of lactation

As the stage of lactation progresses, the activity of the plasmin and PA increases [99,106]. Separate studies showed an increase in γ-casein concentrations and a subsequent reduction in the concentration of β-casein fraction in bovine milk [100]. The concentration of plasminogen also increased from mid-lactation milk to end-lactation milk (almost 2 fold) also indicating more substrate and a bigger proteolytic reservoir [107]. The concentration of the plasminogen activators is at

the highest level during the dry period of the cow. This will result in greater permeability of the blood-milk barrier and as a result there will be more leakage of plasminogen from the blood into the milk where it is converted to plasmin. Not only does the plasmin and proteolytic activity increase with the stage of lactation, but also with the number of lactations and the age of the cow [52,66,107].

4.2.2. Breed of the cow

Plasmin activity can also be correlated to the breed of the cow. The rate of proteolysis by plasmin activity is greater in Friesian milk than in Jersey and Ayrshire milk [116]. Once again the results were correlated with the amount of γ-casein formed after hydrolysis of β-casein, and it was found that Friesian milk has twice the concentration of that of Ayrshire and Jersey milk [114]. Jersey milk exhibits the lowest concentration of γ-casein produced as a result of proteolytic cleavage, due to the activity of plasmin and PA [115]. The results are interesting if one also takes into account the relative differences in protein content between the cows, that of Ayrshire milk and Jersey milk being approximately 0.4-0.5% higher than the protein content in Friesian milk.

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4.2.3. Age of the cow

The plasmin content is affected by the age of the cow. The increase in proteolytic activity is mainly as a result of the higher permeability of the blood-milk barrier in the secretory cells in the udder of the cow. Subsequently there is a bigger transfer of plasmin, plasminogen and PA from the blood to the milk. The plasmin activity also increases with the number of lactations of the cow [115].

4.2.4. Mastitis

Colonisation of the bovine mammary gland by pathogenic bacteria results in a series of events that lead to major alterations in the milk secreted by the bovine mammary glands [67,117-119].

Mastitic infection due to an increase of the pathogenic bacteria in the milk will also result in an increase in the somatic cell count (SCC) of the milk. The effect on lactation and milk composition is a reduction in milk yield and impaired synthetic stability of the secretory tissue. This results in changes in the major and minor constituents in the milk as indicated in Table 4.1 [67,117-121].

Table 4.1: Changes in level of various proteins in milk with mastitic infection [67]

Protein (mg/ml)	**Normal milk**	**Mastitic milk**
Total casein	27.90	22.50
Total whey protein	8.20 – 8.70	13.10 –19.80
Caseins		
α_{s1}-casein	9.50 – 13.30	3.00 – 8.50
β-casein	5.00 – 11.00	0.80 – 6.50
κ-casein	1.60	1.90
Para κ-casein	0	0.50
Whey proteins		
β-lactoglobulin	4.00 – 4.25	2.67 – 4.15
α-lactalbumin	1.03 – 3.20	0.58 – 2.50
Bovine serum albumin	0.10 – 0.30	0.65 – 21.50
Total immunoglobulin	0.25 – 1.33	2.45 – 18.30
Others		
Proteose peptone	1.82	9.24
Lactoferrin	0.10 – 0.20	6.20
Milk fat globule membrane (MFGM) protein (mg.100 g^{-1} fat)	513.70	408.80

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Zachos *et al.* [8] also concluded that the PA activity present in the milk somatic cells increases approximately eight fold during mastitis (increase in SCC from 50,000 – 1,000,000 cells.ml^{-1}). Furthermore, the PA was tissue type rather than urokinase type PA, due to an increase in the activity of the PA in the presence of fibrin. Heegaard *et al.* [20] confirmed an increase in t-PA of 10 to 20 fold subsequent to mastitic inflammation induced by *Staphylococcus aureus*. The increased level of t-PA remained for a period of up to 42 days after infection. In addition to the increase in t-PA, u-PA associated with the bovine cells in the milk also increased.

Somatic cell counts are frequently used in the dairy industry as an indicator of mastitis and the general udder health of the herd. In the milk the SCC can be divided into two distinct groups, namely the macrophages and the polymorphonuclear leukocytes (PMN) [19-21,27,50,57]. These cells are the principle phagocytes and constitute the first line of defence against bacterial infection. These cells induce inflammation due to the secretion of a number of proteins that help the defence against bacterial cells [66,107,117-121].

Macrophages are more prevalent than PMN in the healthy quarters of cows. Upon bacterial infection of the mammary gland, there is a rapid influx of PMN into the milk. Several days after the infection the macrophages again become the predominant cell type in the quarter. The activity detected in the PA from macrophages is not fibrin dependent, and is therefore urokinase type PA [8,50,57,66]. In Table 4.2 the changes of the ratios of SCC of milk with mastitic infection are indicated, the major change with high SCC is the increase in the neutrophil concentration in the milk.

Table 4.2: Changes in ratios of somatic cells in milk with mastitic infection [8]

	Healthy cows	**Mastitis**
Somatic cell count (SCC)	50,000 – 200,000	200,000 – 5,000,000
Lymphocytes	1	1
Neutrophyls (PMN)	1.5	10
Epithelial cells	14	10

4.2.5. Post-harvesting and Storage

In order to prevent the growth of spoilage organisms and to protect milk from any enzymatic activity (bacteriological or native), post harvesting conditions must be optimal. The conversion of plasminogen to plasmin will increase with an increase in temperature. The plasmin activity will be non-significant at a temperature of less than 4°C, but optimal at 37°C. It is also interesting that the activity of plasmin decreases to only between 10-17% with normal pasteurisation treatment (72.5°C for 15 seconds). The longer the storage of pasteurised milk, the higher the plasmin activity due to the inactivation of the PAI. Complete inhibition of plasmin will only occur at sterilisation temperatures of 120°C for 15 minutes, or 142°C for 18 seconds [22,114].

4.2.6. Solubility

At a pH of 4.6, which is the iso-electric point of casein, there is an approximate increase of 4% (from 6% to 10%) in the soluble nitrogen fraction of the milk due to proteolysis [29,66,107]. Ninety four percent of the proteose peptone fraction can be attributed to the plasmin proteolysis of casein [50,57,107,117].

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4.2.7. Ethanol stability

The presence of plasmin induces little if any effect on the alcohol stability in milk [44].

4.3. Cheese

The effects of plasmin on cheese ripening characteristics have also been under investigation and there are numerous articles published on this topic [54,55,67,72,108,111]. With regard to the final quality of the cheese produced with enhanced plasmin activity, the results are, however, contradictory. However, these contradictions should be seen in context as different cheeses will have different organoleptic, chemical and physical properties, and therefore plasmin activity will either enhance or detract from the final product quality, depending on the cheese variant produced. Furthermore the manufacturing parameters are different in different types of cheeses. These parameters include starter culture used, ripening time, rennet addition and quantity, type of rennet, rennet vs. acid coagulation, heating and scalding, pressing, ripening and curing of the cheese.

Fig. 4.1 illustrates the process flow for the basic production of hard and semi-hard cheeses, while Fig. 4.2 illustrates the process flow for the basic production of semi-hard, semi-soft and soft cheese. These process flows explains the difference in production techniques, and will also vary between different varieties produced within the same class designation.

In cheese the effect of plasmin in the different variants can be summarised as follow: Emmental > Blarney and Romano-types > Gouda > Cheddar > Cheshire. The differences in the effect of the plasmin activity can mainly be attributed to the cooking temperatures of the cheese, the pH and the percentage moisture in the

final product. Overall the increase in the plasmin concentration and activity decreases the viscosity of a caseinate solution. In cheese plasmin, plasminogen and PA are incorporated into the curd, while PAI and PI are located mainly in the whey fraction [55,122].

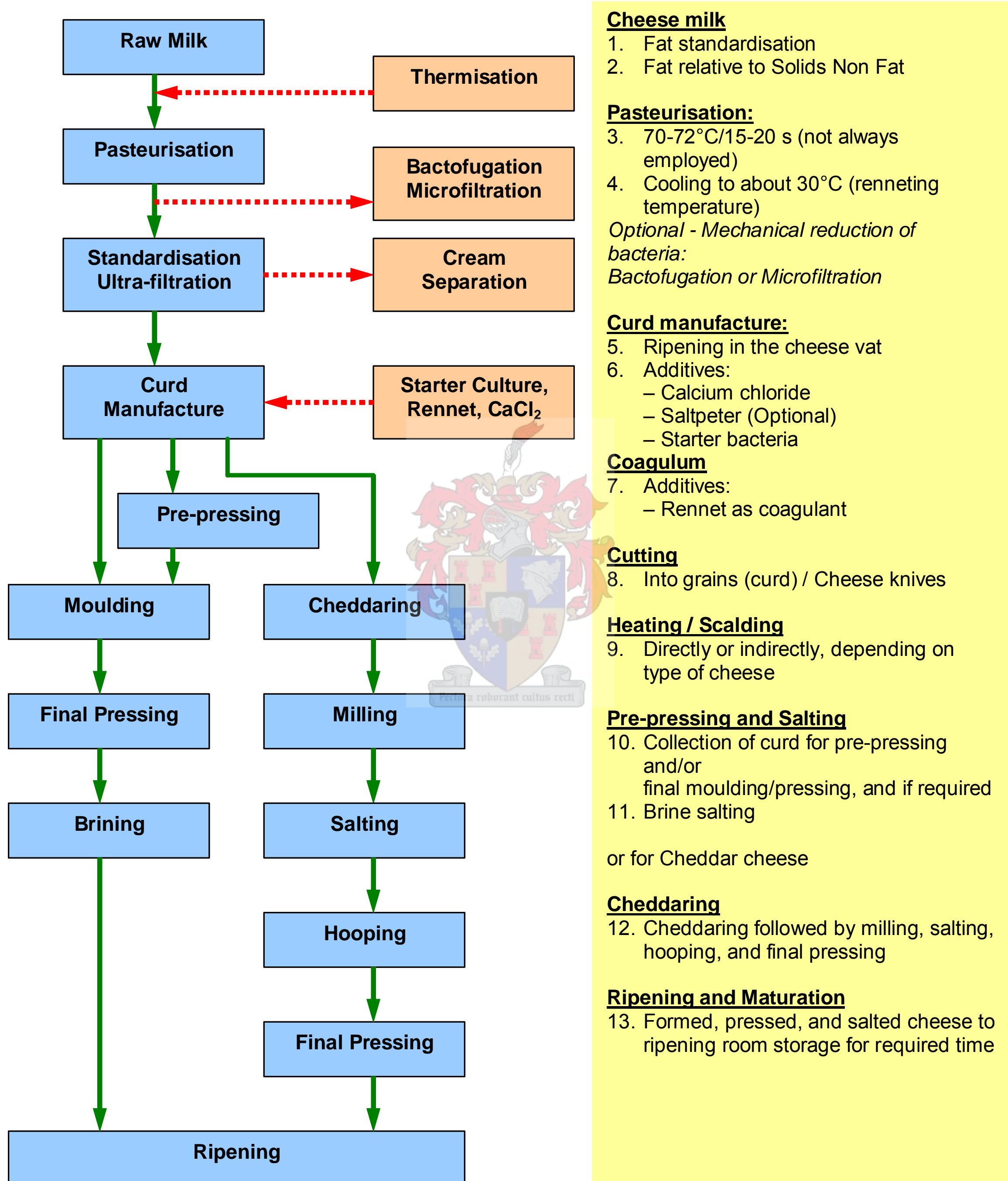
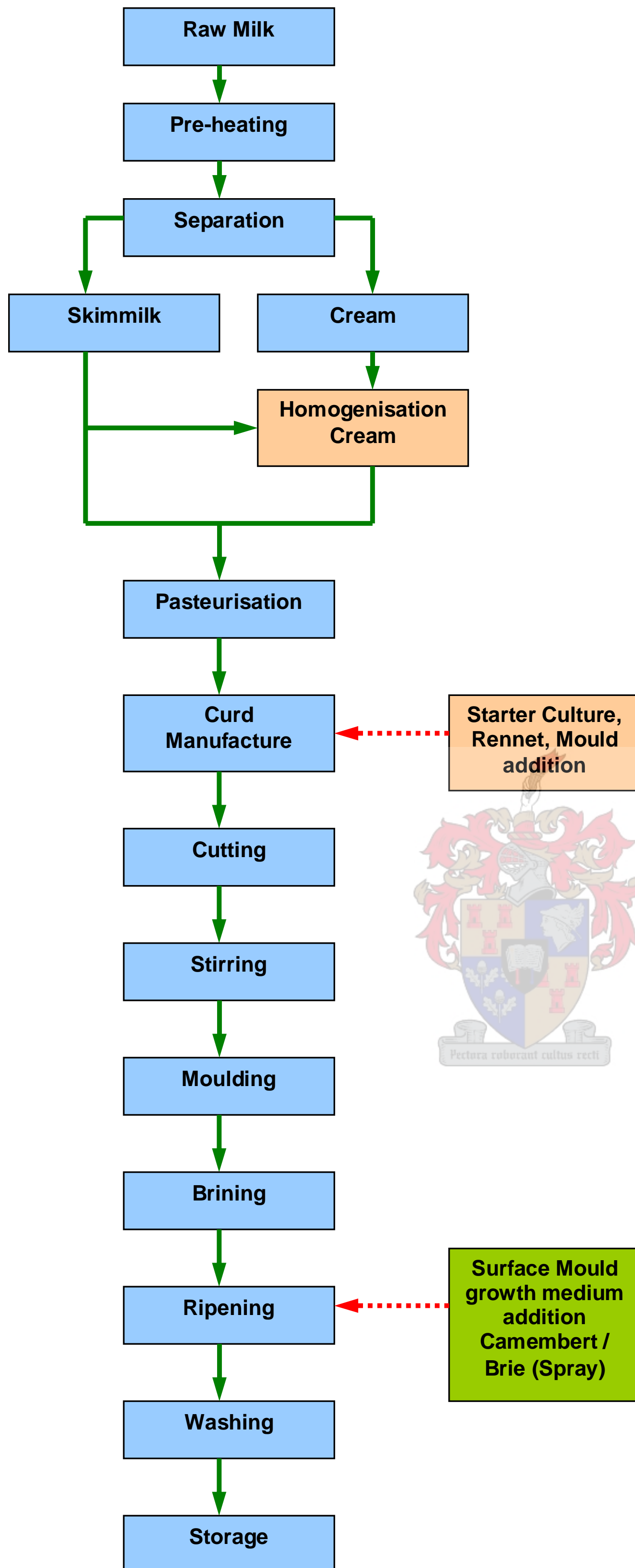


Figure 4.1: Production of hard and semi-hard cheeses.



Cheese milk
1. Fat standardisation
2. Fat relative to Solids Non Fat

Pasteurisation:
3. 70-72°C/15-20 s (not always employed)
4. Cooling to about 32°C (renneting temperature)

Optional - Mechanical reduction of bacteria:
Bactofugation or Microfiltration

Curd manufacture:
5. Ripening in the cheese vat
6. Additives:
   – Calcium chloride
   – Starter bacteria

Coagulum
7. Additives:
   – Rennet as coagulant

Cutting
8. Into grains (curd) / Cheese knives

Stirring
9. Gentle stirring

Moulding
10. Blue Veined: No pressure, 24 hours at 24°C at 90% + humidity. Turning while pressing, approximately 4 – 6 times.
11. Camembert / Brie: No pressure, 20 hours at 24°C at 90% + humidity. Turning while pressing, approximately 4 – 6 times.

Brining
12. Blue Veined: 23% NaCl solution, 48 hours at 15°C
13. Camembert / Brie: 23% NaCl solution, 1 - 2 hours at 15°C

Ripening and Maturation
Blue Veined:
14. Formed, pressed, and salted cheese to ripening room.
15. Maturation at 10 - 12°C for 5 days at 90% + relative humidity. After 5 days pierce wheels – Blue Veined.
16. Wash and wrap in aluminum foil.
17. Storage and distribution at < 5°C.

Camembert / Brie:
18. Maturation at 18°C for 2 days at 80% relative humidity.
19. Maturation at 12°C for 10 - 12 days at 90% relative humidity with frequent turning of moulds.
20. Wash and wrap in aluminum foil.
21. Storage and distribution at < 5°C

Figure 4.2: Typical process flow for the production of semi-hard / semi-soft cheese. The process flow is basically the same for the production of semi-soft / soft cheese.

Proteolysis in cheese ripening can be attributed to two sources, namely the action of the residual rennet in the cheese, of which approximately 6–10% is retained in the final product, and plasmin activity [123-128]. The hydrolysis of α_{s1}- and para-κ-caseins during ripening is mainly associated with the function of the rennet enzyme. Plasmin in cheese is more specific for firstly, β-casein, secondly, α_{s2}-casein and thirdly, α_{s1}-casein. Alpha-lactalbumin and β-lactoglobulin are quite resistant against the hydrolytic action of plasmin [129].

Plasmin is not rate limiting for the development of flavour during cheese ripening, as it is responsible for the formation of rather large to medium-sized peptides (primary proteolysis), which does not contribute to flavour. However, during secondary proteolysis these large to medium-sized peptides may be broken down by proteinases and peptidases from lactic acid starter bacteria, which will result in the formation of the flavour components, consisting of shorter peptides and free amino-acids [129,130,131].

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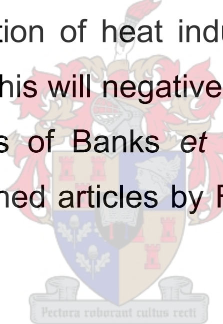
4.3.1. Hard and Semi-hard Cheese

Farkye and Landkrammer [125] reported that proteolysis in the production of Cheddar cheese increased with plasmin activity and resulted in an overall improvement of flavour and quality of the cheese after three and six months of ripening. They also concluded that a 6-fold increase in plasmin activity in the milk did not significantly affect the composition of the cheese produced in terms of moisture, protein and NaCl, although there was a slight increase in moisture and decrease in protein percentages in the final product. They concluded that milk with an approximate 3-fold enhanced level of plasmin resulted in the best overall quality cheddar cheese produced. The increased plasmin activity was obtained by adding plasmin to the milk before ripening and renneting, although it will not be viable in commercial applications due to the cost associated with the purification of bovine plasmin. They suggested that alternative methods should

be investigated such as increasing PA activity to convert native plasminogen to plasmin.

Lau *et al.* [132] produced Cheddar cheese from raw and heated milk (63°C for 30 minutes), and concluded that the cheese produced from the heated milk exhibited a lower proteolysis rate for α_s- and β-caseins than from raw milk used. This was attributed to the lower plasmin activity in the heated milk. In contrast Banks *et al.* [133], Alichanidis *et al.* [109] and Calvo *et al.* [134] applied different heat treatments in milk to produce Cheddar cheese and concluded that the cheese manufactured at higher temperatures (110 °C for 60 seconds) resulted in an increased proteolytic digestion of β-casein as supposed to milk heated at 63 °C for 30 minutes. The cheese of the milk treated with higher temperature will incorporate more denatured whey proteins in the cheese curd and increase cheese yield due to the formation of heat induced complexes between casein and denatured β-lactoglobulin, this will negatively impact on the maturation of the cheddar cheese. The findings of Banks *et al.* [133] and Calvo *et al.* [134] correlate favourably with published articles by Fox *et al.* [70], Farkye *et al.* [108] and Grandison *et al.* [135].



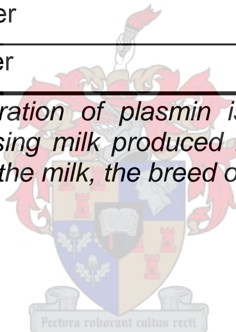
Farkye and Fox [127] produced cheddar cheese with added 6-amino-hexanoic acid which is a PI stimulator. The result was a slower breakdown of β-casein due to an increase in PI activity and a reduction in plasmin activity. Bastian *et al.* [128] produced Cheddar cheese with added urokinase to the milk. The result was an increase in proteolysis in the cheese due to an increase in plasmin activity. The breakdown of β- and α_{s2}-caseins during extended ripening periods resulted in the formation of short peptides which produced a bitter off-flavour. Le Bars *et al.* [136] reported that plasmin proteolysis in the manufacture of Cheddar cheese released astringent and bitter tasting hydrophobic peptides that detracts from the overall quality of the cheese.

In summary the effects of lactation and higher plasmin activity on the manufacture of Cheddar cheese are summarised in Table 4.3. The different stages of lactation will also exhibit different values for plasmin activity as mentioned in paragraph 4.4.1. The main effect of increased plasmin activity is the prolonged rennet coagulating time (RCT) during the manufacturing process of Cheddar cheese.

Table 4.3: Effect of lactation on cheddar cheese manufacturing [59]

Effect[1]	**Mid lactation**	**Late lactation**
Plasmin concentration	Lower	Higher
Rennet coagulation time	Shorter	Longer
Curd strength	Higher	Lower
Syneresis rate	Faster	Slower
Moisture	Lower	Higher

[1] It is also noted that the concentration of plasmin is not the only factor influencing the manufacturing of Cheddar cheese using milk produced in different stages of lactation. Other factors include the mineral balance of the milk, the breed of the cow and the pH of the milk



4.3.2. Swiss Style Cheese

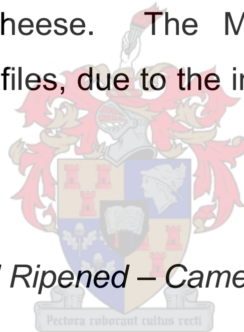
Benfeldt *et al.* [137] studied the effect of heat treatment on cheese milk, which was used for the production of semi-hard Swiss-style cheese, on plasmin activity and proteolysis during cheese ripening. They found reduced plasmin activity in the cheese manufactured from milk treated at higher temperatures (95°C for 15 to 90 seconds) than milk treated at lower temperatures (72°C for 15 to 90 seconds). However, they could not conclude that the thermal treatments had any effect on the endogenous proteolytic enzymes in the milk (such as cathepsin D) the proteolytic enzymes of the starter culture and non-starter bacteria, as well as the proteolytic action of the rennet enzyme used for coagulation. The reduced digestion of β-casein and α_{s2}-casein during ripening can be attributed to the inactivation of plasminogen and plasmin rather than the inactivation of PA due to

the lower decimal reduction values (D-values) of plasminogen and plasmin [109,137-140].

However, plasmin plays a beneficial role in the flavour development of Swiss-type cheeses such as Emmentaler. In Swiss-type cheeses the higher cooking temperatures of the curd destroy the PAI and PI. As a result there is a higher concentration of plasmin present and this result in an increased breakdown of casein during the cheese ripening process.

4.3.3. Pasta filata type cheese – Mozzarella

Late lactation milk with added native bovine plasmin was used for the manufacture of Mozzarella cheese. The Mozzarella cheese had inferior stretchability and meltability profiles, due to the increased breakdown of β-casein [138].



4.3.4. Semi-soft Cheese Mould Ripened – Camembert and Brie

In Camembert, Brie (mould ripened) and Tilset (smear ripened) cheese plasmin activity plays an important role in proteolysis. The catabolism of casein and the deamination of the amino acids produce ammonia (NH_3). Due to the production of ammonia there is an increase in the surface layer pH of these cheeses to a pH of approximately 7.0. The pH in the interior of the cheese also gradually increases due to outward migration of the ammonia and the higher pH facilitates the action of plasmin [67].

4.4. Fermented Products

In contrast to the effect of the fibrinolytic system on cheese manufacture and final product quality, there is limited literature available on the effect of plasmin and plasminogen activators on fermented milk products such as yoghurt. It is hypothesised that the proteolytic effect of the plasmin will be reduced due to the inactivation thereof by the high pasteurisation temperature during yoghurt manufacture (95°C for 5 minutes). Yoghurt is also a short shelf-life product, and therefore the effect of plasmin would not be as severe as in other long-life dairy products. However, it would be interesting to investigate and evaluate the effect of increased PA activity on the quality of the final product, as these fermented milks are acidified milk gels as opposed to rennet induced gels or coagulum.

In Chapter 5 bovine t-PA and u-PA were purified and characterised from bovine milk. In Chapter 6 the practical implication of addition of the purified t- PA to UHT milk, Gouda cheese and yoghurt is quantified in order to evaluate the effect of enhanced plasmin activity on final product quality. The investigation could benefit the primary and secondary dairy industry, as critical control parameters in the production of milk and dairy products could possibly be identified to limit the detrimental affect of endogenous milk proteases to raw milk and ultimately final product quality.

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Chapter 5

ISOLATION AND CHARACTERISATION OF BOVINE PLASMINOGEN ACTIVATOR

5.1. Introduction

Due to the different species of plasminogen activators present in bovine milk, this study concentrated mainly on the isolation and purification of tissue plasminogen activator (t-PA) associated with the casein micelles, rather than urokinase plasminogen activator (u-PA) associated with the somatic cell fraction in the milk. According to Zachos *et al.* [8] t-PA is the major enzyme contributing to the activation of plasmin which leads to the subsequent proteolysis of the β-casein fraction in the milk. t-PA is associated with casein, the major functional component in rennet and acid casein-gels. The influence of t-PA on the stability and functionality of the casein-gels, in products such as cheese and yoghurt, will therefore be an important factor contributing to the characteristics, shelf-stability and quality of the final product.

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Apparent molecular masses of between 75,000 Da to 84,500 Da for t-PA, and 30,500 Da and 47,500 Da for u-Pa have been reported [9,20,56,66]. As indicated in Fig. 5.1, in this study, the PAs in bovine milk have been isolated and partially purified using a combination of centrifugation, fractionation and solubilisation steps, followed by ion-exchange and size exclusion chromatography.

The main goal of the study was to isolate and characterise t-PA associated with the casein fraction in milk. The isolated bovine t-PA was further characterised by SDS-PAGE, high performance liquid chromatography (HPLC) and iso-electric focussing (IEF).

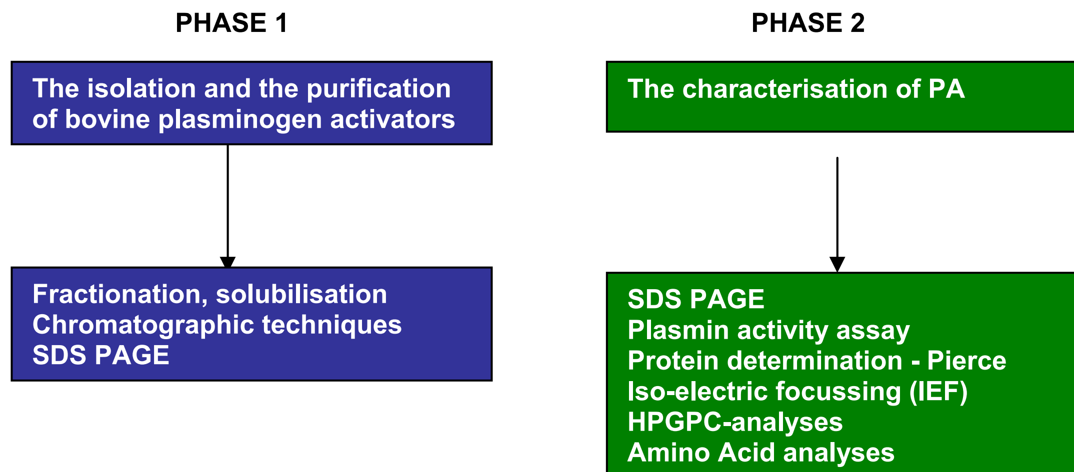

Figure 5.1: Different techniques used to isolate and purify (phase 1) and characterise t-PA (phase 2) from bovine milk.

5.2. Origin and characteristics of milk samples used for the isolation of t-PA

Raw bulk milk samples from morning and evening milk, obtained from a mixed herd of Holstein and Jersey cows, was collected from the Elsenburg Dairy Research Centre of the Department of Agriculture Stellenbosch, South Africa. The average number of days that the cows were in lactation was between 150 and 180 days (mid to late lactation). All samples were kept refrigerated at a temperature below 4°C and were tested within 48 hours of collection.

The milk was subjected to routine tests that would normally be conducted as part of the quality assurance program of milk producers and milk buyers. The tests were done to confirm the values of the following parameters:

1. Standard Plate Count (SPC): to test whether the milk is within acceptable microbiological specifications.

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2. Coliform bacteria: to test standard hygiene practise of milk production and to test whether the milk is within acceptable microbiological specifications.
3. Somatic cell count (SCC): to test herd health and possible infection and/or mastitis.
4. Percentage fat, protein and lactose: to test whether the percentage of total solids (TS) in the milk is within the normal parameters and feeding status of the cows.
5. Freezing point (Fp): to test for unadulterated milk (no added water).
6. Ethanol test (70% (v/v)) : to test for protein stability of the milk.
7. pH: to test if the milk is within acceptable limits and that no microbial growth has occurred (i.e. sour milk).
8. Percentage titratable acidity (%TA): to test if the milk is within acceptable limits and no microbial growth has occurred (i.e. sour milk).
9. Clot-boiling test: to test for protein stability of the milk.

5.3. Determination of PA activity

The PA-activity was determined spectrophotometrically according to the method of Zachos *et al.* [8] using the synthetic substrate, D-Val-Leu-Lys-ρ-nitroanalide (V0882, Sigma Aldrich; millimolar extinction coefficient of 5.57 $mM^{-1}.cm^{-1}$). The progress of the PA catalysed reactions was measured by determining the change in absorbence at 405 nm due to the hydrolysis of the chromatogenic substrate, D-Val-Leu-Lys-ρ-nitroanalide, to yield the free chromophore, ρ-nitroanalide (see Fig. 5.2).

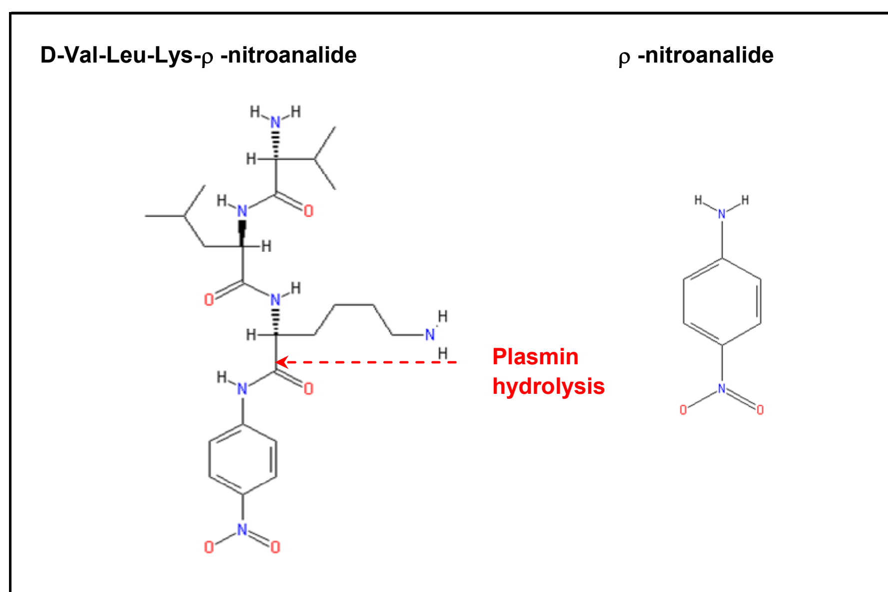

Figure 5.2: Schematic representation of the hydrolysis of the synthetic substrate, D-Val-Leu-Lys-ρ-nitroanalide, by plasmin to yield the chromatophore ρ-nitroanalide in the PA assay.

All assays were performed in triplicate. A typical reaction mixture (final volume 250 µl) contained 100 mM Tris-HCl buffer (pH 8.0), 50 µg.ml^{-1} plasminogen (P5661, Sigma Aldrich), 0.6 mM D-Val-Leu-Lys-ρ-nitroanalide and 5 µl of the PA fraction (see Table 5.1). White *et al.* [56] reported that casein had no influence on PA activity and no corrections for turbidity were therefore made for casein present in the PA fractions investigated in this study.

Table 5.1: Summary of the PA activity assay

Sample preparation before measuring PA activity				
Ingredient	**Control 1**	**Control 2**	**Sample 1**	**Sample 2**
100 mM Tris-HCl buffer, pH 8.0	255 µl	250 µl	250 µl	250 µl
0.6 mM D-Val-Leu-Lys-ρ-nitroanalide	Yes	Yes	Yes	Yes
50 mg.ml^{-1} plasminogen	Yes	No	Yes	Yes
PA fraction	-	5 µl	5 µl	5 µl
TOTAL VOLUME	255 µl	255 µl	255 µl	255 µl

The reaction mixtures were incubated at 37°C in a New Brunswick Scientific Co. G24 Environmental Shaker for 3, 4 and 5 hours respectively. The reaction was stopped by adding 100 µl of 30% (v/v) acetic acid to the samples followed by centrifugation at 8,000 X g for 5 minutes, before reading the absorbance at 405 nm. A sample without added plasminogen served as the control. The activity of the endogenous plasmin was subtracted from total the plasmin activity.

t-PA activity can be distinguished from u-PA activity by adding fibrin (20 µg) and amiloride (20 mM) to the PA-containing fractions. In the presence of fibrin there will be an increase in t-PA activity and in the presence of amiloride a decrease in u-PA activity. t- PA is not affected by amiloride and the activity of u-PA will not be enhanced by the addition of fibrin [140,141].

5.4. The isolation and purification of t-PA from bovine milk

A schematic representation of the isolation and separation of PA from bovine casein is given in Fig. 5.3.

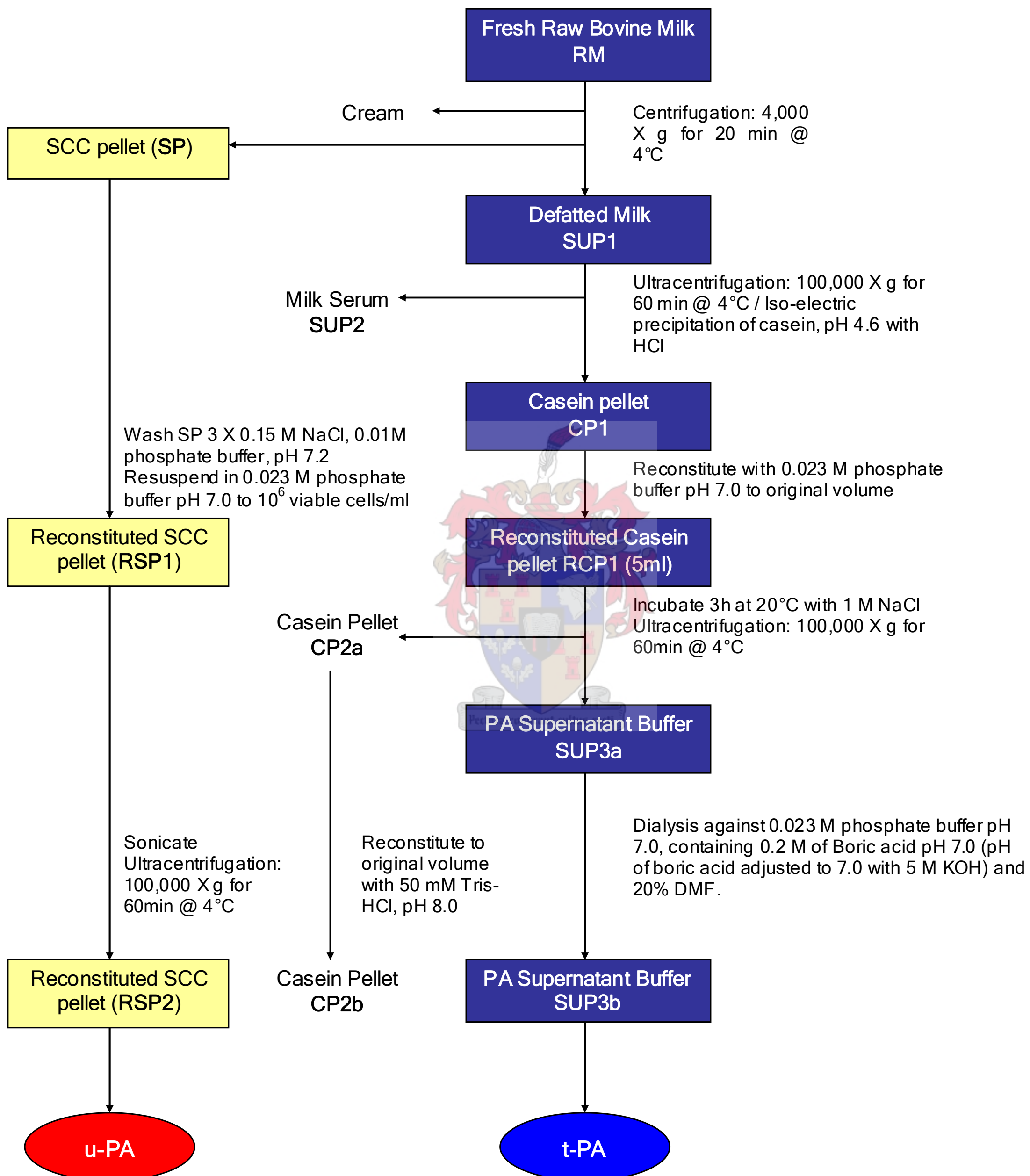

Figure 5.3: Schematic representation of the method for separation of u-PA and t-PA from casein in bovine milk.

The isolation and purification steps were modified from methods used by Politus *et al.* [75], Deharveng and Nielsen [22] and Yamauchi *et al.* [139]. Fresh bovine milk (RM) was centrifuged at 4,000 X g for 20 minutes at 4°C to separate the cream (milk-fat) and somatic cell fraction (SCC pellet - SP) from the casein fraction (milk serum). The skimmed milk, SUP1, was then ultracentrifuged at 100,000 X g for 60 minutes at 4°C to separate the whey proteins (supernatant – SUP2) from the casein pellet (CP1). Alternatively, the casein and whey fractions can also be separated by iso-electric precipitation of casein with acidification to a pH of 4.6 with HCl.

In order to dissociate the PA from the casein micelle structure the casein pellet (CP1) was reconstituted to the original volume (5 ml) with 0.023 mM Phosphate buffer (pH 7.0) and stirred overnight at 4°C (RCP1). NaCl (1 M) was added to the reconstituted casein pellet fraction (RCP1) and incubated for 3 hours at 22°C and subsequently ultracentrifuged at 100,000 X g for 60 min at 4°C. The resulting casein pellet (CP2a) was reconstituted to the original volume (5 ml) with 50 mM Tris-HCl (pH 8.0) and the supernatant fraction (SUP3a) was dialysed for 24 hours against 1000 volumes of 0.023 M phosphate buffer, pH 7.0 containing 0.2 M of boric acid pH 7.0 (the pH of boric acid solution was adjusted to 7.0 with 5 M KOH) and 20% DMF with one change of the external medium. The function of the DMF is to dissociate any κ-casein still present in SUP2.

The SCC pellet (SP), containing PA, obtained after the initial centrifugation step of 4,000 X g for 20 minutes at 4°C, was washed three times with 0.01 M phosphate buffer containing 0.15 M NaCl at (pH 7.2) with centrifugation at 4,000 X g for 20 minutes. The resulting RSP1 pellet was resuspended in 0.023 M phosphate buffer (pH 7.0) to yield at least 10^6 viable cells.ml^{-1}. Tryphan blue (5 µl of a 2.5 g.l^{-1} solution) was added to 20 µl of the SSP fraction. The proportion of cells excluding the dye was counted and sonicated for 45 seconds. The sonicated cell extract was ultracentrifuged at 100,000 X g for 1 hour to remove the entrapped casein (pellet). The SPP extracts (supernatant) were stored at –80°C for further investigation.

5.4.1. Purification of PA using chromatography

A schematic representation of the isolation and purification techniques, utilised for the purification of PA, is given in Fig. 5.4.

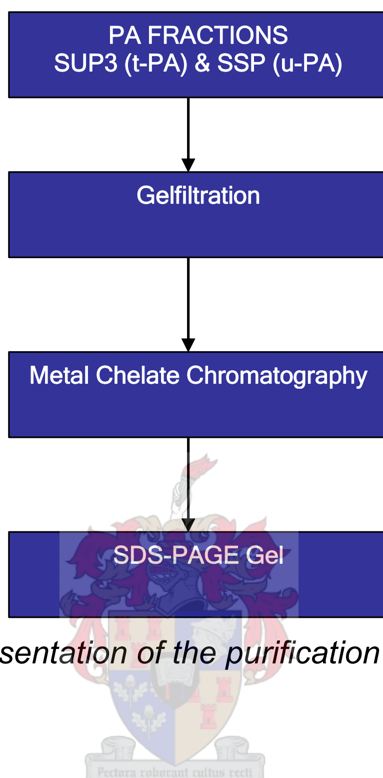

Figure 5.4: Schematic representation of the purification steps followed to purify PA from bovine milk.

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5.4.2. Gel filtration (size exclusion chromatography)

Size exclusion chromatography was carried out to separate PA from other low molecular mass proteins and to determine the apparent molecular mass of the PA. Separation was achieved with a Sephadex G-75 resin in glass column (18 mm ID X 950 mm). The column was equilibrated at a flow-rate of 7.0 $ml.h^{-1}$ with 50 mM Tris-HCl buffer (pH 8.0) containing 0.50 M of NaCl. The two PA containing fractions, RCP2 and SUP3a, were both subjected to gel filtration on the same column. The column was calibrated, using molecular weight standards, and the void (V_0) and total volumes (V_e) were determined by using a mixture of N-2,4-DNP-glycine (Sigma Aldrich) and dextran blue at concentrations of 2 $mg.l^{-1}$. The resulting fractions were collected at 10 minute intervals and the

flow-rate was calibrated for the running of the samples at 5 $ml.h^{-1}$. The eluting fractions were detected with a UV-flow detector set at 280 nm.

5.4.3. Metal chelate (affinity) chromatography

A Zn-chelating column (25 mm x 100 mm inner diameter) was prepared according to the manufacturers instructions using chelating-Sepharose 6B. The resin was equilibrated with running buffer of 20 mM phosphate buffer containing 1 M NaCl, 0.01% (v/v) Tween 80 (pH 7.50). After equilibration the resin was mixed with the PA containing fractions on a shaker plate at 4°C for 3 hours before the mixture was poured into the column.

The column was washed at a flow-rate of 40 $ml.h^{-1}$ with 4 volumes of running buffer. An imidazole gradient of 0-0.1 M was subsequently applied to the column and fractions of 3 ml each were collected. After the gradient was completed an additional 75 ml of 0.1 M imidazole buffer was used to elute all the remaining proteins from the column, while fractions of 5 ml each were collected. All the fractions containing PA activity were pooled, dialysed against deionised distilled water overnight at 4 °C and freeze dried. The protein concentration of each sample was determined spectrophotometrically at 280 nm while the PA-activity was detected using a colorimetric PA assay at 405 nm in the presence of D-Val-Leu-Lys-ρ-nitroanalide.

5.4.4. SDS-PAGE

Gel electrophoresis was carried out using the SDS-PAGE gel system of Life Technologies, South Africa. Stock acrylamide solutions were prepared according to the prescribed method to yield a 10% polyacrylamide resolving gel and a 3.9% polyacrylamide stacking gel solution.

5.4.5. Iso-electric focusing (IEF)

Iso-electric focusing was performed using a PhastGel IEF kit (Amersham Biosciences), pH 3-10. After the separation of the proteins by IEF, the proteins were visualised by silver staining.

5.5. High Performance Gel Permeation Chromatography (HPGPC) analyses

5.5.1. Sample preparation of t-PA for HPGPC analyses

The t-PA fraction obtained after Zn-chelating chromatography still contained plasmin, β-casein and low molecular weight proteins. As a result only Peak 2 of the SUP3b gel filtration step was used for HPLC analyses after SDS-PAGE analyses. For localisation of t-PA activity the lanes of the PAGE-gel were cut into slices of approximately 5 mm^2 and incubated overnight at 4°C in 0.1 M Tris-HCl buffer (pH 8.0) containing 5.5 $g.l^{-1}$ Triton X100, to remove the polyacrylamide. After 2 hours the buffer was discarded and the slices soaked in 0.5 ml fresh 0.1 M Tris-HCl buffer (pH 8.0) for 24 hours to elute the proteins. The buffer containing 125 µg of protein was tested for t-PA activity in the presence of fibrin. The buffer containing t-PA protein was dialysed against deionised distilled water for 24 hours at 4 °C before lyophilisation.

5.5.2. Molecular mass analyses of t-PA

HPGPC of t-PA and protein standards was performed on a 0.75 × 60 cm prepacked TSK G 3000 SW column (Sigma Aldrich). The TSK G 3000 SW column contained a silica-based, hydrophilic bonded phase packing that minimises interaction with proteins. Purified t-PA (200 µg) was analysed by HPGPC on the column equilibrated with 50 mM Tris-HCl, 100 mM NaCl (pH 7.2).

The same buffer was used to elute the protein at a flow rate of 0.4 $ml.min^{-1}$. The column effluent was monitored at 280 nm.

5.5.3. Amino acid analyses of t-PA

A 100 µg sample of lyophilised t-PA was mixed with 1 ml of 6 M HCl in a 5 ml ampoule. The acidified mixture was subsequently flushed with nitrogen under sonication, evacuated and flame-sealed before transfer to a pre-heated oven at 110°C for 24 h. Twenty µl of filtrated ampoule solution was transferred to a test tube and dried under a gentle stream of nitrogen gas. Each dried sample was hydrated with 250 µl of Beckman Na-S dilution buffer and the resulting solution was filtered through a 0.2 µm pore size syringe filter before it was loaded into the amino acid analyser autosampler cartridge. Amino acids were derivatised with *o*-phthaldialdehyde and 2-mercaptoethanol (MCE). The OPA reagent was prepared by combining 25 ml of 100 mM sodium borate, 2.5 ml 20% (w/w) SDS, 40 mg OPA dissolved in 1 ml methanol, and 100 µl MCE, then adjusting the volume to 50 ml with water. Derivatised amino acids were analysed by HPLC on a reverse phase C_{18} column. A tetrahydrofuran/methanol solvent gradient [solvent A, tetrahydrofuran / methanol / 0.1 M sodium acetate, pH 7.2 (5:95:900, by vol.); solvent B, methanol] was used for the separation of amino acids with a flow rate of 2.2 $ml.min^{-1}$. Effluent was monitored by fluorescence at an excitation wavelength of 330 nm and an emission wavelength of 450 nm.

5.6. Results

5.6.1. Chemical and Microbiological analyses of raw milk

Only unadulterated, mid-lactation, raw bulk milk samples with an SPC of less than 50,000 $cfu.ml^{-1}$ and SCC of less than $500,000.ml^{-1}$ were used to isolate and purify the PAs. The SCC was higher than the average for raw bulk milk, an indication of late lactation milk.

All the results obtained from the chemical and physical analyses of the milk were within the normal range associated with raw bulk milk as indicated in Table 5.2, which includes negative results for the clot-boiling and ethanol tests (i.e. no flocculation or precipitation of proteins indicative of protein instability). The pH and %TA values were also within the normal range specification. The results of the chemical and microbiological tests of the raw milk used for PA isolation are summarised in Fig. 5.5 and Fig. 5.6.

Table 5.2: Analyses of raw milk used for partial purification of PA

Sample	SPC (cfu.ml^{-1})	Coliforms (cfu.ml^{-1})	SCC.ml^{-1}	% Fat	% Protein	% Lactose	Fp (-m°C)	pH	%TA
1	42,500	2,850	320,105	3.45	3.32	4.65	523	6.67	0.135
2	23,000	1,400	280,900	3.39	3.28	4.70	526	6.63	0.130
3	33,000	3,800	420,332	3.35	3.20	4.65	522	6.70	0.125
4	49,000	8,250	385,300	3.55	3.40	4.68	530	6.68	0.140

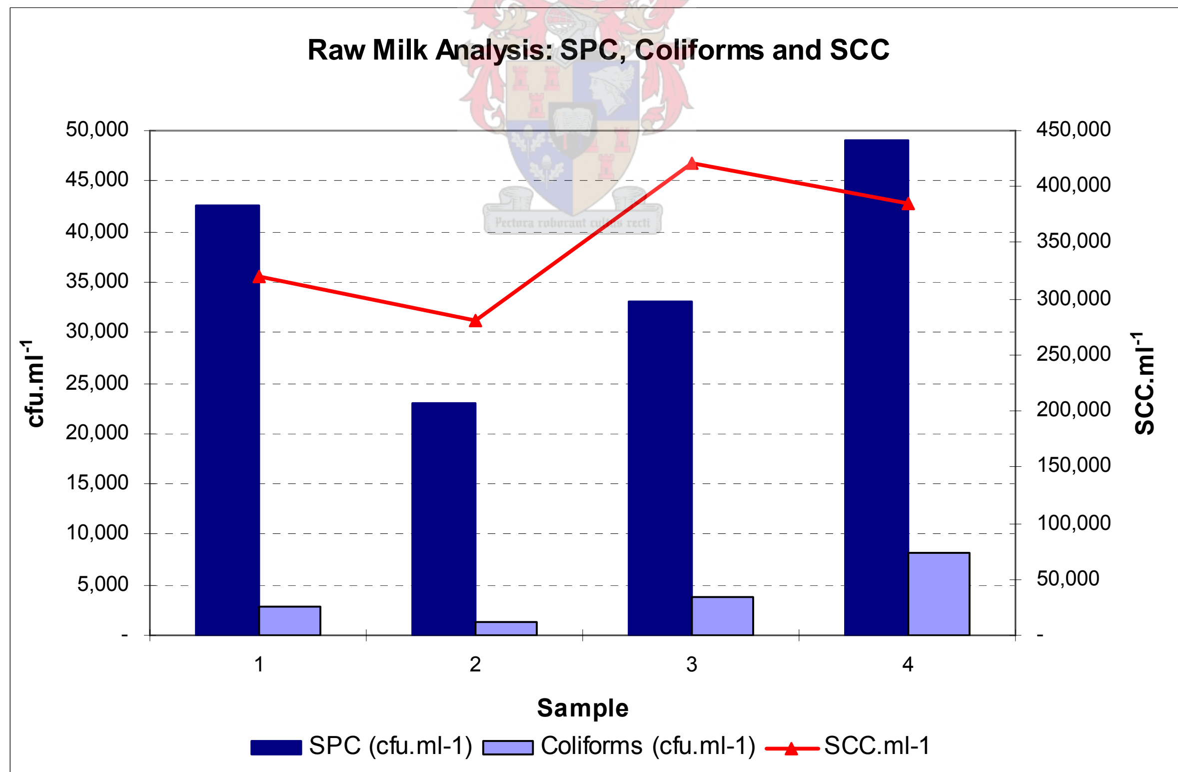

Figure 5.5: Results of Microbiological and SCC analyses of raw milk used for partial purification of PA.

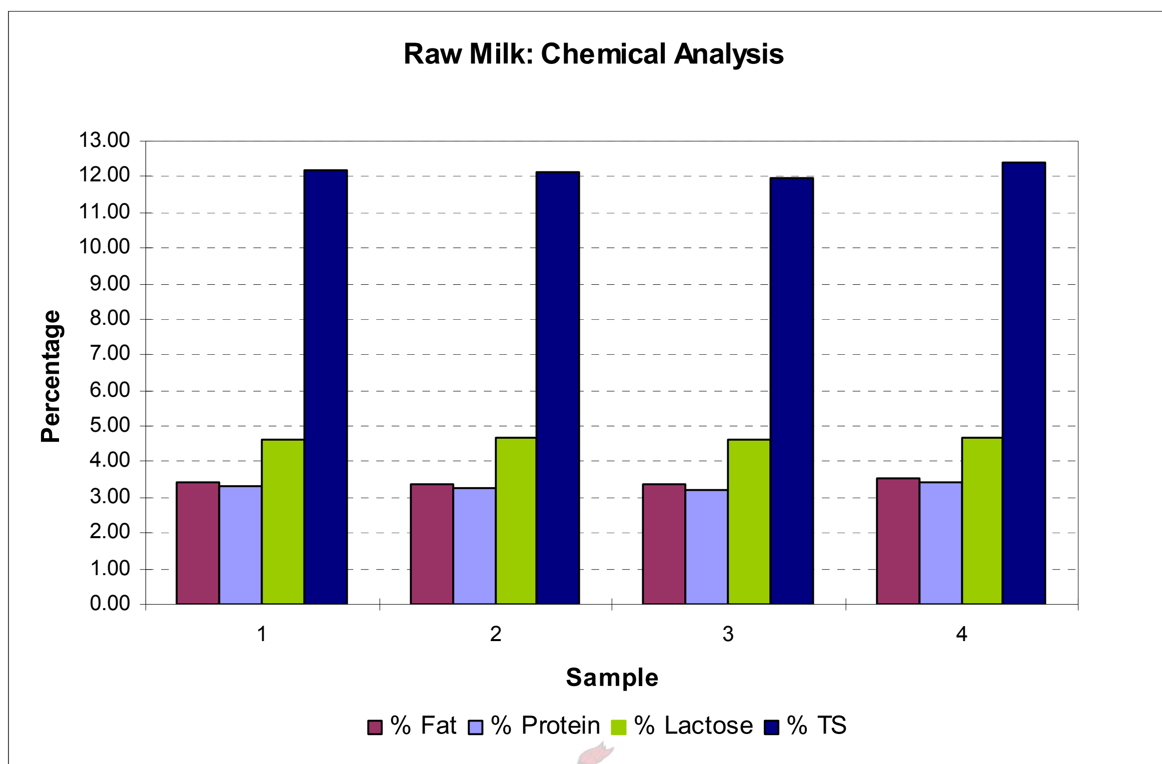

Figure 5.6: Results from the chemical analyses of raw milk used for partial purification of PA: Percentage Fat, Protein, Lactose and Total Solids (TS).

5.6.2. PA activity

The purification of t-PA and u-PA was monitored with the colorimetric PA activity assay described in 5.3. The SCC and casein fractions were tested for PA activity in the absence (sample 1) and the presence of fibrin and amiloride (sample 2) to differentiate between the presence of u-PA and t-PA. Control samples 1 and 2 were also tested for PA activity, control sample 1 was used as blank-control, and control sample 2 was tested without plasminogen to detect endogenous plasmin activity that might occur within the samples as a result of native plasminogen present in the samples. The native plasmin activity was, however, insignificant and therefore no correction for native detected plasmin activity was applied (results not shown).

In my hands the increase in absorbance between 0 to 3 hours was not linear during this pre-incubation period. Linearity in the rate of ρ-nitroanalide formation was calculated from the linear portion of the absorbance versus time curve as indicated in Fig. 5.7, which occurred between 3 to 5 hours. The PA activity detected was expressed in units (U); one unit of enzyme activity was defined as the amount of enzyme that produced a change in absorbance of 0.1 at 405 nm in 60 minutes. The units of PA were calculated from the change in absorbance between 3 hours and 4 hours at 450 nm.

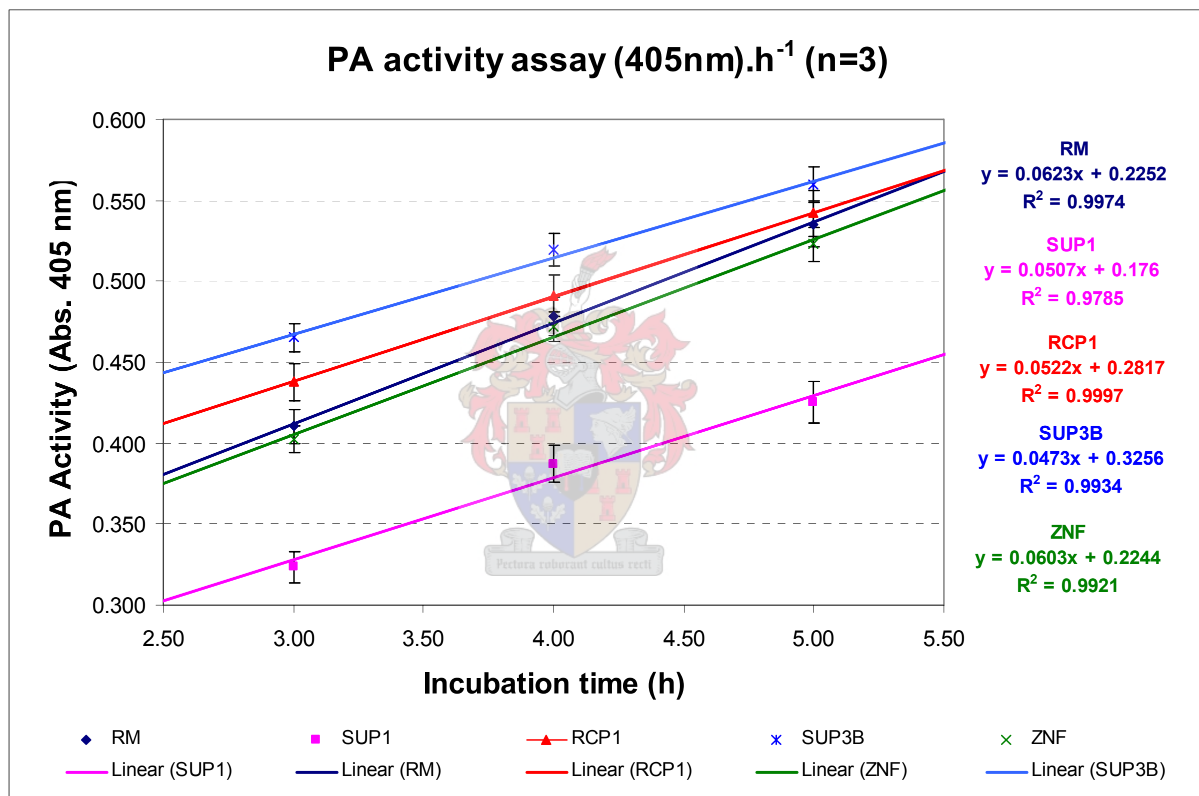

Figure 5.7: Change in absorbance with the PA activity assay at 405 nm for the different fractions isolated.

The PA activity of the casein fractions (RM, SUP1, RCP, SUP3b) showed a significant increase in activity in the presence of fibrin, indicating the presence of t-PA. The average increase of t-PA activity for these samples was 34%; however, the RM fraction had a lower increase in activity (13%) than the other fractions.

In Table 5.3 a summary of the absorbance values of the casein milk fractions obtained are given, as well as the deviation and % deviation between sample 1 (no fibrin and amiloride) and sample 2 (8 µg.ml^{-1} fibrin and 20 mM amiloride added).

Table 5.3: PA activity and PA concentration of casein fractions

PA activity assay (CASEIN FRACTIONS)						
	Δ A_{405nm} 3 h, 4 h @ 37°C					
Casein fractions	**Sample 1**	**Sample 2**	**Dev (±)**	**Dev (%)**	**Units PA**	**Specific Activity (U.g $^{-1}$ prot)**
RM	0.204	0.230	0.026	13%	2.0350	0.0599
SUP1	0.191	0.255	0.064	34%	1.9100	0.0562
RCP1	0.170	0.236	0.066	39%	1.7000	0.0654
SUP3b	0.162	0.234	0.071	44%	1.6200	72.3214
ZNF	0.150	0.212	0.061	41%	1.4950	398.6667

As indicated in Table 5.4 the PA activity measured in the SCC fractions (RSP1, RSP2) showed a decrease in the presence of amiloride, indicating the presence of u-PA. The minor decrease (3%) of PA activity in sample RSP 1 could be contributed to some t-PA being present in the fraction, associated with the casein fragments as reported by White *et al.* [56]. The casein fragments were removed from RSP1 by sonication followed by ultracentrifugation at 100,000 X g for 60 minutes at 4°C. The net effect of the amiloride was enhanced on sample RSP2, indicating the presence of u-PA, therefore it could be concluded that some of the t-PA was removed from fraction RSP1 together with the casein fragments.

In summary it could be concluded that the fractions isolated from casein contained predominantly t-PA activity while the final fractions isolated from the SCC contained predominantly u-PA activity.

Table 5.4: PA activity and PA concentration of SCC fractions

PA activity assay (SCC FRACTIONS)						
	Δ A_{405nm} 3 h, 4 h @ 37°C					
Casein fractions	**Sample 1**	**Sample 2**	**Dev (±)**	**Dev (%)**	**Units PA**	**Specific Activity ($U.g^{-1}$ prot)**
RM	0.204	0.230	0.026	13%	2.0350	0.0599
RSP1	1.860	1.801	-0.056	3%	0.4650	7.7500
RSP2	1.113	0.879	-0.234	21%	0.2783	30.9259

5.6.3. Purification of t-PA and u-PA

The purification tables for the respective PA's isolated from the casein pellet and somatic cell fraction are indicated in tables 5.5 and 5.6 respectively.

Only the SUP3b fraction was subjected to Zn chelating chromatography as the main aim was to isolate and purify t-PA. However, both the SUP3b and RSP1 fraction was further purified using size exclusion chromatography, in an effort to separate u-PA and t-PA from low molecular mass proteins.

During the purification of t-PA (casein fraction) there was a significant increase in the t-PA activity in fractions SUP3a and SUP3b, after incubation of the reconstituted casein pellet fraction (RCP1) with 1 M NaCl followed by ultracentrifugation at 100,000 X g for 60 minutes at 4°C. This indicates that the dissociation of t-PA from casein resulted in an increase in specific activity of the enzyme in the final SUP3b fraction. The percentage yield obtained from the initial purification steps was satisfactory at more than 75% with high specific activity in the SUP3b fraction of 72.32 $U.g^{-1}$ protein. The SUP3b fraction was then further purified using Zn chelating chromatography. The t-PA was purified more than 6,000 times with a final Specific Activity of 398.66 $U.g^{-1}$ protein and a

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final yield of 73% in the ZNF fraction. In Fig. 5.8 the units of t-PA isolated is plotted against Specific Activity of t-PA and %Yield.

Table 5.5: Plasminogen Activator activity from casein pellet in different fractions during isolation and purification steps

Fraction	Total vol. (ml)[1]	[Prot] (mg.ml^{-1})[2]	Tot. Prot. (mg)[3]	Units PA[4]	Specific Activity (U.g^{-1} prot)[5]	% Yield[6]	Fold Purification[7]
RM	1000	34.00	34000.00	2.0350	0.0599	100.00	1.00
SUP1	985	34.50	33982.50	1.9100	0.0562	93.86	0.94
RCP1	30	866.00	25980.00	1.7000	0.0654	83.54	1.09
SUP3b	28	0.80	22.40	1.6200	72.3214	79.61	1208.32
ZNF	5	0.75	3.75	1.4950	398.6667	73.46	6660.77

[1] Total Volume (ml) = Direct volumetric measurement
[2] [Prot] (mg/ml) = Pierce protein measurement
[3] Total protein (mg) = Total volume (ml) X [Prot] (mg.ml^{-1})
[4] Units PA = Spectrophotometric measurement where PA activity detected was expressed in units (U); one unit of enzyme activity was defined as the amount of enzyme that produced a change in absorbance of 0.1 at 405 nm in 60 minutes. The units of PA were calculated from the change in absorbance between 3 hours and 4 hours at 450 nm.
[5] Specific activity = Units PA / Tot. Prot (mg) = U.g^{-1} protein
[6] % Yield = (Units PA in fraction / Units PA in RM fraction)X 100
[7] Purification = Specific Activity PA fraction / Specific Activity fraction RM

Table 5.6: Plasminogen Activator activity from SCC pellet in different fractions during isolation and purification

Fraction	Total vol. (ml)[1]	[Prot] (mg.ml^{-1})[2]	Tot. Prot. (mg)[3]	Units PA[4]	Specific Activity (U.g^{-1} prot)[5]	% Yield[6]	Fold Purification[7]
RM	1000	34.00	34000.00	2.0350	0.0599	100.00	1.00
RSP1	30	2.00	60.00	0.4650	7.7500	22.85	129.48
RSP2	30	0.30	9.00	0.2783	30.9259	13.68	516.69

[1] Total Volume (ml) = Direct volumetric measurement
[2] [Prot] (mg/ml) = Pierce protein measurement
[3] Total protein (mg) = Total volume (ml) X [Prot] (mg.ml^{-1})
[4] Units PA = Spectrophotometric measurement where PA activity detected was expressed in units (U); one unit of enzyme activity was defined as the amount of enzyme that produced a change in absorbance of 0.1 at 405 nm in 60 minutes. The units of PA were calculated from the change in absorbance between 3 hours and 4 hours at 450 nm.
[5] Specific activity = Units PA / Tot. Prot (mg) = U.g^{-1} protein
[6] % Yield = (Units PA in fraction / Units PA in RM fraction)X 100
[7] Purification = Specific Activity PA fraction / Specific Activity fraction RM

Although a relatively low yield of u-PA of 13.68 % was obtained in the final RSP2 fraction, the Specific Activity was relatively high at 30 U.g^{-1} protein. The final purification was lower than that of the t-PA fraction in SUP3b, with u-PA in the RSP2 fraction purified 516 times. The low yield could be attributed to the loss of u-PA during the final reconstitution step of the RSP1 fraction, as some of the u-PA could have been lost in the serum isolated during the separation from casein. During the initial purification step of sample RM, some u-PA could also have remained in the defatted milk fraction (SUP1) and was subsequently removed during the ultracentrifugation step at 100,000 X g for 60 minutes at 4°C from the casein pellet (CP1) and remained in the milk serum supernatant (SUP2). SUP2 did yield some Specific Activity associated with u-PA, 0.014 U.g^{-1} protein, with a low yield of 4.91%. The activity was contributed to u-PA as there was a decrease in absorbance at 405 nm with the PA activity assay in the presence of fibrin and amiloride, indicating the presence of u-PA (results not shown). This

corresponds well with studies from Saksela *et al.* [74] and Precetti *et al.* [78] which concluded that u-PA and PAI activity is associated with the SCC and milk serum fractions in bovine milk.

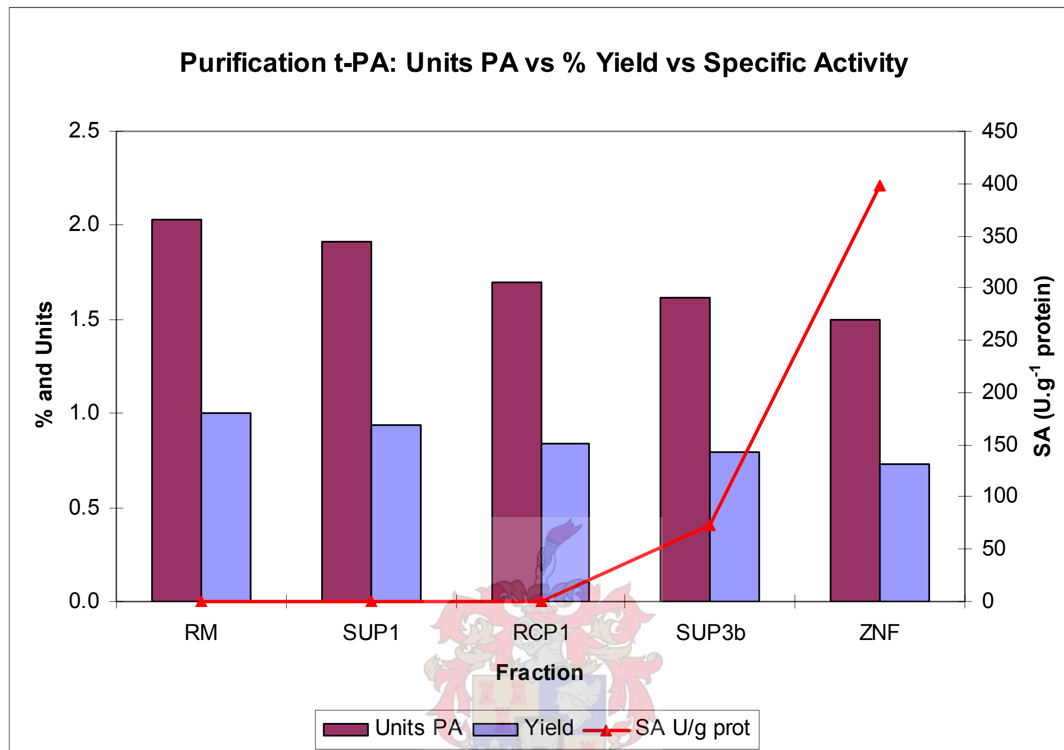

Figure 5.8: Purification of t-PA: Units of PA isolated against Specific Activity of PA and %Yield.

5.6.4. Gel filtration of t-PA (SUP3b) and u-PA (RSP2) fractions

The gel filtration results of the SUP3b and RSP1 fractions are indicated in Fig. 5.9. Absorbance of the column effluent was monitored at 280 nm, while the PA activity assay was used to determine PA activity.

In the SUP3b fraction PA activity was detected in peaks 1 to 4 as indicated in Fig. 5.9. The activity assay of the pooled fractions for SUP 3b, peaks 1–4, are indicated in Table 5.7. Peak 1 (Fig. 5.9) did display some plasmin activity that was enhanced by the addition of fibrin (sample 2). The activity in peak 1 was

attributed to endogenous plasmin activity, as the protein isolated had a molecular mass of 88,500 Da. The molecular mass of bovine plasmin was reported to be between 86,000 – 90,000 Da [70]. PA activity was enhanced by 21% with the addition of fibrin to the pooled fractions of peak 2, which clearly indicated the presence of t-PA. The molecular mass of the t-PA was calculated as 75,000 Da. Peak 3 and 4 also exhibited PA activity that was inhibited by the addition of amiloride, therefore indicating the presence of u-PA. The u-PA in peak 4 was associated with lower molecular mass proteins (estimated Mr between 10,500 – 18,000 Da). The Mr of u-PA in peak 3 and 4 was estimated to be between 30,500 – 47,600 Da.

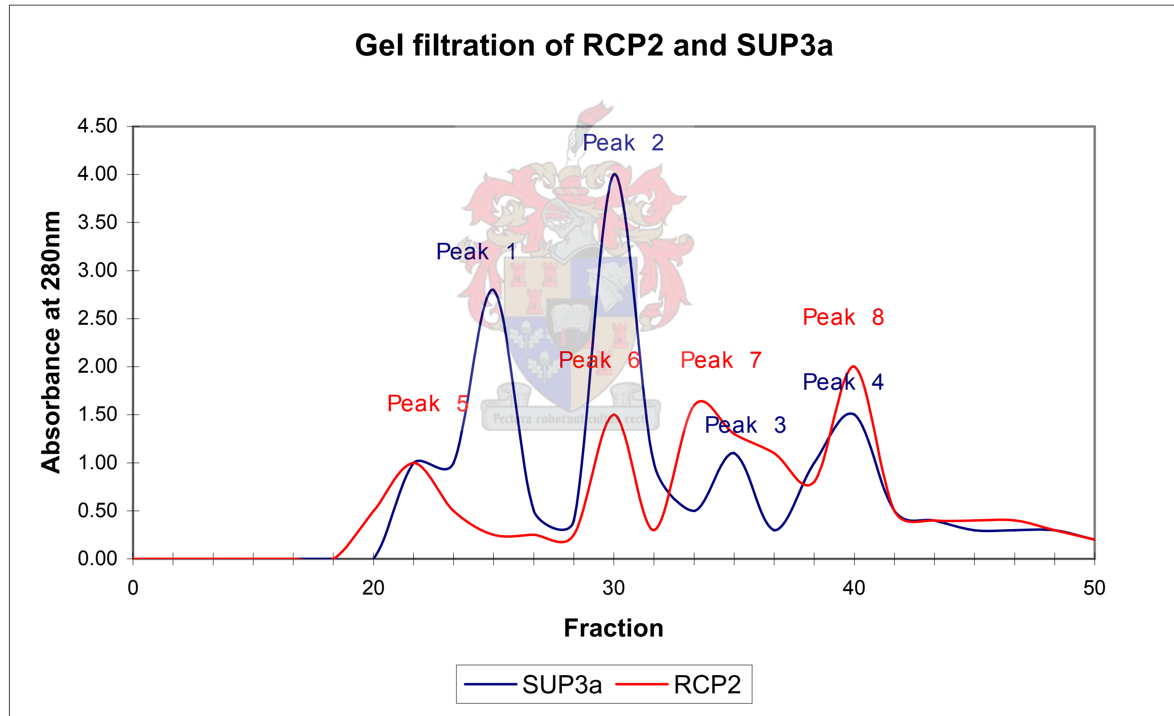

Figure 5.9: Gel filtration elution profiles of RCP and SUP3a PA fractions.

Table 5.7: PA activity of pooled gel filtration fractions for SUP3b

PA activity assay (GEL FILTRATION SUP3b)					
	Δ A_{405nm} 3 h, 4 h @ 37°C				
SUP3b[a]	**Sample 1**	**Sample 2**	**Dev (±)**	**Dev (%)**	**U PA**
Peak 1 (Fractions 25-28)	0.200	0.214	0.014	7%	2.00
Peak 2 (Fractions 29-34)	0.357	0.431	0.074	21%	3.57
Peak 3 (Fractions 36-38)	0.139	0.115	0.023	17%	1.39
Peak 4 (Fractions 43-46)	0.030	0.025	0.005	17%	0.005

In the RSP2 fractions PA activity was detected in peaks 5, 6, 7 and 8 as indicated on the Fig. 5.9. The activity assay of the pooled fractions for RSP2 peaks 5-8 are given in Table 5.8. Low activity was observed in the pooled fractions of peaks 5 and 6; the Mr corresponds well with the Mr of plasmin and t-PA as detected in the pooled fractions of peaks 1 and 2 of the SUP3b fraction (Mr plasmin 88,500 Da and Mr t-PA 75,000 Da). The activity was enhanced with the addition of fibrin by 17% and 57% respectively for pooled fractions of peaks 5 and 6. Pooled fractions of peaks 7 and 8 indicated the presence of u-PA as the activity was inhibited by 15% and 11% respectively with the addition of amiloride. The u-PA in peak 8 was associated with lower molecular weight proteins, estimated Mr between 10,500 – 18,000 Da. The Mr of u-PA in peak 7 and 8 was estimated to be between 30,500 – 47,500 Da.

Table 5.8: PA activity of pooled gel filtration fractions for RSP2

PA activity assay (GEL FILTRATION RSP2)					
	Δ A_{405nm} 3 h, 4 h @ 37°C				
RSP2[a]	**Sample 1**	**Sample 2**	**Dev (±)**	**Dev (%)**	**U PA**
Peak 5 (Fractions 18-23)	0.030	0.035	0.005	17%	0.30
Peak 6 (Fractions 29-34)	0.035	0.055	0.020	57%	0.35
Peak 7 (Fractions 39-41)	0.098	0.083	0.015	15%	0.98
Peak 8 (Fractions 43-46)	0.019	0.017	0.002	11%	0.19

5.6.5. Zinc chelating chromatography of t-PA (SUP3b) fraction

Fig. 5.10 summarises the results obtained when the t-PA-containing fraction of SUP3b was chromatographed on a Zinc chelating resin. Fractions 20 – 26 were pooled and evaluated for t-PA activity. The result of the PA activity assay is given in Table 5.3. The t-PA was purified more than 6,000 times with a final Specific Activity of 398 $U.g^{-1}$ protein. The pooled fractions containing t-PA activity were dialysed for 24 hours against deionised distilled water at 4°C and lyophilised before use as discussed in Chapter 6.

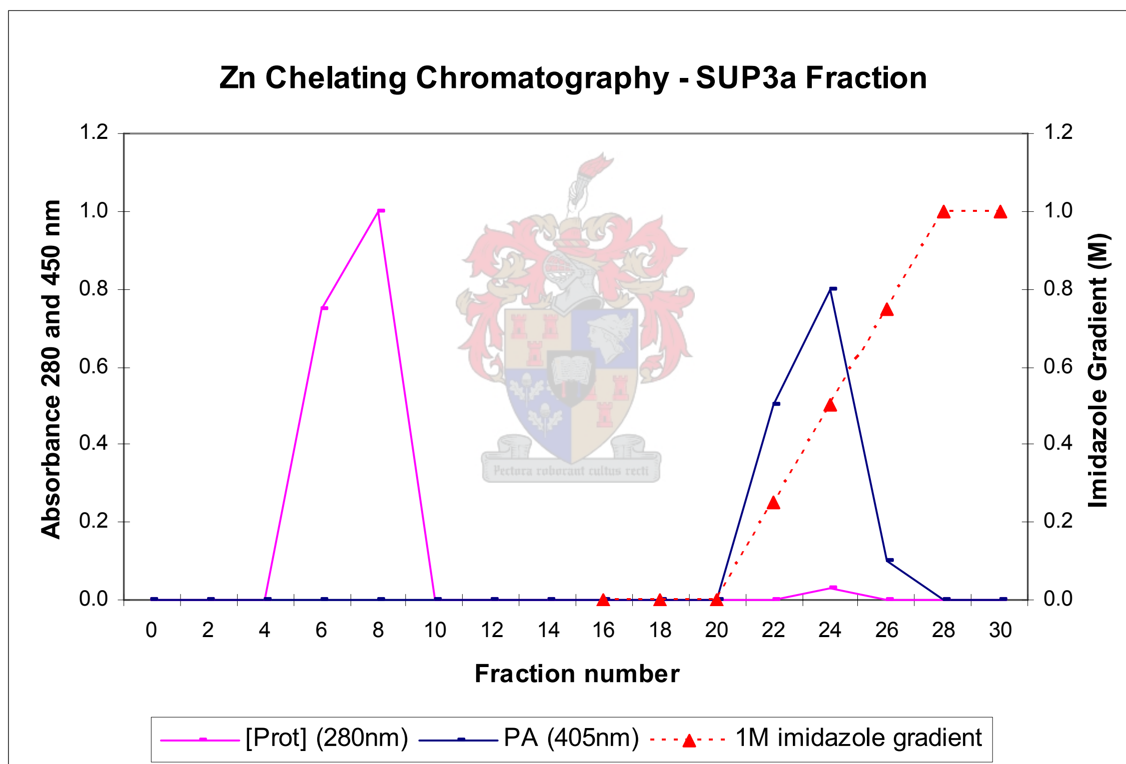

Figure 5.10: Zn chelating chromatography results of the SUP3a fraction run against a 1 M imidazole gradient.

5.6.6. SDS-PAGE

SDS-PAGE analyses were conducted on the following purified samples: (i) gel filtration sample of peak 2 (Fractions 29-34) in lanes 2 and 3, (ii) Zn-chelating fraction in lane 5 and (iii) RSP2 fraction in lane 6 as indicated in Fig. 5.11.

The gel filtration sample loaded in lanes 2 and 3 of the SDS-PAGE gel showed 4 distinct bands. Band 1 corresponded to an Mr 85,000 Da and appeared to be plasmin, band 2 corresponded to an Mr of 73,000 Da and appeared to be t-PA, band 3 corresponded to an Mr of 25,500 Da and appeared to be β-casein. Band 4 was an idistinct smear corresponding to an Mr 10,000 – 18,000 Da and appeared to be lower molecular mass proteins. The Zn-chromatography fraction loaded in lane 5 showed one distinct band which corresponded to an Mr of 73,000 Da and appeared to be t-PA.

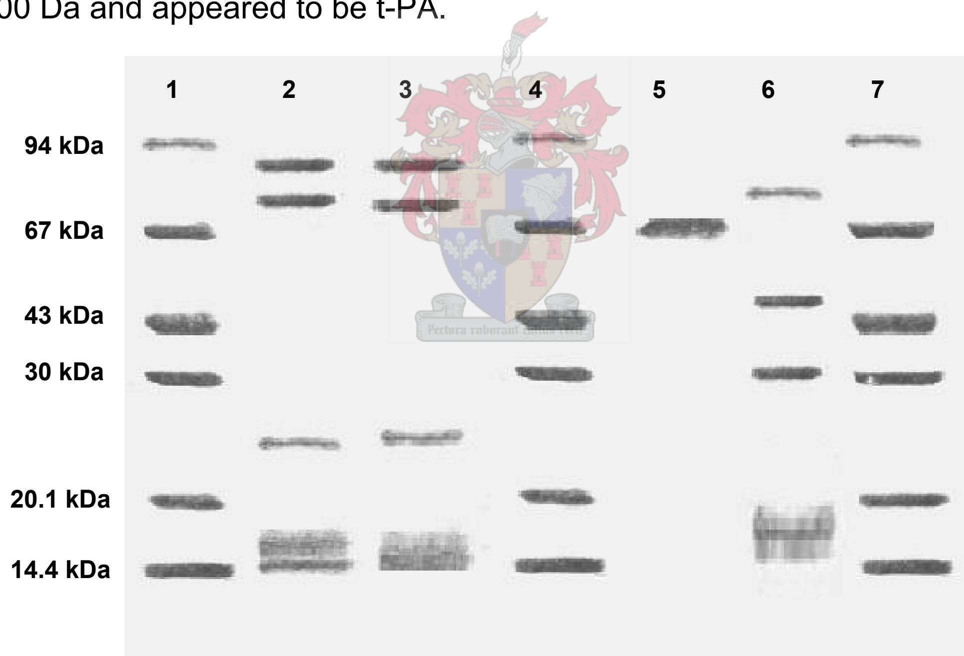

Figure 5.11: SDS-PAGE analyses of the pooled samples of peak 2 after gel filtration, Zn-chelating chromatography fraction and RSP2 fraction; Lane 1 – Molecular Marker (94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa and 14.4 kDa); Lane 2 – Gel filtration peak 2; Lane 3 – Gel filtration peak 2; Lane 4 – Molecular Marker; Lane 5 – Fraction from Zn-chelating chromatography (t-PA); Lane 6 – RSP2 fraction; Lane 7 – Molecular Marker.

The results of the SDS-PAGE analyses compared favourably with the results obtained from the gel filtration and Zn-chelating chromatography steps. Peak 2 of the SUP3b gel filtration analyses was tested for t-PA activity (lanes 2 and 3). For localisation of t-PA activity the electrophoretic lanes were cut into slices of approximately 5 mm^2 and incubated overnight at 4°C in 0.1 M Tris-HCl (pH 8.0) buffer containing 5.5 $g.l^{-1}$ Triton X100 to extract the proteins from the gel. After 2 hours the buffer was discarded and the gel slices were soaked in 0.5 ml fresh 0.1 M Tris-HCl (pH 8.0) buffer for 24 hours to elute the proteins. 125 µl of the buffer containing protein was tested for t-PA activity in the presence of fibrin. The SDS-PAGE isolated fraction had PA activity which was enhanced in the presence of fibrin indicating t-PA. The molecular mass of the isolated t-PA, according to SDS-PAGE analyses, was calculated as 73,000 Da. The u-PA active RSP2 fraction in lane 7 appeared to have some t-PA protein associated with it (band 1, Mr 73,500 Da) while the u-PA protein was associated with bands 2 and 3 (Mr 33,500 and 50,000 Da).

5.6.7. Iso-electric focusing (IEF)

The IEF analyses yielded 3 distinct pI values for the purified t-PA of 6.60, 6.85 and 7.30 respectively as indicated in Fig. 5.12. These results corresponded well with results obtained by Okamoto *et al.* [36] on the pI values for purified t-PA from human milk.

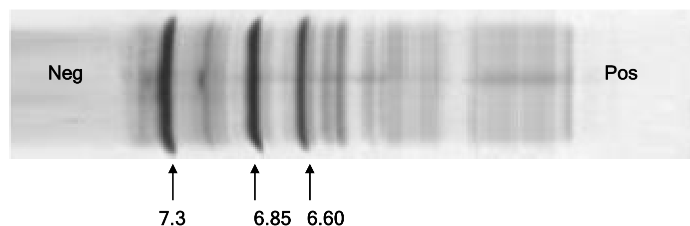

Figure 5.12: IEF electropherogram of purified t-PA.

5.6.8. HPGPC analyses of purified t-PA

The determination of the molecular mass of purified bovine t-PA with HPGPC analyses confirmed a Mr of 74,500 Da, which compares well with the Mr obtained by SDS-PAGE and gel-filtration. The recovery of the protein was high and less than 10% of the t-PA activity was lost after HPGPC analyses (results not shown). The chromatogram of t-PA and the standard proteins is shown in Fig. 5.13.

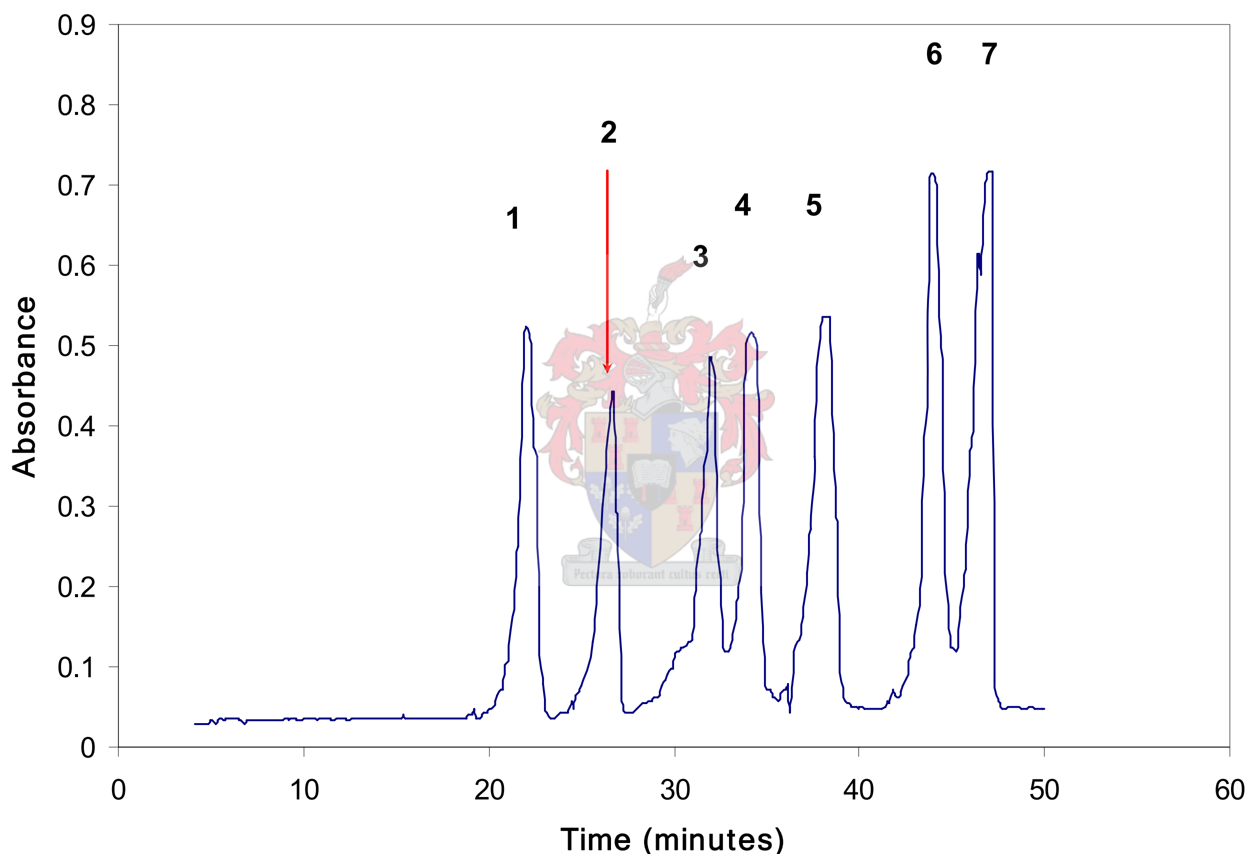

Figure 5.13: HPGPC of purified t-PA analysed together with molecular mass protein standards; 1. Phosphorylase B (94 kDa); 2. Purified t-PA indicated by a red arrow (75,5 kDa); 3. BSA (66 kDa); 4. ovalbumin (45 kDa); 5. carbonic anhydrase (29 kDa); 6. chymotrypsinogen (24 kDA) and 7. cytochrome C (12,5 kDa). The column was equilibrated and subsequently eluted with 50 mM Tris-HCl, 100 mM NaCl, pH 7·2.

5.6.9. Amino acid analyses of purified t-PA

Acid hydrolysis considerably influences amino acid recovery. During acid hydrolysis Trp and Cys are destroyed, Ser and Thr are partially lost and Met can undergo oxidation. Gly and Ser are common contaminants, therefore average responses were subtracted for blank runs. Pre-column derivitisation of amino acids with OPA, in the presence of MCE, yield isoindolic derivatives in a quick and simple reaction. However, secondary amino groups, such as proline and hydroxyproline do not react with OPA, and were also not detected. Some OPA-amino acid derivatives are unstable, therefore it is very important to control both the reaction and injection times [142]. The elution profile of the amino acid analyses of bovine t-PA is represented in Fig. 5.14. Amino acid concentrations were calculated based on Beckman amino acid standards, which had been treated similarly to the samples. An internal standard of 7.5 mM norvaline was added to the samples and amino acid standards prior to analyses as a reference and in order to compare linearity and range of analyses. Linear regression analyses confirmed a squared correlation coefficient (R^2) of greater than 0.989 for detection of norvaline. Amino acid analyses of the bovine t-PA hydrolysate indicated that the bovine protein consisted of 478 amino acids.

The amino acid composition of bovine t-PA is summarised and compared to that of humans and Vampire bats in Table 5.9 [143-146].

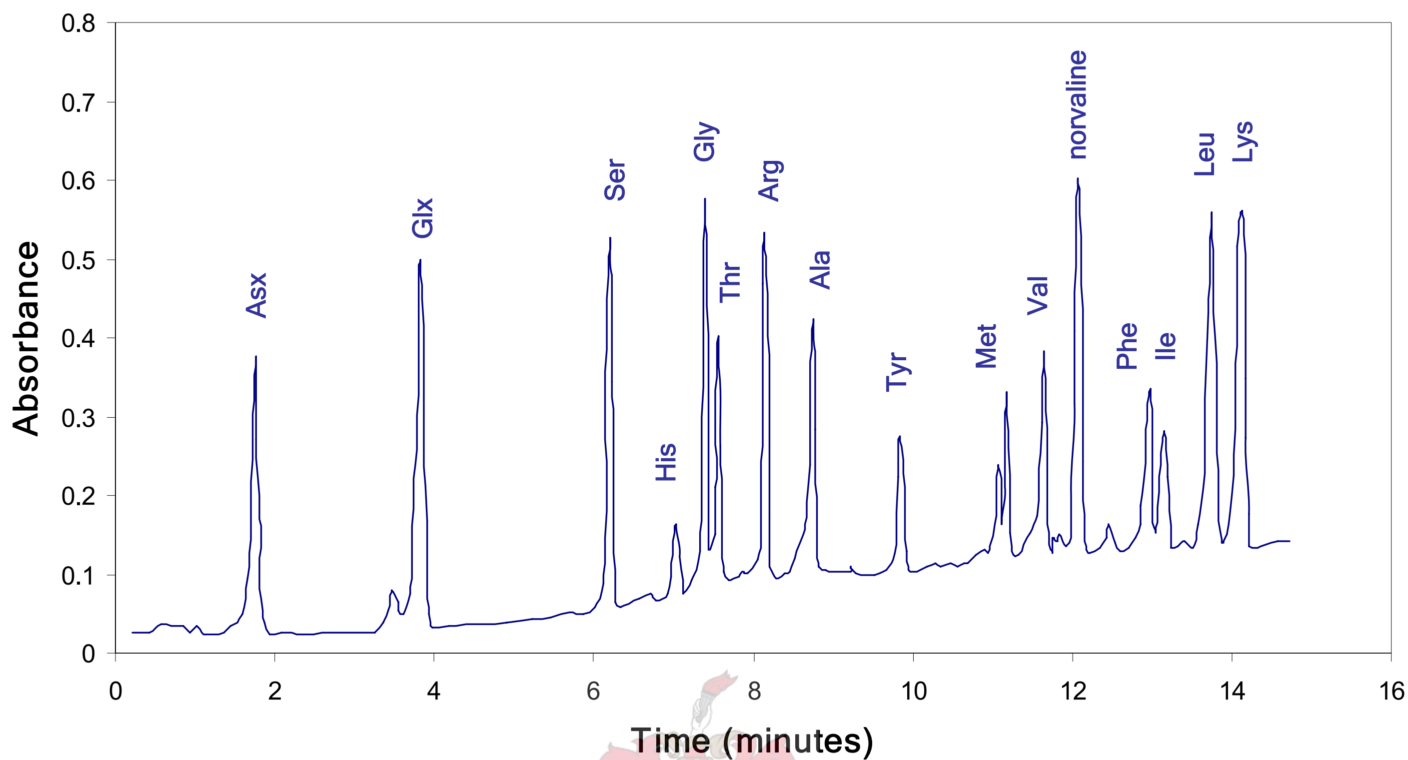

Figure 5.14: Amino acid analyses of bovine t-PA hydrolysate. Separation was achieved on a reverse phase C18 HPLC column. A tetrahydrofuran/methanol solvent gradient [solvent A, tetrahydrofuran / methanol / 0.1 M sodium acetate, pH 7.2 (5:95:900, by vol.); solvent B, methanol] was used for the separation of amino acids with a flow rate of 2.2 ml.min^{-1}.

Table 5.9: Comparison of the amino acid composition of purified bovine t-PA with other species

AMINO ACID COMPOSITION : t-PA			
Amino Acid	**Bovine *(Bos taurus)***	**Human *(Homo sapiens)***	**Vampire Bat *(Desmodus rotundus)***
Asx	53	-	-
Asn	-	22	23
Asp		29	20
Glx	46	-	-
Gln	-	27	21
Glu	-	27	26
Ser	43	50	36
His	20	17	12
Gly	47	46	38
Thr	31	25	31
Arg	40	40	31
Ala	35	36	25
Tyr	23	24	21
Val	27	28	31
Met	7	7	8
Phe	19	18	15
Ile	16	20	14
Leu	44	43	37
Lys	27	22	25
Trp	*Not detected*	13	10
Cys	*Not detected*	38	30
Pro	*Not detected*	30	23
TOTAL	**478**	**562**	**477**

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5.7. Discussion

t-PA and u-PA were successfully isolated and purified from a mixed herd of Jersey and Friesland cows at Elsenburg Dairy Laboratory, Stellenbosch. A combination of treatments were used to isolate the t-PA, including defatting of the milk, concentration of the casein by ultracentrifugation, salting out and finally dissociation of the t-PA from the casein micelles, yielding the t-PA containing fraction SUP3B. u-PA was isolated from the somatic cell fraction in the milk, the final fraction obtained after isolation and purification being RSP2.

The resulting t-PA obtained in the SUP3B fraction was purified 1200 times from the raw milk, with a specific activity of 72 $U.g^{-1}$ protein and a yield of 79%. Further purification of t-PA was obtained by using a combination of gel filtration and metal chelate (affinity) chromatography. The u-PA in the RSP2 fraction obtained was purified more than 516 times from the raw milk, with a specific activity of 30 $U.g^{-1}$ protein and a yield of 13%. No further purification of the u-PA fraction was conducted in this study.

The t-PA obtained after zinc chelating chromatography was purified more than 6,000 times with a final Specific Activity of 398 $U.g^{-1}$ protein and a final yield of 73%. The t-PA was then characterized by SDS-PAGE, iso-electric focusing (IEF) and HPGPC. The Mr of t-PA after gel filtration was estimated at 75 kDa, and this result correlated well with the Mr obtained from the SDS-PAGE, which was estimated at 73 kDa, and the chromatogram of the HPGPC, which confirmed a Mr of 74,5 kDa. The IEF analyses yielded 3 distinct pI values for the purified t-PA of 6.60, 6.85 and 7.30 respectively. The t-PA was analysed for amino acid composition and sequence compared to the t-PA of humans and Vampire Bats. The significance of the comparison is found in the extensive review of the human fibrinolytic system and the role of t-PA in haemostasis and thrombosis, as described in Chapter 2. Reuters reported in January of 2003 that t-PA (desmoteplase) in Vampire Bats has been studied by researchers in Australia,

who reported that the t-PA isolated from the Vampire Bats and administered to patients could decrease the mortality rate of patients that have suffered from strokes. According to Dr. Robert Medcalf of Monash University, the t-PA isolated from *Desmodus rotundus* assists with the solubilisation of fibrin clots in the blood, and will have advantages over commercially available drugs currently used due to the increased specific activity of the t-PA isolated.

Chapter 6 describes the study of the effect of the addition of the purified t-PA to milk. The investigation focussed on the t-PA and plasmin activity achieved with addition of t-PA, as well as on the extent of proteolysis that occurred during the normal shelf-life of value added products such as UHT milk, Gouda cheese and fermented milks (yoghurt).

Chapter 6

THE EFFECT OF t-PA ADDITION TO DIFFERENT DAIRY PRODUCTS

6.1. Introduction

Protease hydrolysis of micellular and casein dispersions in milk by the fibrinolytic system, of which t-PA is a key element, causes multiple changes in the functional properties of milk and dairy products. These changes directly influence the quality of milk as basis for all dairy products [12-16]. In this chapter the experiments investigating the effect of the addition of t-PA on the final quality of various milk products are described.

A number of factors determine the levels of (native) plasmin activity in fresh milk. These factors are: (i) the milk yield of cows, as they should be dried off at low milk yields; (ii) animal health, as high somatic cell counts (SCC) result in increased plasmin activation; (iii) nutritional status of the animals, as animals on poor nutritional rations are more susceptible to producing abnormal milk, (iv) seasonality of production and supply and (v) the degree of dilution of milk at the milk collection facility.

The knowledge gained from the isolation and purification of t-PA described in Chapter 5 was, as illustrated in this chapter, put into practice by manufacturing milk products with increased levels of plasmin activity. It is important to understand the effect of t-PA on the biochemical and physical properties of manufactured dairy products, as this will determine the overall quality, the organoleptic profile and shelf-life of the final products. These factors play an important role in producing products, in both the primary and secondary dairy

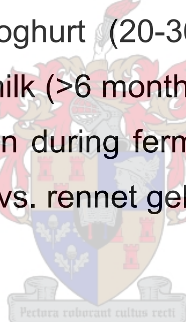
industries, that are economically viable and conform to consumer expectations and demand.

The milk products selected for this investigation included; (i) ultra-high temperature (UHT) milk, (ii) Gouda cheese and (iii) yoghurt. The rationale behind this selection was that these dairy products are representative of different manufacturing protocols within the dairy industry. The different manufacturing protocols contribute to the unique characteristics of each of the products, the main differences between the products selected for evaluation being:

(i) pH of the final product; neutral pH vs. low pH, in this instance UHT (pH 6.70) milk vs. Gouda cheese and yoghurt (<pH 4.60)

(ii) shelf-life of the final product; short shelf-life vs. extended shelf-life, in this instance yoghurt (20-36 days) vs. Gouda cheese (>3 months) and UHT milk (>6 months)

(iii) type of gel formation during fermentation, in this instance acid gel formation (yoghurt) vs. rennet gel formation (Gouda cheese)



6.2. Ultra-High Temperature (UHT) milk

6.2.1. Introduction

The gelation of UHT milk is a result of proteolysis either by native proteases such as plasmin, or heat resistant bacterial proteases in the milk [13,14,53,147,148] and is a well-documented phenomenon in the dairy industry,. It has been reported that there is no correlation between the degree of proteolysis of casein and the formation of non-casein nitrogen and non-protein nitrogen compounds, due to the difference in the activity of proteolytic enzymes as well as the metabolic activity of the bacteria present [149]. However, a decrease in the casein nitrogen content of the curd or the presence of short peptides or amino acids (proteose peptones) in milk serum is a good estimate of protease levels.

An increase in protease activity will result in breakdown of casein and subsequent protein destabilisation in UHT milk and other dairy products.

Onset of gelation in UHT milk is affected by various factors, such as the heat treatment, homogenisation, sequence of processing steps, milk solids content, composition of milk, quality of milk and storage temperature [150,151]. Age gelation has been attributed to various changes in UHT milk during storage and to various conditions that alter the gelation time. In general, gelation occurs when casein micelles lose colloidal stability and form a three-dimensional gel network [149,150]. The stability of casein micelles has been attributed to the presence of κ-casein, colloidal calcium phosphate, a high zeta potential (-18 mV) and steric stabilisation (prohibition of aggregation of submicelles by steric repulsion) by the hydrophilic C-terminals of κ-casein or "hairy layers" [146][1]. Gelation is preceded by changes at the surface of the casein micelles. These changes enhance interaction between micelles and can be categorised as changes that arise from protease activity [42,151].

The ambient storage temperature of the UHT milk influences the rate of proteolysis [148]. The proteolytic cleavage of β-casein is more rapid than that of α_{s1}-casein with the subsequent production of the γ-casein. The gelation pattern of UHT milks correlates well with the formation of γ-casein and therefore relates directly to the plasmin activity in the milk. Several experimental data sets confirm these results [6,14,15,103]. In experiments where serine-protease inhibitors that inhibit PA activity and thereby reduce the formation of γ-casein had been added to UHT milk, the onset of gelation was reduced or completely inhibited as opposed to the control samples without added serine-protease inhibitors [53]. It is also interesting to note that the general plasmin activity was higher in UHT milk produced with the indirect method than with the direct infusion or injection methods [14,15,53]. In UHT, sterilised, unconcentrated milks the onset of gelation is preceded by proteolysis, which is not the case for concentrated

[1] Refer to Chapter 3, pp 32-33

sterilised UHT milks. The gelation of the latter is a result of a nonenzymatic physico-chemical process (dissociation of proteins from the casein micelles and association of the proteins to the micelle surface; aggregation or gel formation occurs through association of micelle surface proteins).

6.2.2. Experimental protocol

The objective of this study was to evaluate the effect of added t-PA to UHT milk on the shelf-life of the product. Commercial full cream UHT treated milk (indirect heating 141.5°C with 4 seconds holding time) was collected from Parmalat Cape Town, South Africa, 7 days post production. Twenty units of the purified t-PA, obtained after Zinc chelating chromatography, as described in the previous chapter, was then added aseptically to 1000 ml milk in the presence of fibrin at a concentration of 8 $\mu g.ml^{-1}$ of final product. The milk was then evaluated over a 6-month period for changes in appearance, pH, apparent viscosity, gel formation, enzymatic activity, and casein breakdown.

The lyophilised t-PA enzyme, isolated after Zn-chelating chromatography, was resuspended in 100 mM Tris-HCl buffer (pH 8.0) and injected aseptically into the commercial containers, in a laminar flow cabinet. Twenty-four 1 litre containers of UHT milk were injected with t-PA and fibrin solution, and an equal number of samples were kept as controls. The area of injection was swabbed with ethanol (95% v/v) prior to inoculation. The t-PA / fibrin solution was cold filter sterilised through an 0.2 µm pore size filter into a sterile test tube before injection with sterile disposable needles and syringes. After injection the cartons were immediately sealed with an adhesive. After the adhesive had set (approximately 1 h), each container was gently rotated by hand for about 20 seconds. When the adhesive had hardened (after 24 hours), all containers (injected milk cartons and controls) were placed in enclosed cabinets for storage at an average temperature of 23°C.

Pectora roborant cultus recti

The milk was evaluated for appearance and colour, pH, gel formation, sediment, viscosity, PA activity and proteolysis after 30, 60, 90, 120, 150 and 180 days.

Appearance, colour and sediment were visually investigated. Gel formation and apparent viscosity were measured with a Brookfield viscometer at 20°C (spindle 1 at a speed of 60 rpm). Triplicate readings were taken directly in centipoise when the spindle had been rotating for 30 s. Protein analyses of the samples were done according to standard IDF procedure (IDF, 1964) by Kjeldahl analysis of the total protein (TP), non-protein nitrogen (NPN) and non-casein nitrogen (NCN). Casein nitrogen (CN) was calculated as the difference between NCN and NPN. All nitrogen determination were done in triplicate and results were expressed as protein equivalent using a conversion factor of 6.38. The UHT milks were analysed for the presence of short peptides and amino acids, which would indicate casein breakdown and protease activity. The UV absorption at 280 nm of milk serum, isolated from the experimental milk samples, was determined in order to detect the aromatic amino acid side chains of short peptides originating from hydrolysed casein [106]. An increase in absorbance at 280nm indicates the presence of the liberated breakdown products (proteose peptones) of casein in the serum. The PA assay was performed spectrophotometrically according to the method of Zachos *et al.* [8] using a synthetic substrate, D-Val-Leu-Lys-ρ-nitroanalide (V0882, Sigma Aldrich; millimolar extinction coefficient of 5.57 $mM^{-}1.cm^{-1}$).

6.2.3. Results

Raw milk normally contains approximately 0.3 $mg.l^{-1}$ plasmin and up to nine times more plasminogen [7]. Some of the plasmin and plasminogen is inactivated during heat treatment of milk. However, the potential for plasmin activation remains if t-PA is still active after heat treatment. PAs are more resistant to heat treatment than plasmin and plasminogen [151-154]. Table 6.1 summarises the

reported decimal reduction time (D-values) for plasmin, plasminogen and PA reported by various researchers.

Table 6.1: D-Values for plasmin, plasminogen and PA

D[1]-VALUES OF PLASMINOGEN, PLASMIN AND PA		
D-Value	**Enzyme**	**Reference**
35.7 min at 72.5°C	PL	Driessen and van der Waals (1978) [153]
12.4 min at 72.5°C	PL	Driessen and van der Waals (1978) [153]
10 sec at 142,5°C	PL	Driessen and van der Waals (1978) [153]
2 min at 85°C	PL and PG	Alichanidis *et al.* [109]
17 sec at 130°C	PL and PG	Alichanidis *et al.* [109]
7 sec at 140°C	PL and PG	Alichanidis *et al.* [109]
1.5 min at 85°C	PL and PG	Rollema and Poll [71]
15 sec at 110°C	PL and PG	Rollema and Poll [71]
6 sec at 140°C	PL and PG	Rollema and Poll [71]
109 min at 72.5°C	PA	Dongjin, Lu and Nielsen [152]
32 sec at 140°C	PA	Dongjin, Lu and Nielsen [152]

[1] D-value refers to decimal reduction time. This is the amount of time that it takes at a certain temperature to inactivate 90% of the enzyme being studied

The milk containers with added t-PA began to form a gel after 60 days of storage, and this gelation was accompanied by an increase in apparent viscosity (Table 6.2 and Fig. 6.1)[2]. The PA activity was constant throughout shelf-life, and there was an approximate increase in absorbance at 280 nm of 0.12 in milk with added t-PA as compared to that of the control milk (Table 6.3).

[2] In the samples with added t-PA, activity was detected using the PA activity assay as described in Chapter 5.

Table 6.2: Apparent increase in viscosity of UHT milk with added t-PA during 180 days shelf-life

VISCOSITY OF UHT MILK (BROOKFIELD, *cpi*- 20°C)[1]						
Day	**Control 1**	**Control 2**	**Control AVE**	**t-PA 1**	**t-PA 2**	**t-PA AVE**
0	2.30	2.40	2.35	2.35	2.40	2.38
30	2.40	2.60	2.50	2.40	2.20	2.30
60	3.00	2.80	2.90	3.50	3.20	3.35
90	3.20	3.00	3.10	18.00	21.00	19.50
120	2.40	2.50	2.45	32.00	29.00	30.50
150	3.40	3.40	3.40	38.00	42.00	40.00
180	3.00	3.00	3.00	45.00	43.00	44.00

[1] *Measured at 21°C with spindle 1 at 60 rpm*

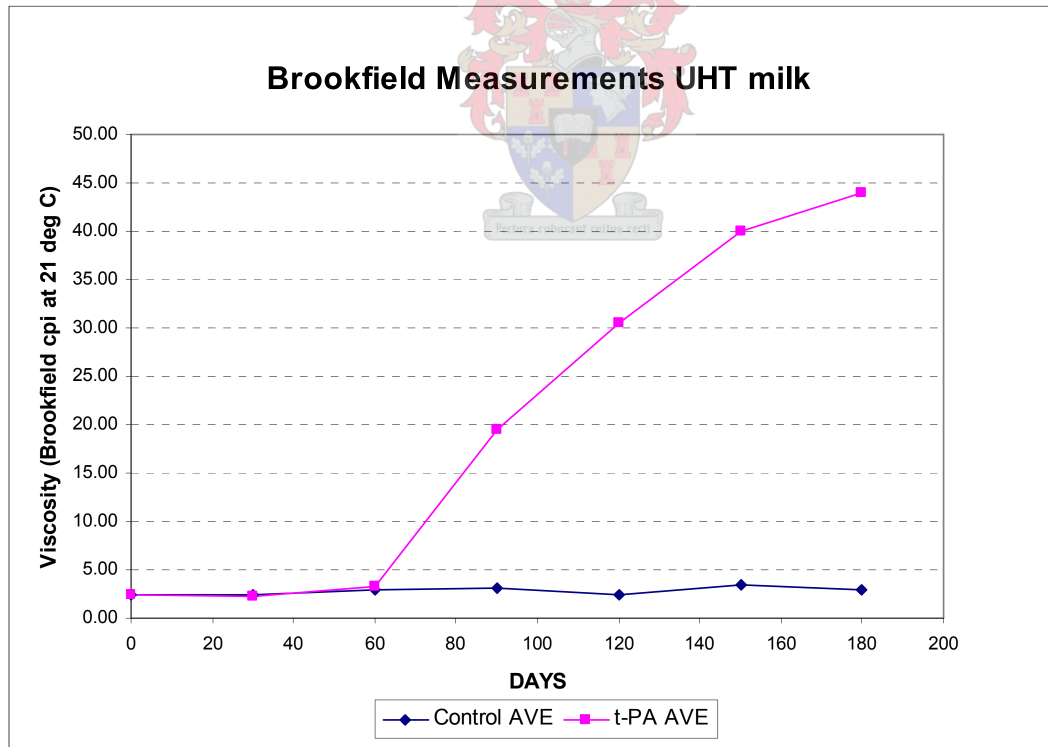


Figure 6.1: Apparent increase in viscosity of UHT milk with added t-PA during 180 days shelf-life.

Table 6.3: PA activity of UHT milk with added t-PA during 180 days shelf-life

PA ACTIVITY ASSAY (UHT MILK)			
	Δ A_{405nm} 3 h, 4 h @ 37°C		
Day	**Control[a]**	**t-PA sample[a]**	**Difference: Control – t-PA**
Day 0	0.153	0.289	0.136
Day 30	0.156	0.279	0.123
Day 60	0.165	0.270	0.105
Day 90	0.183	0.312	0.129
Day 120	0.180	0.302	0.122
Day 150	0.173	0.276	0.103
Day 180	0.155	0.273	0.118
Average	**0.166**	**0.286**	**0.119**

[a] *Fractions were diluted 1:2 before activity assay, therefore a 3-fold dilution*

Casein breakdown occurred, as indicated by Kjeldahl analysis of the total protein (TP), non-protein nitrogen (NPN), casein nitrogen (CN) and non-casein nitrogen (NCN) fractions (Table 6.4 and Figs. 6.2, 6.3 and 6.4). Between day 0 and day 180 casein decreased by 0.14%, and this correlated with an increase in the proteose peptone (PP) fraction of 0.12% over the same period of time, indicating the breakdown of casein into smaller peptides and amino acids. This was also confirmed by the increase in absorbance at 280 nm of milk serum as detected by the method of Richardson *et al.* [106]. The casein breakdown metabolites of the t-PA inoculated UHT milk, as measured by the absorbance at 280 nm, was approximately 9 fold higher than that of the control samples (Table 6.5 and Fig. 6.5). Literature reveals that absorption values in UHT milk serum exceeding 0.9 lead to gel formation of UHT milk upon storage [154].

Table 6.4: Kjeldahl analysis of total nitrogen (TN), total protein nitrogen (TPN) and casein nitrogen (CN) of UHT milk with added t-PA during 180 days shelf-life

KJELDAHL PROTEIN ANALYSIS (UHT MILK)							
Day	**TN[1]**	**TPN[2]**	**CN[3]**	**NCN[4]**	**WPN[5]**	**NPN[6]**	**PP[7]**
CONTROL							
0	3.440	3.260	2.620	0.820	0.630	0.180	0.010
30	3.435	3.255	2.600	0.835	0.640	0.180	0.015
60	3.416	3.226	2.580	0.836	0.630	0.190	0.015
90	3.410	3.230	2.590	0.820	0.630	0.180	0.010
120	3.427	3.232	2.590	0.837	0.630	0.195	0.012
150	3.438	3.258	2.620	0.818	0.620	0.180	0.018
180	3.386	3.206	2.570	0.816	0.625	0.180	0.011
t-PA							
0	3.440	3.260	2.620	0.820	0.630	0.180	0.010
30	3.475	3.265	2.600	0.875	0.640	0.210	0.025
60	3.435	3.235	2.540	0.895	0.640	0.200	0.055
90	3.403	3.233	2.510	0.893	0.620	0.170	0.103
120	3.472	3.252	2.490	0.982	0.610	0.220	0.152
150	3.462	3.268	2.450	1.012	0.640	0.194	0.178
180	3.430	3.207	2.389	1.041	0.625	0.223	0.193

[1]TN Total Nitrogen = TPN + NPN
[2]TPN Total Protein Nitrogen = CN + NCN
[3]CN Casein Nitrogen = TPN - NCN
[4]NCN Non-casein Nitrogen = TPN - CN
[5]WPN Whey Protein Nitrogen = NCN – (NPN + PP)
[6]NPN Non-Protein Nitrogen = TN - TPN
[7]PP Proteose Peptones = NCN – (WPN + NPN)

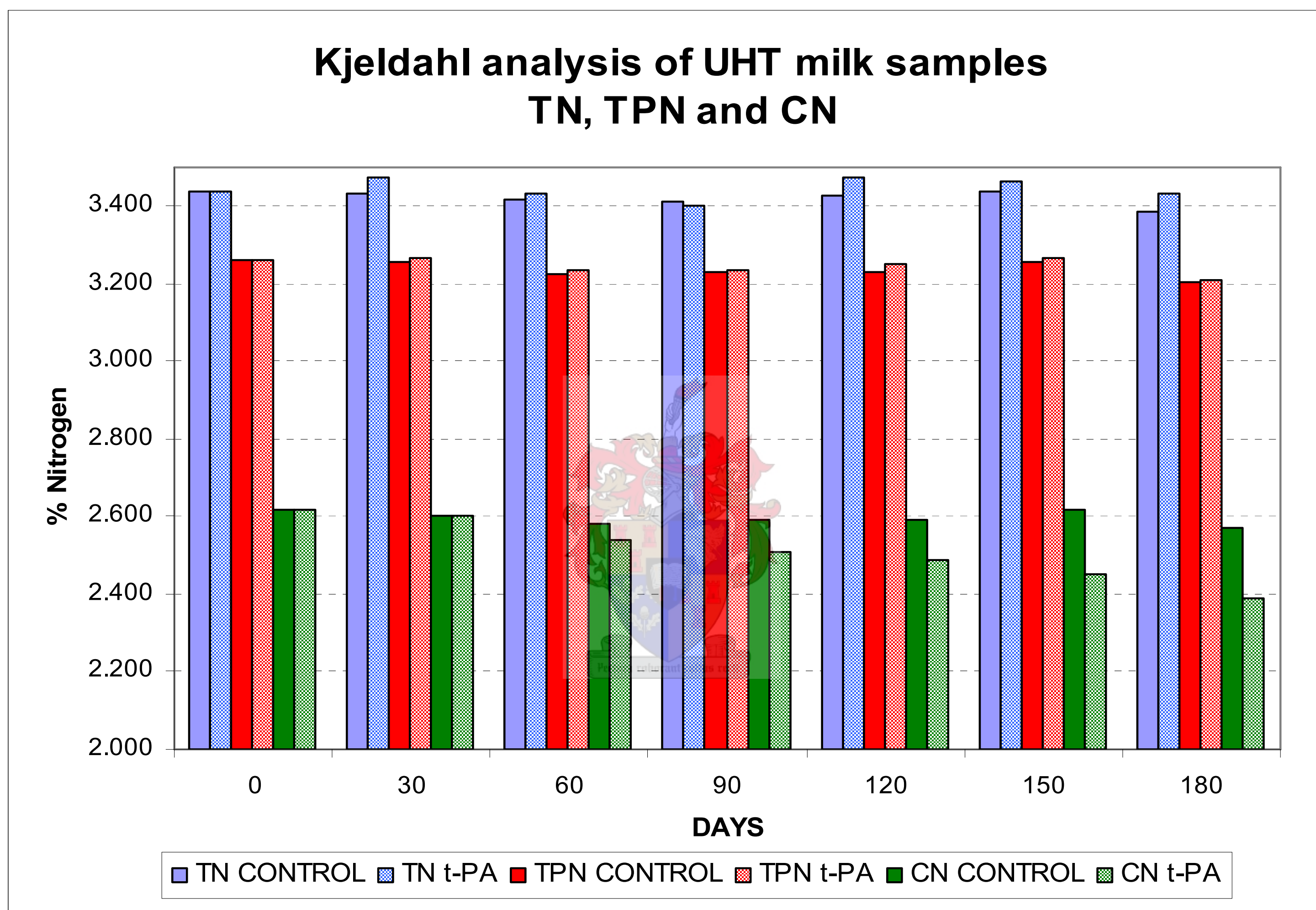


Figure 6.2: Kjeldahl analysis of total nitrogen (TN), total protein nitrogen (TPN) and casein nitrogen (CN) of UHT milk with added t-PA during 180 days shelf-life.

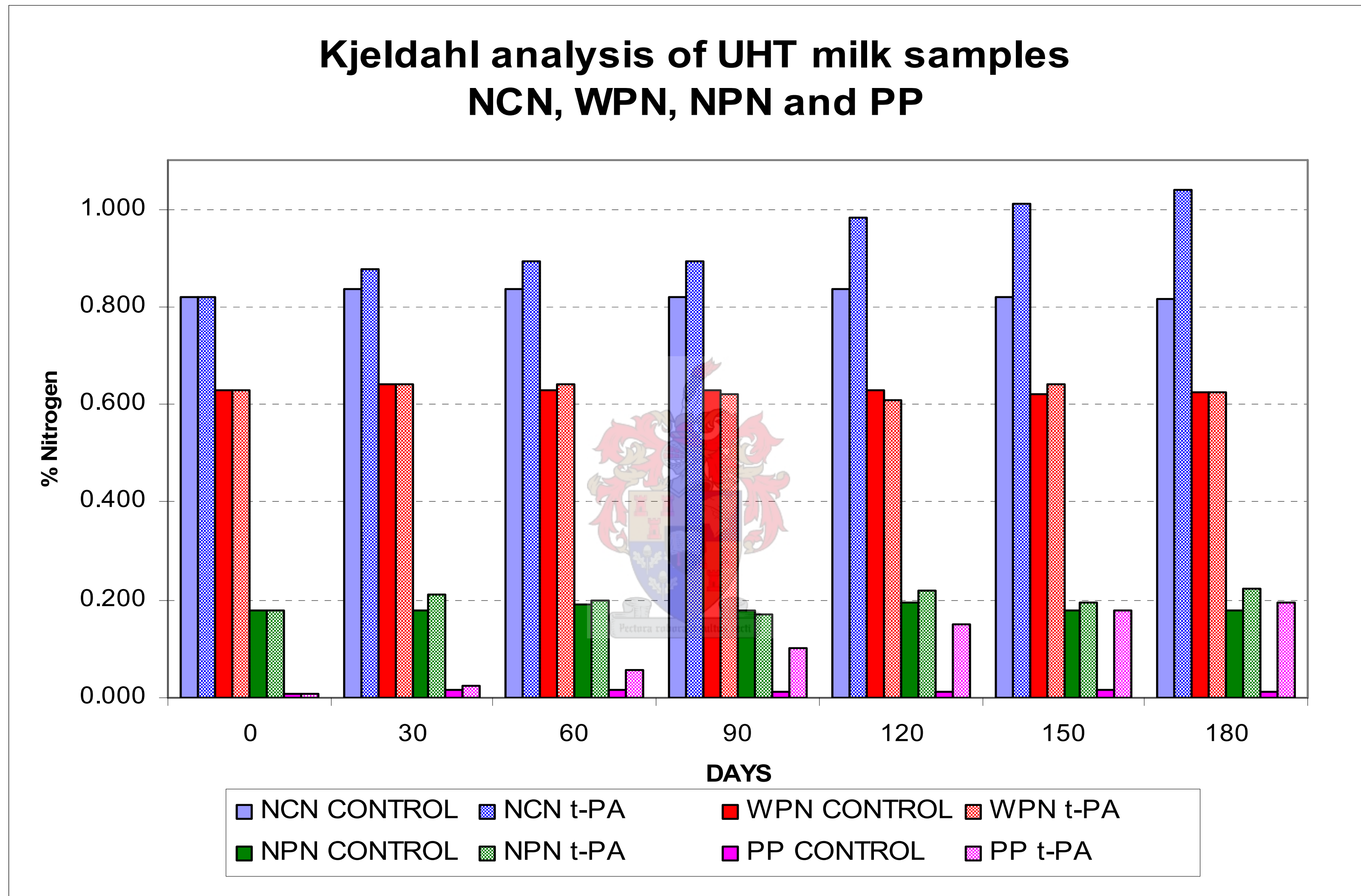


Figure 6.3: Kjeldahl analysis of non-casein nitrogen (NCN), whey protein nitrogen (WPN), non-protein nitrogen (NPN) and proteose peptones (PP) of UHT milk with added t-PA during 180 days shelf-life

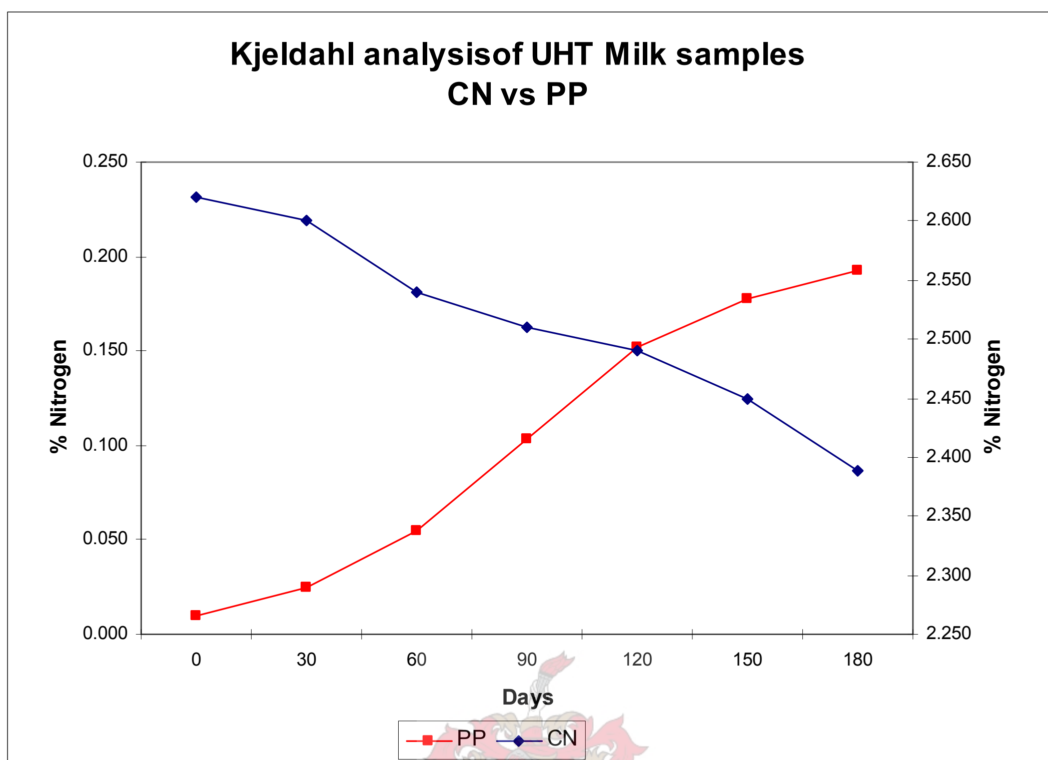


Figure 6.4: Kjeldahl analysis of proteose peptones (PP) vs. casein nitrogen (CN) of UHT milk with added t-PA during 180 days shelf-life.

Table 6.5: Spectrophotometric analysis of UHT milk serum at 280 nm with added t-PA during 180 days shelf-life

ABSORBANCE 280 NM (UHT MILK SERUM)						
Day	**Control 1**	**Control 2**	**Control AVE**	**t-PA 1**	**t-PA 2**	**t-PA AVE**
0	0.132	0.143	**0.138**	0.135	0.138	**0.137**
30	0.137	0.139	**0.138**	0.240	0.240	**0.240**
60	0.102	0.130	**0.116**	0.475	0.532	**0.504**
90	0.133	0.122	**0.128**	0.847	0.878	**0.863**
120	0.145	0.139	**0.142**	1.000	0.923	**0.962**
150	0.212	0.260	**0.236**	1.128	1.133	**1.131**
180	0.233	0.200	**0.217**	1.283	1.199	**1.241**

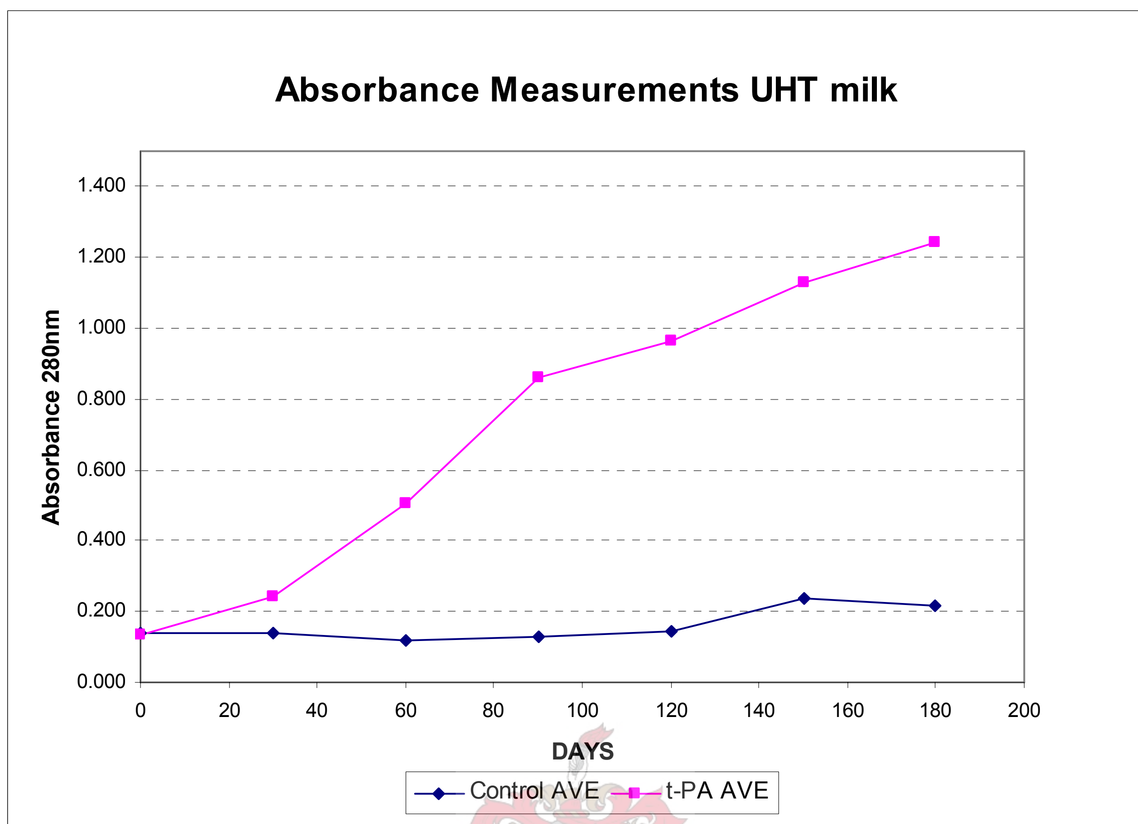


Figure 6.5: Spectrophotometric analysis of UHT milk serum at 280 nm with added t-PA during 180 days shelf-life.

There was no relationship between the decrease in pH and the onset of gelation in UHT milk as indicated in Table 6.6 and Fig.6.6. This finding correlates well with previous results obtained by Andrews *et al.* [155], which proposed that the decrease in pH during storage is due to loss of positive charges on the protein molecule, caused by the reaction of free amino groups in Maillard-type reactions [156].

Visual and sensory analyses of the samples revealed a severe bitter off-flavour in the coagulated milks. A typical visual distinction between bacterial protease in milk is the formation of sediment, while endogenous proteases cause a clear serum layer at the bottom of the container [60]. Prior to gelation at day 90, there was also a visible increase in severity of small white specs on the surface of the UHT milks.

Table 6.6: pH measurement of UHT milk serum at 280 nm with added t-PA during 180 days shelf-life

pH (UHT MILK)						
Day	**Control 1**	**Control 2**	**Control AVE**	**t-PA 1**	**t-PA 2**	**t-PA AVE**
0	6.66	6.66	6.66	6.66	6.65	6.66
30	6.63	6.64	6.64	6.60	6.62	6.61
60	6.62	6.61	6.62	6.59	6.58	6.59
90	6.60	6.61	6.61	6.57	6.55	6.56
120	6.60	6.61	6.61	6.50	6.52	6.51
150	6.58	6.57	6.58	6.50	6.51	6.51
180	6.56	6.55	6.56	6.48	6.49	6.49

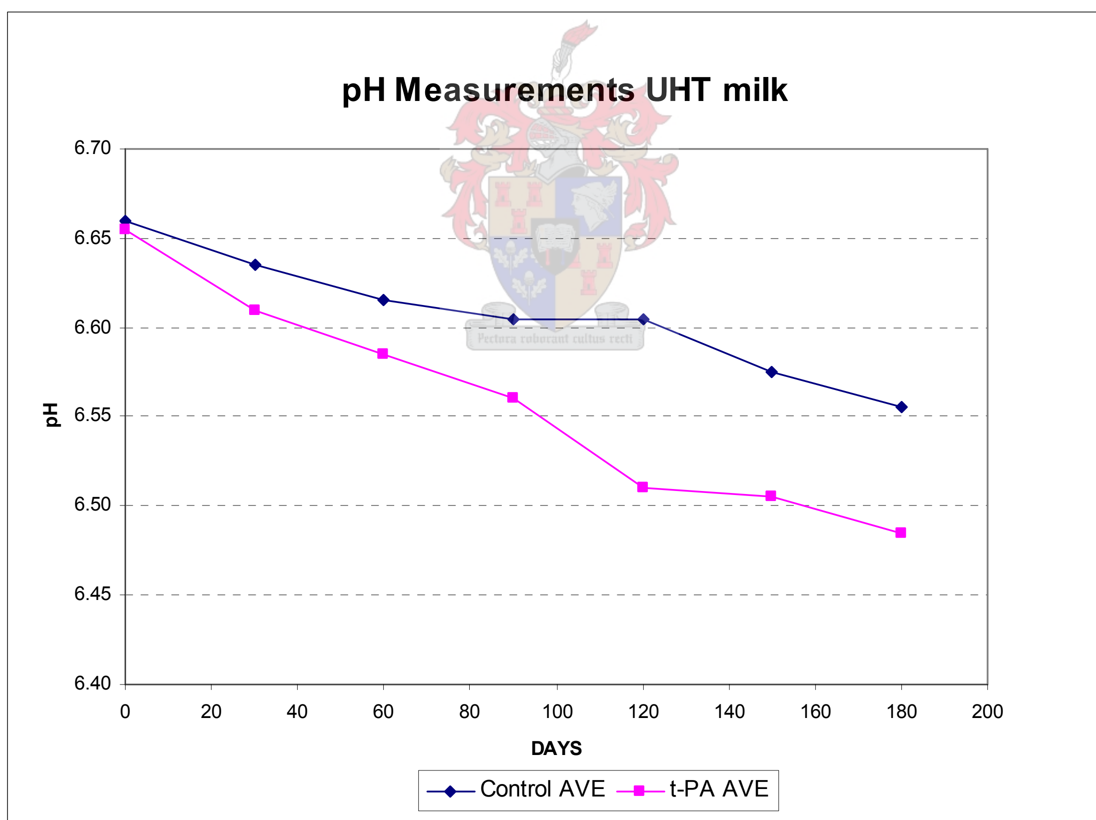


Figure 6.6: pH measurement of UHT milk serum at 280 nm with added t-PA during 180 days shelf-life.

6.2.4. Discussion

The results obtained from this study confirmed previous reports of an apparent link between proteolysis, an increase in apparent viscosity, and the formation of a gel and confirmed that there is a relationship between a low level of plasmin activity induced by the addition of t-PA and the gelation of UHT milk. In the study of Rollerma *et al.* [58], it was suggested that the added plasmin preferentially attacked β and α-caseins over κ-casein. Venkatachalam *et al.* [157] concluded that age gelation involves dissociation of proteins from the casein micelles and their formation on the micelle surface as protuberances and tendrils. Aggregation of the protein particles occurs through these appendages, not through the original micelle surface as in rennet or acid coagulation of milk.

6.3. Gouda Cheese

6.3.1. Introduction

Gouda cheese first appeared in the Netherlands in the sixteenth century. Gouda cheese was produced on farms near the village Gouda, approximately 33 km from Rotterdam.

South Africa produces about 65,000 metric ton of cheese per year from 650 millions litres of milk. About 60% of this quantity is in the form of Cheddar and Gouda cheese and the balance consists of other types, headed by Feta and Mozzarella. Of the 65,000 metric ton of cheese produced, it is estimated that 15,600 metric ton produced is Gouda. The per capita consumption of cheese in South Africa has increased from 1 to 1.5 kilograms per year since 1995, but is still far less than France who top the list at 25 kilograms per year, followed by Greece and Germany with 20 kg and the Netherlands with 18 kg per year.

South Africa produces fine quality cheeses, but consumer demand is forcing manufacturers to constantly improve the quality their products. Cheesemakers are well aware of the fact that inferior quality milk with high somatic cell count, reduces cheese yield and affects the characteristics of the final product. This has been confirmed in research and numerous publications over the years [158-160]. Internationally cheesemakers and milk buyers encourage farmers to produce milk with a low SCC in order to ultimately improve the quality of the final products. Milk buyers enforce penalties on farmers with high SCC milk and pay premiums for low SSC milk. In South Africa premiums can be as much a 2-4 cents per litre for milk with low SCC (<500,000.ml^{-1}), and penalties can be as much as 6 cents per litre for milk with high SCC (>500,000.ml^{-1}).

Summer *et. al.* at the University of Wisconsin have reported that milk production decreases linearly with an increase in SCC [159]. It is reported that in Wisconsin mastitis alone costs the dairy farmers more than $100 million per year as a result of reduced milk production. High levels of SCC cause a decrease in cheese yield; the relationship is, however, not linear. The decrease in cheese yield can be as high as 1% for SCC that increases to 100,000.ml^{-1}. If the increase of SCC exceeds 1,000,000 SCC.ml^{-1} the rate of decrease in cheese yield is less; however, it can be as much as an additional 2% loss [159].

Verdi and Barbano [19] reported that the major enzyme responsible for the proteolytic activity in milk is the native serine protease plasmin. Plasmin levels are elevated during mastitis, resulting in the hydrolysis of casein in the milk. This disproves previous theories that the loss in cheese yield with high SCC is as a result of the cow producing milk with an increased whey protein to casein ratio.

In addition to reduced cheese yield, hydrolysis of casein negatively affects the rheological properties of rennet-induced milk gels resulting in an increase in rennet coagulation time, an increase in moisture content in the final cheese, and an increase in fat losses in the whey [158-162]. Plasmin readily hydrolyses β- and α_s-caseins to form peptides and amino acids [68]. This negatively impacts

on the formation of the casein matrix during renneting, forming a weaker gel that deteriorates during cooking and stirring of the curd. The lower calcium in milk with a high SCC also contributes to a more fragile curd during renneting.

6.3.2. Gouda Characteristics

To evaluate the effect of t-PA addition to milk, prior to the manufacture of Gouda cheese it is important to understand typical product characteristics.

6.3.2.1. Composition of Gouda Cheese

The typical chemical composition of Gouda is indicated in Table 6.7.

Table 6.7: Gouda Cheese Standards (Chemical and Microbiological) [163-168]

GOUDA CHEESE STANDARDS		
CHEMICAL STANDARDS	**GOUDA ROUND**	**GOUDA BLOCK**
pH	5.0 – 5.20	5.0 – 5.45
% Moisture	38 – 43	38 – 43
% Fat	29 – 34	29 – 34
% Fat in Dry Matter (FDM)	48 – 60	48 – 60
% Salt	0.9 – 1.6	1.0 – 2.0
Maturing period	≥ 4 weeks	≥ 4 weeks
MICROBIOLOGICAL STANDARDS	**GOUDA ROUND**	**GOUDA BLOCK**
Coliforms (cfu.g^{-1})	≤ 500	≤ 500/g
E.coli (cfu.g^{-1})	Negative	Negative
Yeast and Moulds (cfu.g^{-1})	≤ 300	≤ 300

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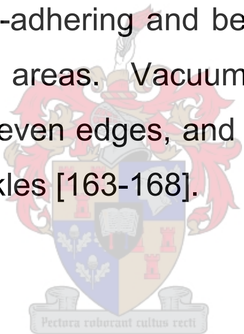
6.3.2.2. Colour

Gouda cheese may be uncoloured or coloured. The colour is white to yellow, not waxy but dull, clear and uniform throughout. Discoloured spots or patches are not tolerated [163-168].

6.3.2.3. Appearance

The cheese must be well moulded and the surface without cloth creases or cracks. In the case of round cheeses the top and bottom surfaces should be smooth, closed, flat and parallel. The side surface of the cheese is evenly rounded without sharp corners. If coated for example with wax, the coating should be clean, uniform, close-adhering and be free of blisters, scales, cracks, mould, and rot spots or soiled areas. Vacuum packed block cheeses should have flat parallel sides, square even edges, and evenly folded, neat, close-fitting wrapper which is free from wrinkles [163-168].


Pectora roborant cultus recti

6.3.2.4. Body and Texture

The body must be waxy, reasonably solid, reasonably spreadable, elastic, easy to slice at ±15 °C, not too soft and pasty, not too hard and not too crumbly or "short".

The texture must be close-knit with the exception for a few typical round Gouda "eyes". Undesired gas-openings are not permissible [163-168].

6.3.2.5. Flavour

Clean typical Gouda cheese flavours are; pleasant, nutty, mild becoming piquant, but not sour or too salty. No off-flavours should be detectable [163-168].

Controlled proteolysis is important for the flavour development in cheese, on the contrary uncontrolled proteolysis can cause detrimental effects in dairy products, such as the gelation of ultra high temperature (UHT) milk, the manufacturing of poor quality cheeses, poor ripening of cheese, decline cheese yield, degradation in stored casein products (decrease in viscosity) and decreased heat and ethanol stability of raw and processed milks, to name but a few.

6.3.3. Experimental Protocol

The object of the study was to evaluate the effect of added t-PA on Gouda cheese over shelf-life. Purified t-PA, obtained after Zinc chelating chromatography as described in Chapter 5, was added to milk (20 $U.l^{-1}$ milk) and incubated at 4°C for 24 hours prior to the manufacturing of the Gouda cheese in the presence of fibrin (concentration of 8 $\mu g.ml^{-1}$ milk). The cheese was then evaluated over a 6-month period for changes in flavour, appearance, enzymatic activity, and casein breakdown.

The freeze-dried t-PA enzyme was resuspended in 100 mM Tris-HCl buffer (pH 8.0) and added to the raw milk; this was done at Elsenburg Agricultural College, Stellenbosch, using morning and evening milk obtained of the mixed Jersey and Friesland herd. After addition of t-PA, the milk was incubated at 4°C for 24 hours prior to cheese manufacture. Gouda cheese was then manufactured on small scale (20 litres of milk was used) at Elsenburg Dairy Laboratory according to the flow-diagram in Fig. 6.7. The production sheet of the Gouda manufactured (with added t-PA) is indicated in Fig. 6.8. A control batch was also produced using the same milk and process on the same day as indicated in the production sheet in Fig. 6.9.

After 60, 90, 120, 150 and 150 days the cheese was evaluated for flavour, appearance and colour, PA activity and proteolysis. Absorption at 280 nm of whey after the first drain was measured in order to detect the aromatic amino acid side chains of short peptides of hydrolysed casein molecules [106]. An

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increase in absorbance would indicate the presence of the liberated breakdown products (proteose peptones) of casein in the serum. The whey was analysed for whey protein nitrogen (WPN), non-protein nitrogen (NPN) and total protein (TP) using the standard International Dairy Federation procedure (IDF, 1964) by Kjeldahl analysis. All nitrogen results were expressed as protein equivalent using a conversion factor of 6.38 and were analysed in triplicate. Mojonnier analyses (ether extraction method) were done in order to determine the % fat in the whey.

Sensory evaluations were performed by trained panellists in accordance with the guidelines of the American Dairy Science Association (ADSA) as described by Bodyfelt *et al.* [165]. Protein analyses of the samples were done according to standard IDF procedure (IDF, 1964) by Kjeldahl analysis of the total protein (TP), non protein nitrogen (NPN) and noncasein nitrogen (NCN). Casein nitrogen (CN) was calculated as the difference between NCN and NPN. All nitrogen results were expressed as protein equivalent using a conversion factor of 6.38 and were the result of triplicate analyses. Cheese for PA activity were grated and 10 g samples were mixed with 90 ml of 0.1 M trisodium citrate, containing 13 $mmol.l^{-1}$ ε-amino caproic acid, and dissolved by stirring for 1 hour 30 minutes at ambient temperature. The samples were then centrifuged at 25,000 X g for 20 minutes after which the fat was removed and the remaining supernatant (milk serum) used for PA analysis. The PA activity was determined spectrophotometrically as previously described [8].

The water soluble N (WSN) extracts of the cheeses, after pressing (day 1) and at 30, 60, 90, 120, 150 and 180 days of ripening, were prepared by the method of Kuchroo and Fox [169]. The N content of the extracts was determined by the Kjeldahl method and WSN was expressed as a percentage of TN. Total free amino acid levels were determined by a modified Cd-ninhydrin reactive (CdN) method. The CdN method is a photometric method, specific for free amino acids and spectrophotometrically measured at an absorbance of 507 nm. Cd-ninhyrdin reacts with alpha-amino acids, and changes from a yellow to a deep purple

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colour in solution. Results are expressed as free amino acids in millimoles of Leucine [170].

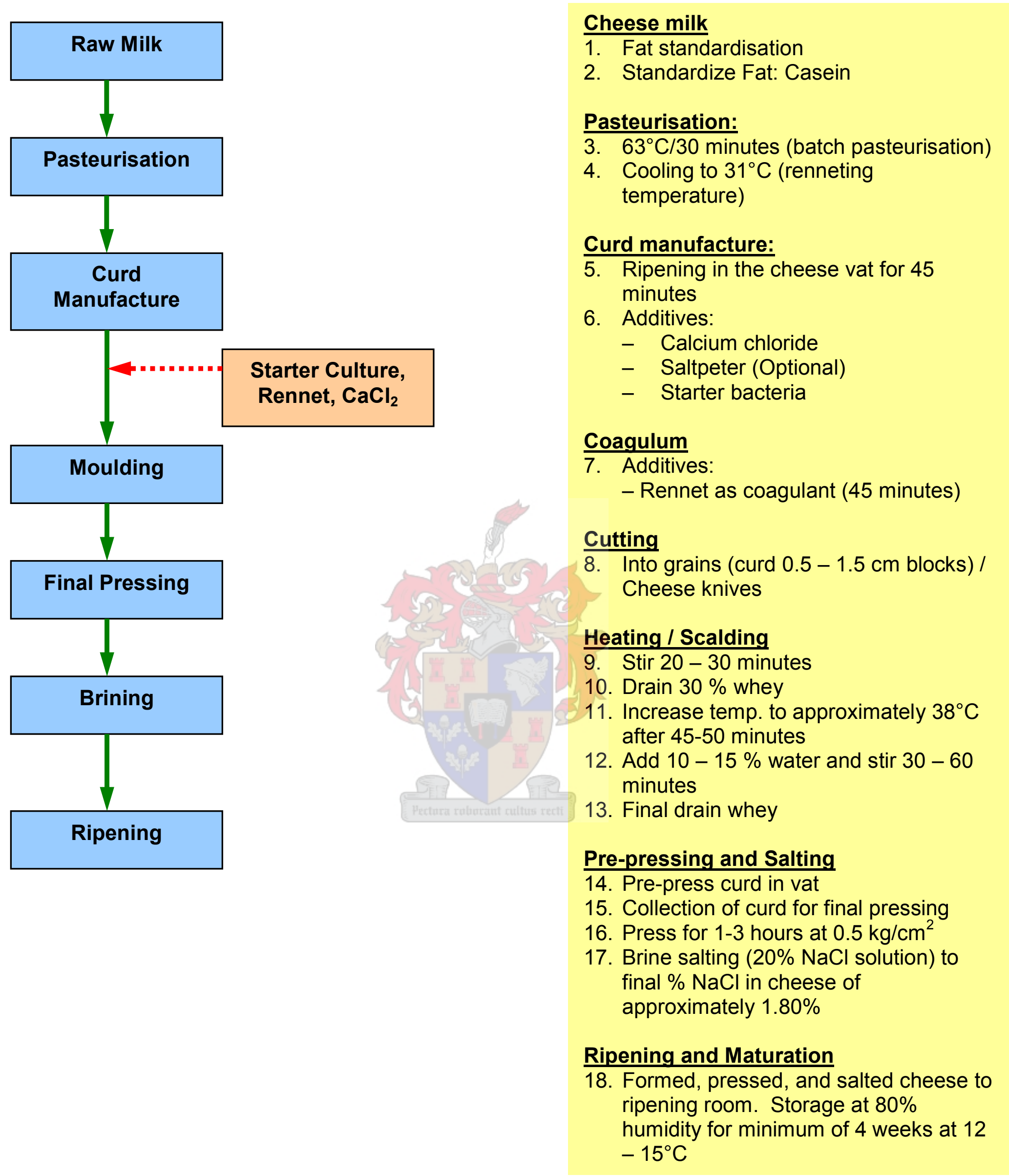

Figure 6.7: Process-flow diagram of Gouda manufacture.

GOUDA CHEESE MANUFACTURING RECORD

DATE: 15/12/2003
BATCH: PA1

MILK ANALYSIS	DATE	TIME	TEMP. (°C)	% TA	pH
	15/12/2003	12H00	4°C	0.15%	6.71
	% FAT	% PROT	% CASEIN	% LACTOSE	CASEIN:FAT
	3.70	3.51	2.79	4.55	0.75 : 1
	DATE	TIME	TEMP. (°C)	% TA	pH
	16/12/2003	12H00	4°C	0.17%	6.67
	% FAT	% PROT	% CASEIN	% LACTOSE	CASEIN:FAT
	3.72	3.49	2.68	4.53	0.72 : 1
MANUFACTURE	**DATE**	**TIME**	**TEMP. (°C)**	**% TA**	**pH**
HOMOGENISE	No				
PASTEURISE	16/12/2003	12H30	63°C / 30 min	0.17	6.66
STARTER ADDED	16/12/2003	13H10	31°C	0.17	6.66
TYPE	MAO (homofermentative)				
QUANTITY	2 units per 20 l milk				
$CaCl_2$ ADDED	16/12/2003	13H10	31°C	0.17	6.66
TYPE					
QUANTITY (ml)	4 ml				
ANNATO ADDED	16/12/2003	13H10	31°C	0.17	6.66
TYPE					
QUANTITY (ml)	1 ml				
RENNETING	16/12/2003	13H55	31°C	0.12	6.68
TYPE	**Chymax (microbial) Liquid**				
QUANTITY (ml)	1.2 ml				
CUTTING OF CURD	16/12/2003	14H50	31°C	0.13	6.68
FIRST DRAINING	16/12/2003	15H35	31°C	0.13	6.65
WATER ADDED	16/12/2003	15H55	31°C	0.10	6.57
FINAL DRAIN	16/12/2003	17H30	37.5°C	0.13	6.42

CALCULATIONS AND ANALYSIS		COMMENTS
L MILK	20.00	
KG MILK	20.60	SCC/ml of milk = 183,000
KG CHEESE	2.05	
% ACTUAL YIELD	9.95%	
% THEORETICAL YIELD	12.06%	
KG CHEESE (4 WEEKS)	1.99	
% FAT	31.20%	
% FAT IN DRY MATTER	57.25%	
% MOISTURE	45.50%	
% TOTAL SOLIDS	54.50%	
% SALT	1.63%	
% SALT IN MOISTURE	3.58%	

Figure 6.8: Production sheet of Gouda with added t-PA and fibrin.

GOUDA CHEESE MANUFACTURING RECORD

DATE: 15/12/2003
BATCH: CONTROL

MILK ANALYSIS	DATE	TIME	TEMP. (°C)	% TA	pH
	15/12/2003	12H00	4°C	0.15%	6.71
	% FAT	% PROT	% CASEIN	% LACTOSE	CASEIN:FAT
	3.70	3.51	2.79	4.55	0.75 : 1
	DATE	TIME	TEMP. (°C)	% TA	pH
	16/12/2003	12H00	4°C	0.16%	6.67
	% FAT	% PROT	% CASEIN	% LACTOSE	CASEIN:FAT
	3.72	3.5	2.80	4.53	0.75 : 1
MANUFACTURE	**DATE**	**TIME**	**TEMP. (°C)**	**% TA**	**pH**
HOMOGENISE	No				
PASTEURISE	16/12/2003	12H35	63°C / 30 min	0.16	6.67
STARTER ADDED	16/12/2003	13H15	31°C	0.16	6.66
TYPE	MAO				
QUANTITY	2 units per 20 l milk				
$CaCl_2$ ADDED	16/12/2003	13H15	31°C	0.16	6.67
TYPE					
QUANTITY (ml)	4 ml				
ANNATO ADDED	16/12/2003	13H15	31°C	0.16	6.67
TYPE					
QUANTITY (ml)	1 ml				
RENNETING	16/12/2003	14H00	31°C	0.125	6.68
TYPE	**Chymax (microbial) Liquid**				
QUANTITY (ml)	1.2 ml				
CUTTING OF CURD	16/12/2003	14H30	31°C	0.13	6.68
FIRST DRAINING	16/12/2003	15H05	31°C	0.13	6.65
WATER ADDED	16/12/2003	15H20	31°C	0.09	6.55
FINAL DRAIN	16/12/2003	16H00	37°C	0.135	6.4

CALCULATIONS AND ANALYSIS		COMMENTS
L MILK	19.98	
KG MILK	20.58	SCC/ml of milk = 183,000
KG CHEESE	2.23	
% ACTUAL YIELD	10.84%	
% THEORETICAL YIELD	11.41%	
KG CHEESE (4 WEEKS)	2.17	
% FAT	32.50%	
% FAT IN DRY MATTER	54.83%	
% MOISTURE	40.73%	
% TOTAL SOLIDS	59.27%	
% SALT	1.50%	
% SALT IN MOISTURE	3.68%	

Figure 6.9: Production sheet of control Gouda.

6.3.4. Results

The results obtained indicated a definite difference between the processing parameters and final product quality of the Gouda cheese processed with the control milk (control Gouda) and that of the Gouda cheese processed with the t-PA inoculated milk (t-PA Gouda).

The milk was analysed prior to cheese manufacture after 24 hours of incubation for TPC, Coliforms, Psychrotrophs, Spores and SCC. The milk was of good bacterial quality and average SCC (APC 13,500 $cfu.ml^{-1}$, Coliforms 4 $cfu.ml^{-1}$, Psychrotrophs 1,250 $cfu.ml^{-1}$, Spores 2 $cfu.ml^{-1}$ and SCC 183,000 $SCC.ml^{-1}$)

After 24 hours of incubation, plasmin activity was already detected in the milk with added t-PA; plasmin activity was defined as the difference between the native plasmin activity in the control milk vs. the plasmin activity in the t-PA added milk. Activation of plasmin, achieved by the addition of t-PA and fibrin, resulted in the breakdown of casein (0.11%) and an increase in the proteose peptone fraction (0.10%) of the milk. No effect on the TN, TPN and NPN components were noted as indicated in Table 6.8 and Figs. 6.10 to 6.12. Native plasmin and plasminogen were present in the milk, and as reported earlier in normal milk the plasminogen concentration can be up to 9 times that of plasmin. Therefore the addition of t-PA did activate the conversion of plasminogen (as the "proteolytic reservoir") to plasmin, increasing the potential for proteolysis not only after 24 hours of incubation, but also during further ripening.

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Table 6.8: Protein analysis of Gouda milk after milking and after 24 hour incubation at 4°C

KJELDAHL PROTEIN ANALYSIS MILK (GOUDA CHEESE)								
HOURS	**TN**	**TPN**	**CN**	**CN/TPN**	**NCN**	**WPN**	**NPN**	**PP**
CONTROL								
0	3.670	3.510	2.790	79.49%	0.880	0.700	0.160	0.020
24	3.667	3.502	2.810	80.24%	0.857	0.670	0.165	0.022
t-PA GOUDA								
0	3.686	3.511	2.790	79.46%	0.896	0.700	0.175	0.021
24	3.653	3.490	2.680	76.79%	0.973	0.690	0.163	0.120

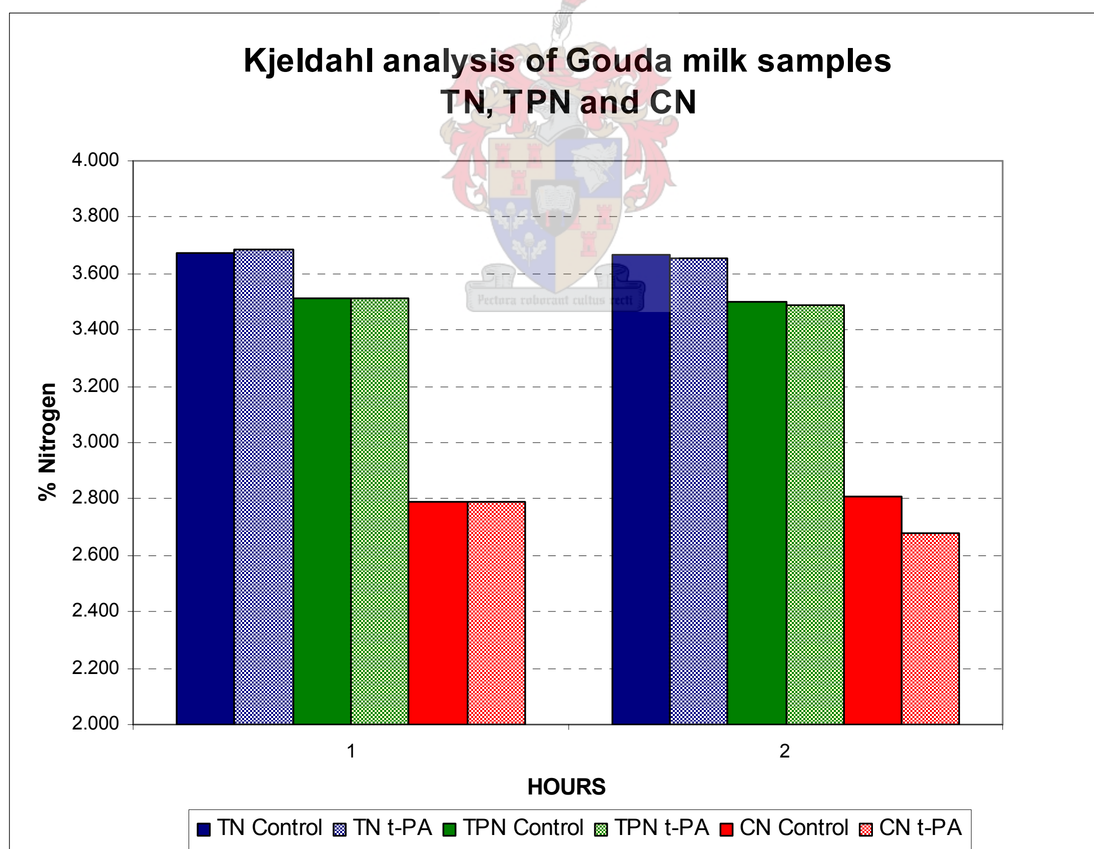

Figure 6.10: Total nitrogen (TN), total protein nitrogen (TPN) and casein nitrogen (CN) of Gouda milk, after milking and 24 hour incubation.

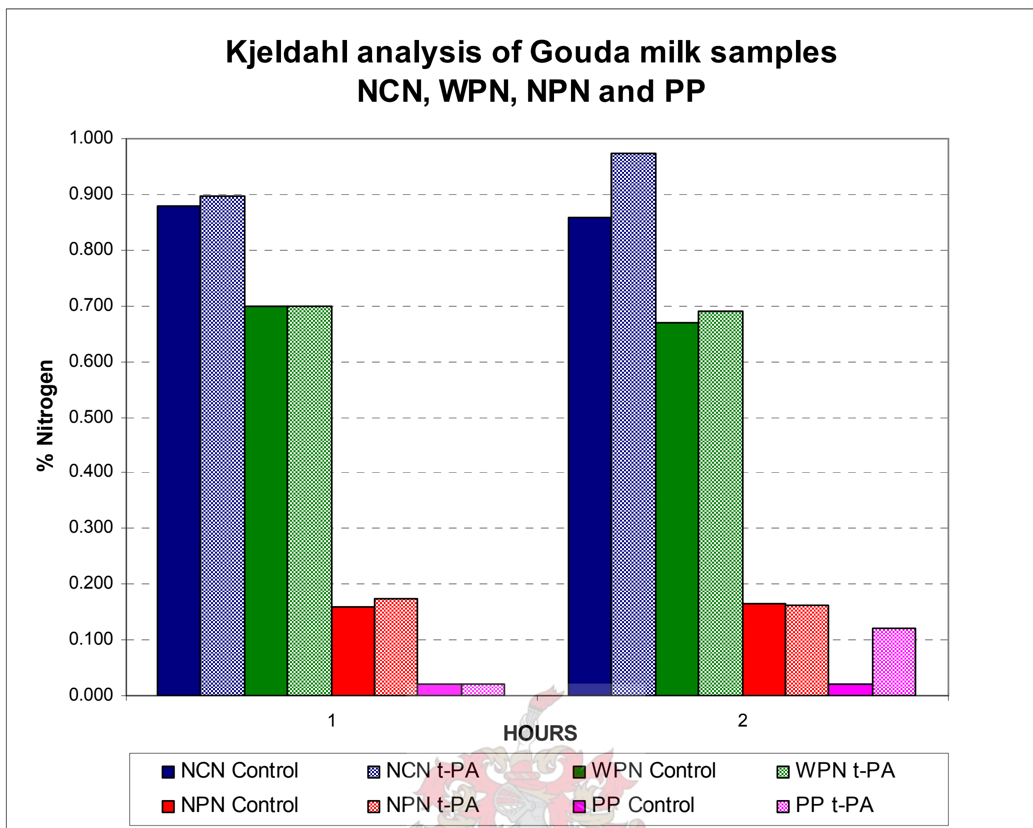

Figure 6.11: Non-casein nitrogen (NCN), whey protein nitrogen (WPN), non-protein nitrogen (NPN) and proteose peptones (PP) of Gouda milk, after milking and 24 hour incubation.

The PA activity in the t-PA Gouda remained constant throughout shelf-life and was significantly higher than that of the control Gouda. No retardation in activity was noted after 180 days which emphasises that the enzymatic activity of plasmin remains, and PA will therefore affect extended shelf-life products, such as UHT milk and matured cheese. Accelerated plasmin activity was noted, as there was an increase in the absorbance of 0.043 for the control Gouda compared to the 0.120 increase in absorbance of the t-PA Gouda (as indicated in Table 6.9 and Fig. 6.13 and measured by the PA assay). It is expected that there should be a decrease in plasmin activity after a certain period of time, however, this was not indicated in this study and has not been reported either. Future analysis should investigate the "saturation" point for plasmin and PA activity in ripened cheese.

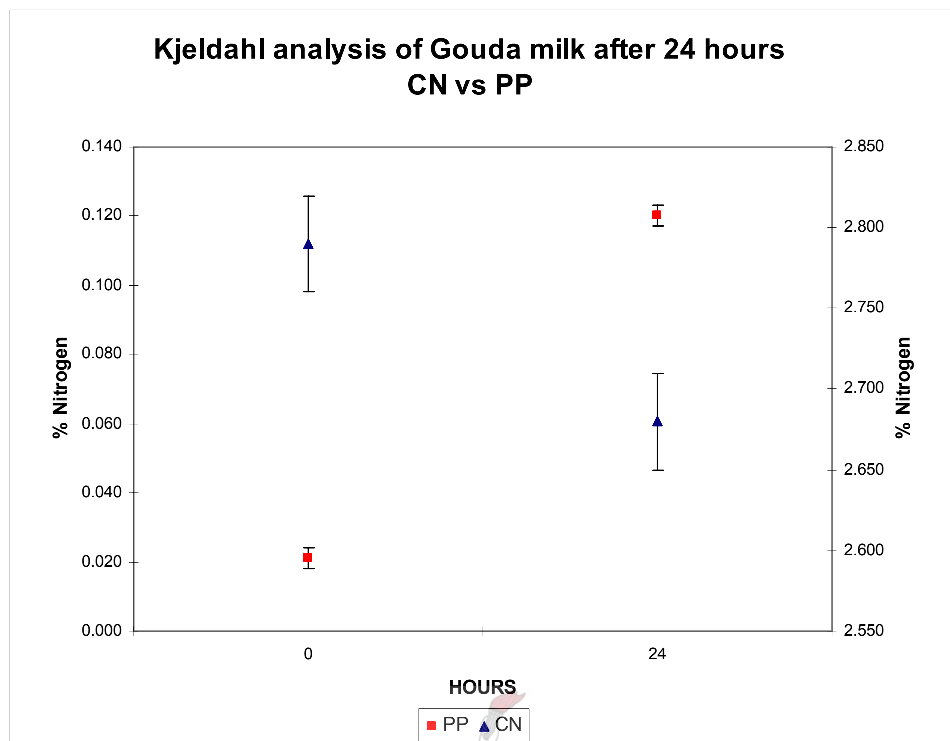

Figure 6.12: Casein nitrogen (CN) vs. proteose peptones (PP) of Gouda milk, after milking and 24 hour incubation with t-PA.

Table 6.9: PA activity assay of serum isolated from Gouda cheese 0 to 180 days post production

ABSORBANCE GOUDA SERUM Δ A_{405nm} 3 h, 4 h @ 37°C[1]						
Day	**Control 1**	**Control 2**	**Control AVE**	**t-PA 1**	**t-PA 2**	**t-PA AVE**
0	0.123	0.120	**0.122**	0.610	0.630	**0.620**
30	0.132	0.111	**0.122**	0.623	0.634	**0.629**
60	0.144	0.139	**0.142**	0.611	0.607	**0.609**
90	0.154	0.180	**0.167**	0.701	0.698	**0.700**
120	0.150	0.170	**0.160**	0.713	0.722	**0.718**
150	0.190	0.123	**0.157**	0.732	0.742	**0.737**
180	0.165	0.165	**0.165**	0.730	0.749	**0.740**

[1] *Fractions were diluted 1:2 before activity assay, therefore a 3-fold dilution*

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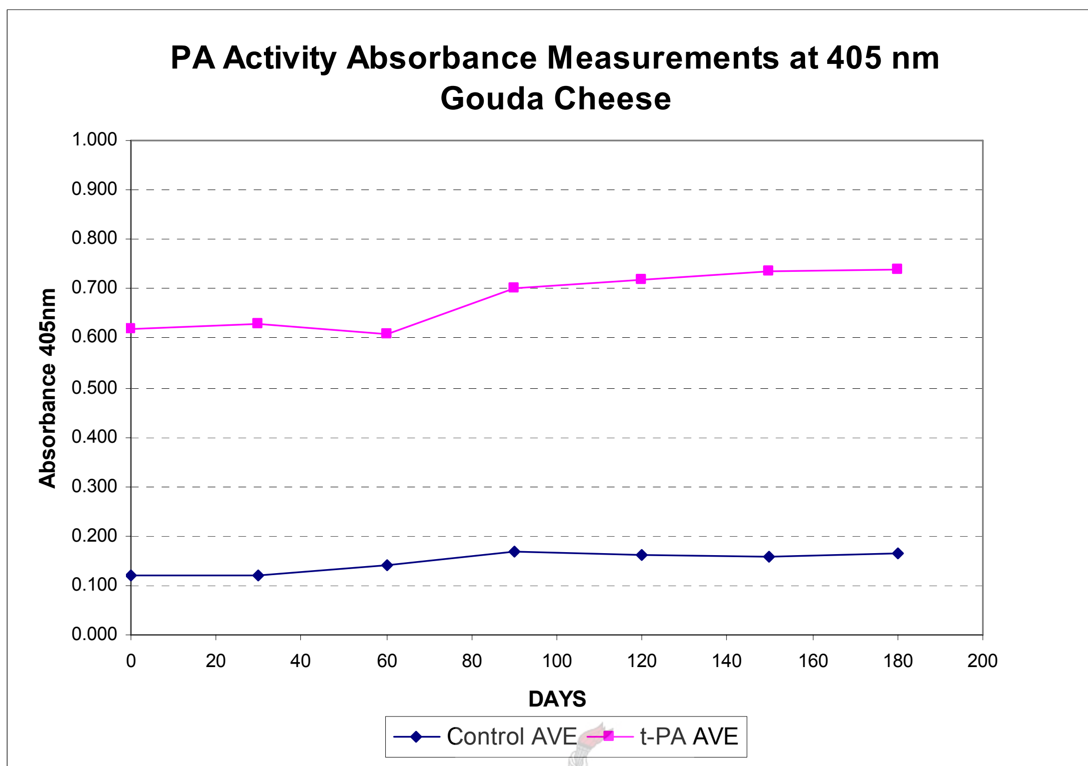

Figure 6.13: PA activity assay of serum isolated from Gouda cheese (t-PA Gouda vs. control Gouda) 0 to 180 days post production.

The chemical analyses results of the Gouda cheeses, after 4 weeks storage, are summarised in Table 6.10. The final product yield of the t-PA Gouda was less than the control Gouda and the theoretical yield, calculated according to the formula of Van Slyke and Price [171-172] (Table 6.11).

During the manufacturing of the Gouda cheese (with and without added t-PA), the t-PA Gouda had a weaker gel formation and a much softer curd (higher moisture content) at final drain. The renneting time of the t-PA milk was almost 55 minutes as opposed to that of the control batch of 30 minutes, indicating an increase of 83% in rennet coagulation time. The curd took longer to work dry in the vat, as normal production time after cutting for Gouda cheese is typically 1 to 1 ½ hours. From cutting the curd to final drain of the control cheese it took approximately 1½ hours in comparison to the t-PA added milk which took 2½ hours, as indicated in the production sheets. The results correlate well with the

whey analysis during production and the final moisture analysis of the cheese after week 4. The moisture of the t-PA Gouda was 10.48% higher than the control Gouda at 45.50% after week 4, as opposed to that of the control cheese with 40.73% moisture.

Table 6.10: Chemical analysis of Gouda manufactured after 4 weeks

CHEMICAL ANALYSIS GOUDA AFTER 4 WEEKS OF RIPENING		
	t-PA	**CONTROL**
KG CHEESE (4 WEEKS)	1.99	2.17
% FAT	31.20%	32.50%
% FAT IN DRY MATTER	57.25%	54.83%
% MOISTURE	45.50%	40.73%
% TOTAL SOLIDS	54.50%	59.27%
% SALT	1.63%	1.50%
% SALT IN MOISTURE	3.58%	3.68%

Table 6.11: Theoretical vs. Actual Gouda cheese yields

GOUDA CHEESE YIELDS					
	CALCULATED	**WEEK 1**	**% Calc - Actual**	**WEEK 4**	**% Calc - Actual**
Control	11.41%	10.84%	0.57%	10.54%	0.87%
TPA	12.06%	9.95%	2.11%	9.68%	2.39%

The results of the whey analysed after first drain also correlates well with the observations during the Gouda manufacturing as indicated in Table 6.12. Kjeldahl analysis indicated that the TN in the whey of the t-PA Gouda (TN 0.973%) was approximately 13.50% higher than the control whey (TN 0.857%). The calculated PP fraction after Kjeldahl analysis was almost 3-fold higher than that of the control, and this correlated with the results of the spectrophotometric analysis at 280 nm. The samples of the whey analysed also showed a significant increase in PA activity of the t-PA whey compared to that of the control whey. The fat loss in the t-PA Gouda whey was 85% higher than the fat loss in the control whey, which positively correlates with the reduction in cheese yield. Normally fat losses in whey can be attributed to poor handling of the curd during

manufacture, such as too much stirring, excessive pumping of the milk, excessive heating of the milk during pasteurisation, too high an initial fat percentage in the milk, or commencing stirring too quickly after cutting (therefore not allowing the curd to cure).

Table 6.12: Whey analysis during Gouda cheese manufacture

WHEY ANALYSIS FIRST DRAIN							
	% FAT[1]	% TN[2]	% WP[2]	% NPN[2]	PP[3]	Δ A280nm[4]	PA ACTIVITY[5]
Control	0.34%	0.86%	0.62%	0.17%	0.07%	0.099	0.232
TPA	0.63%	0.97%	0.60%	0.17%	0.21%	0.253	0.748

[1] Mojonnier analysis
[2] Kjeldahl analysis
[3] Calculated value
[4] Absorbance at 280 nm
[5] Absorbance at 405 nm

The protein analysis of the Gouda during maturation as indicated in Table 6.13 gave interesting results. Both cheeses had constant TN values throughout shelf-life, as expected; however, there was a definite increase in WSN fractions as measured by the Kjeldahl method. The increase in WSN and total free amino acids formed in the control cheese can be attributed to a combination of factors such as proteolysis by plasmin, residual rennet activity and normal proteases produced by the homo-fermentative MAO starter culture used. This is necessary for normal flavour development in cheese, especially matured cheese where controlled proteolysis gives a certain character to the cheese. The WSN, expressed as a percentage of TN, was significantly higher in the t-PA Gouda as opposed to that of the control Gouda. There was a positive correlation in both batches of cheese produced between WSN, expressed as a percentage of TN, and the total free amino acids formed (mM Leucine.l^{-1}). The t-PA Gouda exhibited a 3-fold increase in % WSN: TN and free amino acids formed as supposed to in the control Gouda, which proves accelerated proteolysis due to enhanced plasmin activity, as residual rennet activity and starter culture proteolysis should be seen as a constant in both sets of Gouda. The results obtained contradict previous reports by Farkye and Fox [108], who noted that a 6-fold increase in plasmin activity in Cheddar cheese did not significantly

increase the % WSN:TN compared to the control cheese without accelerated plasmin activity. However, the results of this study do agree with their findings that an increase in % WSN:TN occurs during normal maturation, with higher values obtained after 3 and 6 months. According to Farkye and Fox [108] the plasmin alters the peptide profile of the WSN fraction in the cheese, which contributes to further breakdown of peptides in the WSN as a result of plasmin activity. The results, summarised in Fig. 6.14, indicate a linear relationship between maturation time, % WSN:TN (R^2 = 0.985 t-PA and R^2 = 0.955 Control) and total free amino acids formed (R^2 0.989 = t-PA and R^2 = 0.957 Control).

Table 6.13: Protein analysis of standard and t-PA augmented Gouda during shelf-life with specific mention of the water soluble nitrogen (WSN) as a percentage of total nitrogen (TN), and the total free amino acids as determined by the modified Cd-ninhydrin (CdN) reactive method

KJELDAHL PROTEIN ANALYSIS (GOUDA CHEESE)				
DAY	**TN**	**WSN[1]**	**WSN:TN**	**CdN AMINO GROUP (mM)**
CONTROL				
0	26.330	0.798	3.03%	0.20
30	26.300	1.022	3.89%	0.20
60	26.320	1.987	7.55%	0.24
90	26.400	2.489	9.43%	0.25
120	26.360	3.833	14.54%	0.27
150	26.330	4.232	16.07%	0.30
180	26.330	4.289	16.29%	0.30
t-PA GOUDA				
0	26.730	0.810	3.03%	0.20
30	26.700	2.030	7.60%	0.30
60	26.720	3.855	14.43%	0.40
90	26.720	4.234	15.85%	0.45
120	26.710	5.550	20.78%	0.60
150	26.730	7.470	27.95%	0.69
180	26.740	8.790	32.87%	0.83

[1] *As percentage of TN*

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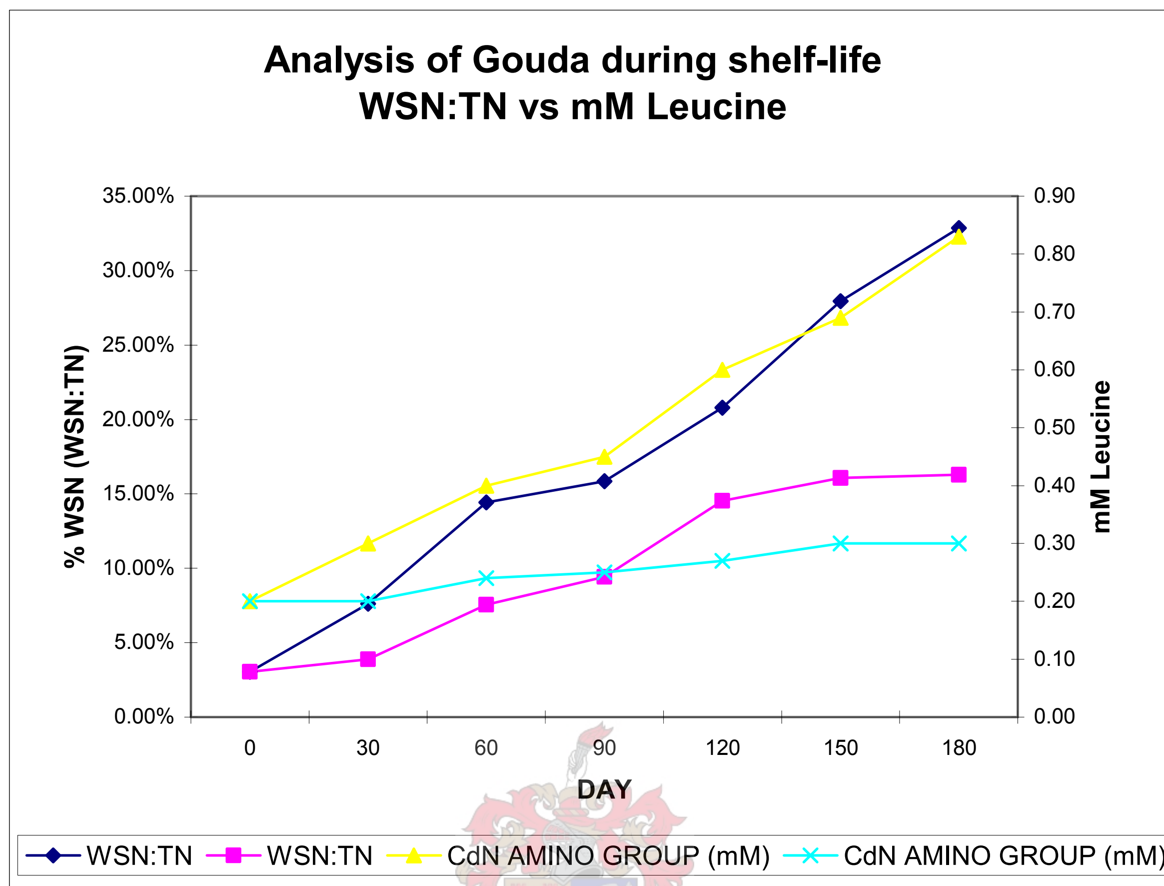

Figure 6.14: Analyses of water soluble nitrogen (WSN), total nitrogen (TN) and mM Leucine in Gouda cheese from day of production to 180 days.

The results of the sensory analysis correlated well with the chemical analysis and estimation of the degree of proteolysis that occurred during maturation. However, the cheese was only evaluated after day 30 and day 150 by 5 individual trained panelists. The control cheese was superior in terms of appearance, body and texture and final flavour delivery. Overall, t-PA Gouda scored lower for these attributes, and this could be attributed to the higher final moisture in the cheese and the enhanced "uncontrolled" proteolysis induced by the addition of t-PA to the cheese milk. The t-PA cheese appeared slightly more bleached, with a shorter body and brittle texture. The control batch had a homogenous even colour, with no specs or discolouration and exhibited good elasticity. The t-PA cheese had a bitter flavour that increased in severity during

shelf-life. The most common descriptors used by the panelists could be summarised as follows:

- Control Gouda (day 30): Neutral colour, creamy, nutty taste, good mild flavour
- t-PA Gouda (day 30): White spots, bleached appearance, pasty, slight bitterness, high acid, astringent
- Control Gouda (day 150): Neutral colour, creamy, nutty taste, good mild flavour
- t-PA Gouda (day 150): White spots, bleached appearance, pasty, severe bitterness, high acid, astringent

The results of the tasting panel are summarised in Table 6.14. Scores were allocated as follow: Maximum 2 points for appearance, maximum 7 points for body and texture, maximum 11 points for flavour to give a final total out of 20 points.

Table 6.14: Sensory scores of Gouda cheese after 1 month and 5 months

GOUDA SENSORY ANALYSIS - Average Scores (n=5)				
DAY	**Appearance (2)**	**Body and texture (7)**	**Flavour (11)**	**Score (20)**
t-PA				
30	1.330	4	7.8	13.130
150	1.130	4	7	12.130
CONTROL				
30	1.500	6	9.66	17.160
150	1.500	6.2	10	17.700

6.3.5. Discussion

The results obtained from this study indicated that the extent of the breakdown of α_s- and β-caseins increased with incubation time. Snoeren and van Riel reported that κ-casein was not degraded by plasmin [12]. In this study the Gouda cheese manufactured with added t-PA exhibited a reduced cheese yield of almost 1.0% in comparison with the control Gouda cheese. Hydrolysis of casein negatively affected the rheological properties of rennet-induced milk gel in this experiment, increasing rennet coagulation time (83%), increasing moisture in the final cheese (10.48%) and increasing fat losses in the whey (83%).

From the results of this study it can therefore be deduced that the elevated plasmin activity in milk from cows that are in late lactation or experiencing mastitis, can alter the rennet coagulation properties of milk and ultimately lead to inferior final cheese quality.

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6.4. Fermented Dairy Products - Low fat yoghurt

6.4.1. Introduction

Acidified or fermented milk products are one of the oldest and most popular foodstuffs known to man. The use of fermented milk dates back centuries, although no precise record of the date when it was first made is available. It is likely that the origin of yoghurt was in Asia in the 8th century, where it was first produced by the Turks who lived as nomads. It is estimated that today the world-wide production of fermented milk products exceeds 20 million tons, of which approximately 145,000 tonnes (less than 1%) is produced in South Africa.

The popularity of fermented milks, including yoghurt, is due to the association of these products with numerous proven health benefits, the main benefit being to

gastro-intestinal health. In addition to providing good nutritional benefits, it is also widely used as a suitable vehicle (“carrier’) for fortification and added nutritional, functional ingredients. The basic ingredients of yoghurt are milk and microflora. Milk is converted into yoghurt by cultivating in it specific lactic acid bacteria and souring it under defined conditions.

6.4.2. Yoghurt characteristics

According to the FAO/WHO (1977) the definition of yoghurt is as follow:

“Yoghurt is a coagulated milk product obtained by lactic acid fermentation, through the action of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, from milk and milk products (pasteurized and/or concentrated milk), with or without optional additions (milk powder, skim milk powder, whey powder etc.). The micro-organisms in the final product must be viable and abundant.”

Yoghurt is distinguished by a typical and pleasant aroma attributable to the presence of sufficient quantities of acetaldehydes as the principle aroma compounds. Yoghurt flavour relies on a good balance between the volatile fatty acids and is to some degree enhanced by the presence of diacetyl. The milk acid and refreshing taste of yoghurt is attributable to the presence of lactic acid. The aroma and taste combines to produce a flavour that can be described as walnut-like.

The method of gel formation and acidification is different to the coagulum formation in cheese, which is dependant on enzymatic action (rennet). The production of lactic acid by lactic acid bacteria induces the process of coagulation, initiated at a pH of 5.2 - 5.3 and is completed at a pH of 4.6 - 4.7. The casein micelle is freed from bound salts, of which calcium is the main element, at its iso-electric point. Removed calcium combines with lactic acid to form calcium lactate. The solubilisation of the colloidal calcium phosphate (CCP) alters the charge on individual caseins, and results in an increase in ionic

strength of the solution. The proteins coagulate and represent a coprecipitate of casein and denatured whey proteins. The visible manifestation is the formation of semisolid gel entrapping fat globules and serum with dissolved constituents. The gel is of a white opaque appearance, gentle and smooth with a smooth custard like consistency and viscosity.

6.4.3. Experimental protocol

The objective of the study was to evaluate the effect of added t-PA to milk used for the production of yoghurt (t-PA yoghurt), and to evaluate the effect of the addition on shelf-life over a period of 30 days. Yoghurt manufactured from the same milk with no t-PA addition prior to fermentation served as a control (control yoghurt). Twenty Units of the purified t-PA, obtained after Zinc chelating chromatography as described in Chapter 5, was added per 1000 ml of milk in the presence of fibrin at a concentration of 8 $\mu g.ml^{-1}$ of final product. The yoghurt was evaluated over 30 days for changes in appearance, pH, apparent viscosity, gel formation, enzymatic activity, and casein breakdown.

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The freeze-dried t-PA enzyme was resuspended in 100 mM Tris-HCl buffer (pH 8.0) and added to the raw milk (morning and evenings milk of the mixed Jersey and Friesland herd at Elsenburg Agricultural College, Stellenbosch). The milk with the added t-PA was then incubated in a cold room (4°C) for 24 hours prior to yoghurt manufacture.

Yoghurt was manufactured on small scale (20 litres of milk) at Elsenburg Dairy Laboratory according to the flow-diagram in Fig. 6.15. The yoghurt was manufactured using the basic yoghurt manufacturing process followed in most commercial factories in South Africa. The main difference to the pilot plant samples produced was the time-temperature combination of pasteurisation (85°C with a holding time of 30 minutes as supposed to 95°C with a holding time of 5 minutes). No commercial available stabiliser was added to properly evaluate the effect of the added t-PA to the acid gel. However, the percentage milk solids non

fat was increased by the addition of 2% skim milk powder (low-heat treated) to yield a final calculated protein value of 4.60%. The reason for the addition of skim milk powder as supposed to milk solids such as whey protein concentrate, was to keep the casein: whey protein ration as close as possible as to that found in fresh milk. The milk was analysed prior to manufacturing after 24 hours of incubation for TPC, Coliforms, Psychrotrophs and Spores by direct plating methods and SCC by laser spectroscopy. The culture for fermentation was a typical heterofermentative yoghurt culture from Danisco Cultor which consisted of *Lactobacillus delbreuckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The production sheet of the yoghurt manufactured (with added t-PA) is indicated in Fig. 6.16. A control batch was also produced using the same milk and process on the same day as indicated in the production sheet in Fig. 6.17. The yoghurt was hygienically filled and sealed with aluminium foil in 175 ml cups and stored at 4°C for the duration of shelf-life and evaluation. Preservative, 50% lactose: 40% natamycin was added at a dosage level of 10 ppm to the final product in order to prevent yeast and mould growth.

After 1, 5, 10, 15, 20 and 30 days the yoghurt was evaluated for appearance and colour, pH, gel formation, sediment, viscosity, PA activity and proteolysis.

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Appearance, colour and sediment were visually investigated. Gel formation and apparent viscosity were measured with a Brookfield viscometer at 20°C at 4°C. Triplicate readings were taken directly in centipoises (cpi) when the spindle had been rotating for 30 seconds. Protein analyses of the samples were done according to standard IDF procedure (IDF, 1964) by Kjeldahl analysis of TP, NPN and NCN. CN was calculated as the difference between NCN and NPN. All nitrogen results were expressed as protein equivalent, using a conversion factor of 6.38, and were analysed in triplicate.

The yoghurt samples were analysed for the presence of short peptides and amino acids, indicating casein breakdown and protease activity. Milk serum (whey), isolated from the experimental and control milk, was tested to estimate

casein hydrolysis as previously described [106]. The PA activity was determined by the method of Zachos *et al.* [8], as previously described. If no whey formation was present in a given yoghurt sample, 10 grams of the sample was centrifuged at 25,000 X g for 20 minutes after which the remaining supernatant (milk serum or free whey) used for casein hydrolysis and PA analysis.

Sensory evaluations were performed by trained panellists in accordance with the guidelines of the American Dairy Science Association (ADSA) as described by Bodyfelt *et al.* [165].

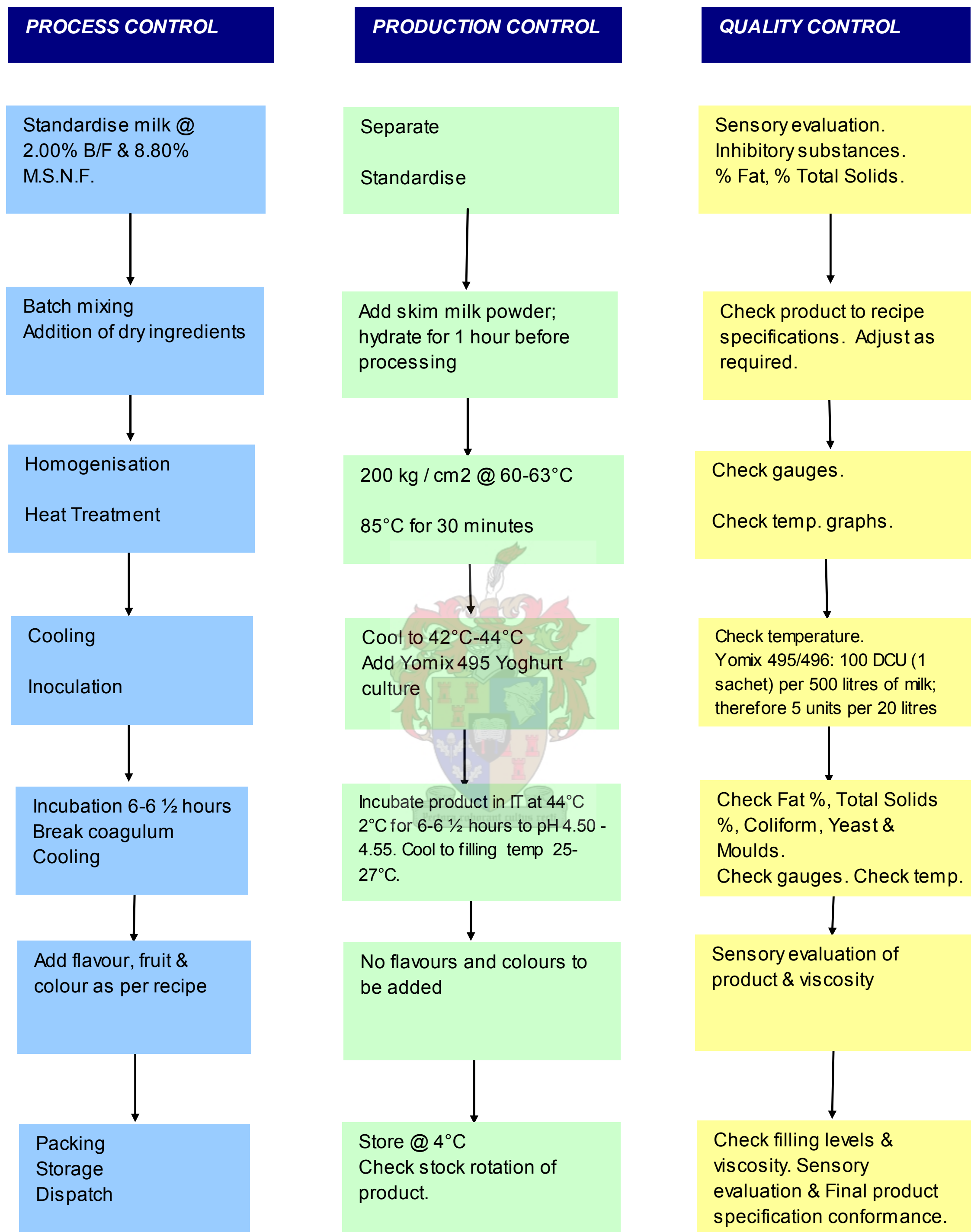

Figure 6.15: Yoghurt production process-flow

YOGHURT PRODUCTION SHEET

LOW FAT YOGHURT PA1

DATE

MILK VOLUME: 19.42 L

BATCH NUMBER

PRODUCT RECIPE				UNITS
	L	KG	%	
STANDARDISED LOW FAT MILK	19.42	20.000	97.790%	
SKIMMILK POWDER		0.450	2.200%	
Culture Yo-Mix 495 (100DCU per 1000 liter)		0.002	0.010%	5 UNITS
Total		**20.45**	**100.000%**	
SPECIFIC GRAVITY (SG)	1.039			
LITERS FROM 1000 KG	962.567			
INGREDIENTS	**QTY**	**BATCH CODE**	**CHECKED BY**	**SIGN**
MILK				
SKIM MILK	**20.000**			
FULL CREAM MILK	**0 L**			
CREAM	**0 L**			
%BUTTERFAT	2.10			
%TOTAL SOLIDS	11.00			
°BRIX	-			
RESAZURIN	PASSED			
pH	6.67			
ALCOHOL TEST 68% PASSED	**YES**			
STABILISER				
SUGAR				
OTHER INGREDIENTS: SKIMMILK POWDER	2.00 KG			
%BUTTERFAT	2.08			
%TOTAL SOLIDS	13.00			
°BRIX	13.00			
TASTE	GOOD			
ODOUR	GOOD			
CULTURE				
Yomix 495	5 UNITS			
PROCESSING	**TEMPERATURE**	**pH**	**TIME STARTED**	**TIME FINISHED**
BATCHING	**5 °C**			
HYDRATION TIME / SWELLING	**1 H 00**			
HOMOGENISE	**63.5 °C**			
PRESSURE	**200 BAR**			
PASTEURIZE	**85°C / 30 MIN**			
COOLING YOGHURT MILK TO INCUBATION TEMP	**42.5 °C**			
INOCULATION				
%BUTTERFAT	**2.08%**			
%TOTAL SOLIDS	**12.98%**			
°BRIX	**13 °B**			
BREAK COAGULUM		**4.55**	**6 H 00 FERMENTATION**	
COOLING				
FILLING	**QTY**	**BATCH CODE**	**TIME STARTED**	**TIME FINISHED**
FRUIT TYPE	0			
YOGHURT BASE	0			
FINAL FACTORY QUALITY CONTROL RESULTS (RELEASING RESULTS)				
	VALUE	**SPEC**	**SIGN**	**COMMENTS**
pH	**4.55**	4.2- 4.5		
VISCOSITY				
%TOTAL SOLIDS (PLAIN BASE)	**13.04**	12 - 14%		

Figure 6.16: Production control sheet of yoghurt with t-PA addition.

YOGHURT PRODUCTION SHEET

LOW FAT YOGHURT CONTROL

DATE	
MILK VOLUME: 19.42 L	BATCH NUMBER

PRODUCT RECIPE				UNITS
	L	KG	%	
STANDARDISED LOW FAT MILK	19.42	20.000	97.790%	
SKIMMILK POWDER		0.450	2.200%	
Culture Yo-Mix 495 (100DCU per 1000 liter)		0.002	0.010%	5 UNITS
Total		**20.45**	**100.000%**	
SPECIFIC GRAVITY (SG)	1.039			
LITERS FROM 1000 KG	962.567			
INGREDIENTS	**QTY**	**BATCH CODE**	**CHECKED BY**	**SIGN**
MILK				
SKIM MILK	**20.000**			
FULL CREAM MILK	**0 L**			
CREAM	**0 L**			
%BUTTERFAT	2.10			
%TOTAL SOLIDS	11.00			
°BRIX	-			
RESAZURIN	PASSED			
pH	6.67			
ALCOHOL TEST 68% PASSED	YES			
STABILISER				
SUGAR				
OTHER INGREDIENTS: SKIMMILK POWDER	2.00 KG			
%BUTTERFAT	2.08			
%TOTAL SOLIDS	13.00			
°BRIX	13.00			
TASTE	GOOD			
ODOUR	GOOD			
CULTURE				
Yomix 495	5 UNITS			
PROCESSING	**TEMPERATURE**	**pH**	**TIME STARTED**	**TIME FINISHED**
BATCHING	**5 °C**			
HYDRATION TIME / SWELLING	**1 H 00**			
HOMOGENISE	**63 °C**			
PRESSURE	**200 BAR**			
PASTEURIZE	**85°C / 30 MIN**			
COOLING YOGHURT MILK TO INCUBATION TEMP	**42.5 °C**			
INOCULATION				
%BUTTERFAT	**2.08%**			
%TOTAL SOLIDS	**12.98%**			
°BRIX	**13 °B**			
BREAK COAGULUM		**4.55**	**5 H 30 FERMENTATION**	
COOLING				
FILLING	**QTY**	**BATCH CODE**	**TIME STARTED**	**TIME FINISHED**
FRUIT TYPE	0			
YOGHURT BASE	0			
FINAL FACTORY QUALITY CONTROL RESULTS (RELEASING RESULTS)				
	VALUE	**SPEC**	**SIGN**	**COMMENTS**
pH	**4.56**	4.2- 4.5		
VISCOSITY				
%TOTAL SOLIDS (PLAIN BASE)	**13**	12 - 14%		

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Figure 6.17: Production control sheet of control yoghurt.

6.4.4. Results

The raw milk used for yoghurt manufacture was of good bacterial quality and average SCC (APC 18,900 cfu.ml^{-1}, Coliforms 2 cfu.ml^{-1}, Psychrotrophs 1,877 cfu.ml^{-1}, Spores 1 cfu.ml^{-1}and SCC 142,000 SCC.ml^{-1}).

The viscosity measured followed the same trend for both the control and t-PA yoghurt samples. There was a definite loss in viscosity after approximately 20 days of shelf-life, although the initial viscosity of the t-PA yoghurt was also lower. The results are shown in Table 6.15 and Fig. 6.18.

Table 6.15: Viscosity measurement of yoghurt over a 30 day storage period
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YOGHURT VISCOSITY (BROOKFIELD, *cpi* at 4 °C)[1]						
Day	**Control 1**	**Control 2**	**Control AVE**	**t-PA 1**	**t-PA 2**	**t-PA AVE**
1	1823	1825	**1824**	1798	1791	**1795**
5	1833	1835	**1834**	1807	1806	**1806**
10	1842	1841	**1842**	1811	1813	**1812**
15	1843	1839	**1841**	1808	1810	**1809**
20	1839	1838	**1839**	1811	1815	**1813**
25	1827	1825	**1826**	1801	1798	**1800**
30	1820	1828	**1824**	1790	1793	**1792**

[1] *Measured at 4°C with spindle 3 at 60 rpm*

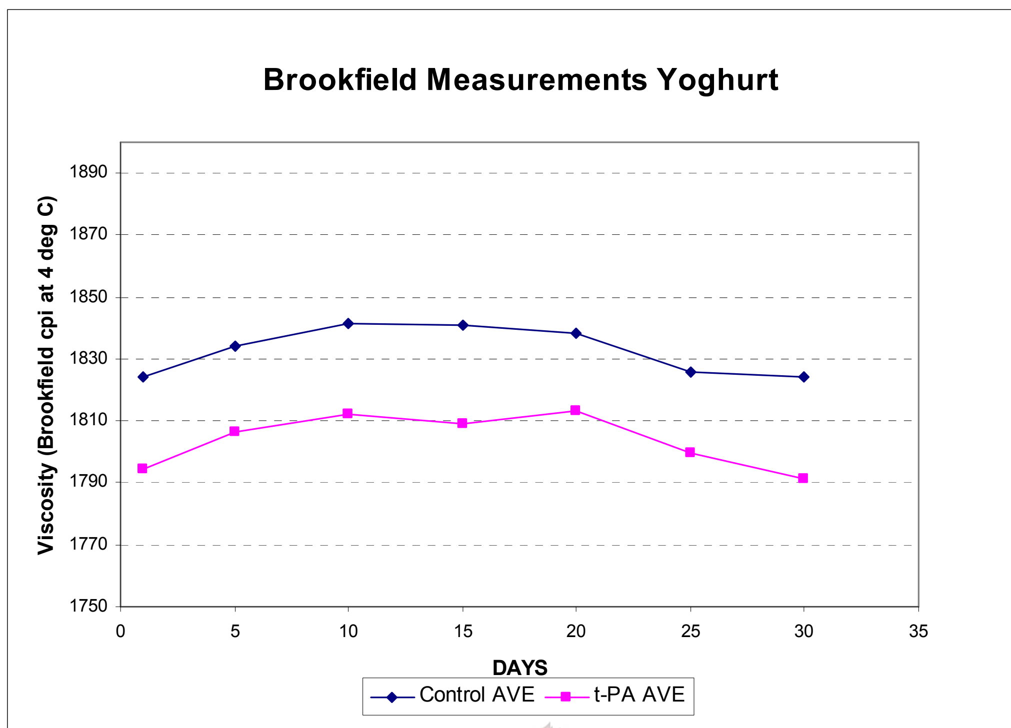

Figure 6.18: Viscosity measurement of yoghurt during a 30 day storage period.

The PA activity of the t-PA yoghurt was 3-fold higher than the PA activity in the control sample, but no significant increase in plasmin activity was measured over the 30 day storage period (Table 6.16 and Fig. 6.19).

Table 6.16: PA activity of serum from day of production to 30 days

PA ACTIVITY ASSAY (YOGHURT)			
Day	**Δ A_{405nm} 3h, 4h @ 37°C**[1]		
	Control	**t-PA sample**	**Dev (±)**
0	0.059	0.198	0.139
5	0.048	0.178	0.130
10	0.063	0.175	0.112
15	0.033	0.161	0.128
20	0.047	0.184	0.137
25	0.075	0.189	0.114
30	0.066	0.200	0.134
Average	**0.056**	**0.184**	**0.128**

[1] *Fractions was diluted 1:2 before activity assay, therefore a 3-fold dilution*

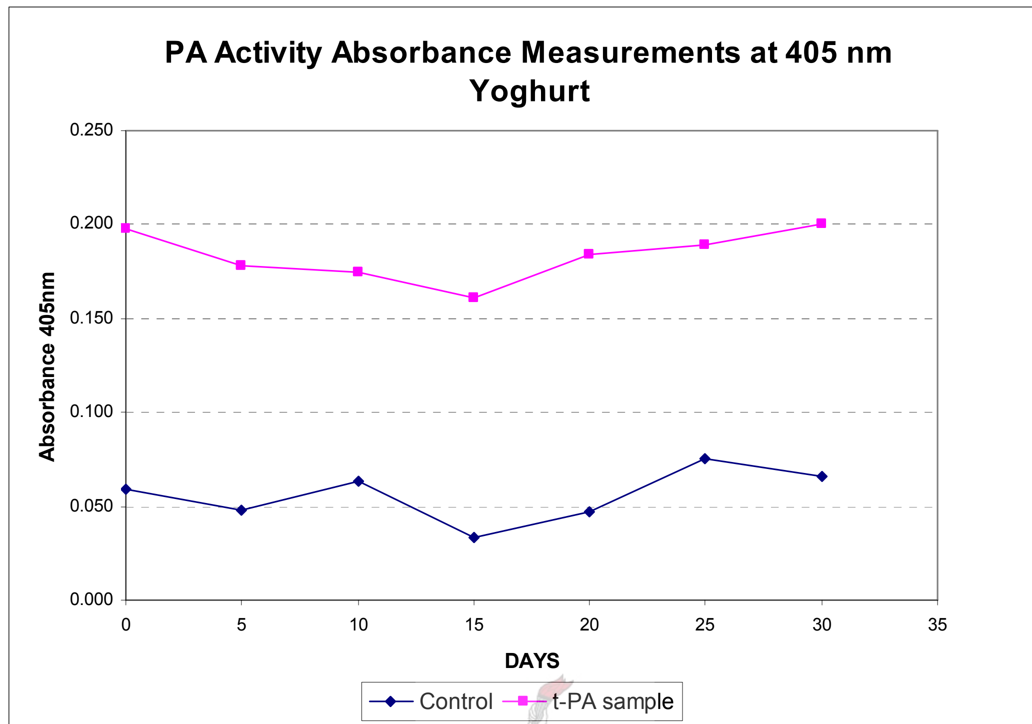

Figure 6.19: PA activity of serum isolated from t-PA and control yoghurt from day of production to 30 days.

Kjeldahl analyses of the yoghurt samples showed no significant change in TN, TPN, NPN and WPN as indicated in Table 6.17 and Figs. 6.20 and 6.21. There was a slight increase in the NCN of the t-PA yoghurt and a decrease in the CN in comparison to the control yoghurt, as a result of the increase of the PP (3-fold) as indicated in Table 6.17 and Figs. 6.22 and 6.23. However, the reduction in CN was within the standard error range for analytical method used (0.03%) and therefore not significant. The main observation was a positive correlation between the increase in the PP fraction and the increase in absorbance at 280 nm as indicated in Table 6.18 and Fig. 6.24. The $A_{280\ nm}$ of the t-PA yoghurt on day 15 was significantly lower than that on day 10 and did not follow the trend of the rest of the analyses throughout the storage period. This can possibly be attributed to an experimental error or incorrect dilution prior to analysis.

The pH measurements (Table 6.19 and Fig. 6.25), of both the control and t-PA yoghurt samples, proved to be within the acceptable limits for yoghurts and no discrepancies were noted in this regard. There was a slight acidification noted throughout shelf-life, but this can be attributed to the normal lactic acid production by the viable cultures added.

Table 6.17: PA activity assay of serum isolated from yoghurt at 405 nm from day of production to 30 days

KJELDAHL PROTEIN ANALYSIS (YOGHURT)								
Day	**TN**	**TPN**	**CN**	**CN/TPN**	**NCN**	**WPN**	**NPN**	**PP**
CONTROL								
1	4.763	4.563	3.640	79.77%	1.123	0.910	0.200	0.013
5	4.754	4.534	3.630	80.06%	1.124	0.890	0.220	0.014
10	4.777	4.567	3.625	79.37%	1.152	0.930	0.210	0.012
15	4.803	4.573	3.640	79.59%	1.163	0.920	0.230	0.013
20	4.765	4.545	3.650	80.32%	1.115	0.880	0.220	0.015
25	4.756	4.556	3.633	79.74%	1.123	0.910	0.200	0.013
30	4.751	4.541	3.599	79.26%	1.152	0.930	0.210	0.012
t-PA								
1	4.763	4.563	3.640	79.77%	1.123	0.910	0.200	0.013
5	4.751	4.531	3.625	80.00%	1.126	0.890	0.220	0.016
10	4.760	4.550	3.604	79.20%	1.156	0.930	0.210	0.016
15	4.766	4.526	3.590	79.32%	1.176	0.920	0.240	0.016
20	4.745	4.525	3.620	80.00%	1.125	0.880	0.220	0.025
25	4.761	4.511	3.570	79.14%	1.191	0.910	0.250	0.031
30	4.772	4.542	3.580	78.82%	1.192	0.930	0.230	0.032

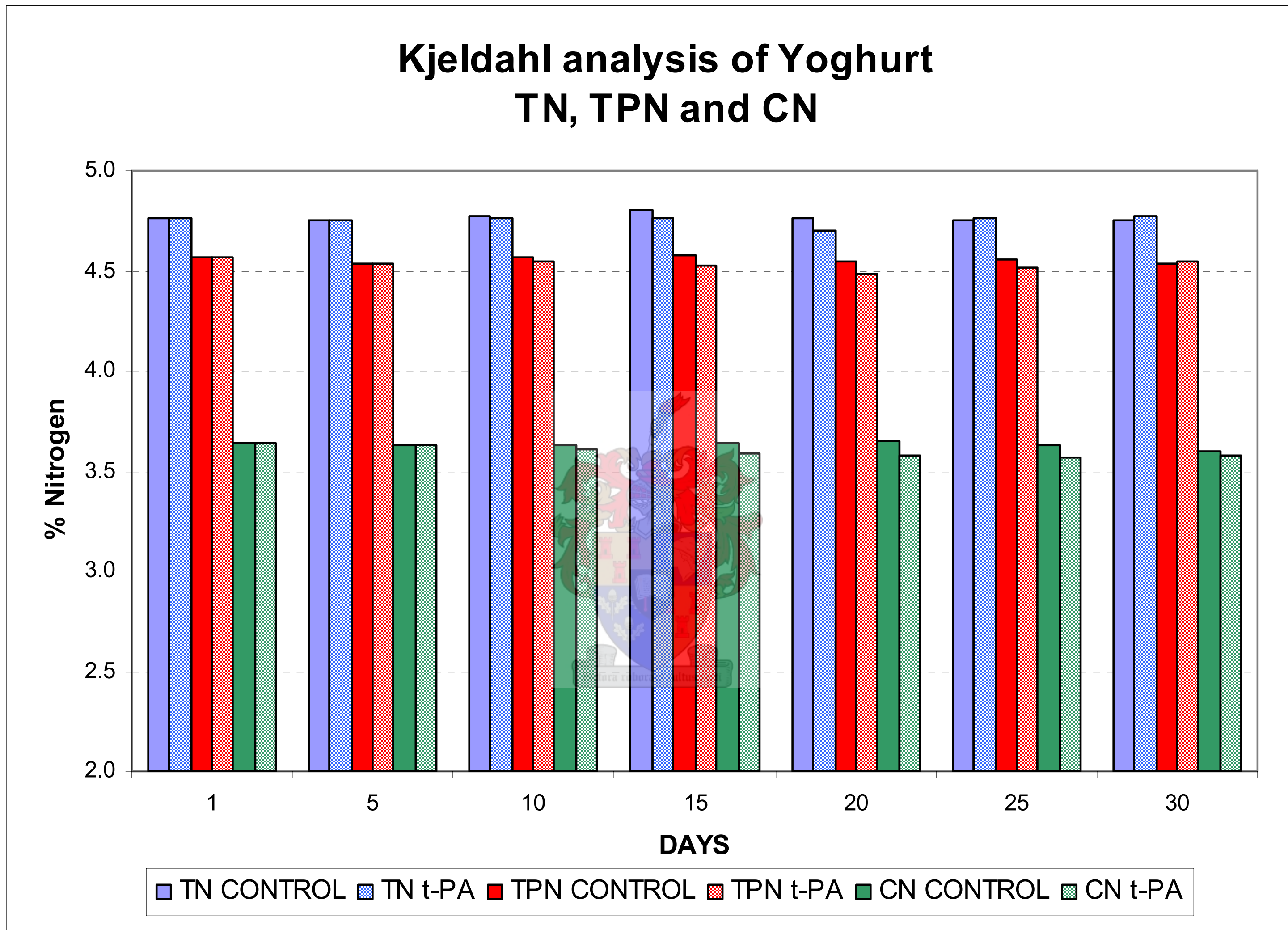

Figure 6.20: Kjeldahl analysis of TN, TPN and CN in yoghurt samples throughout 30 days shelf-life.

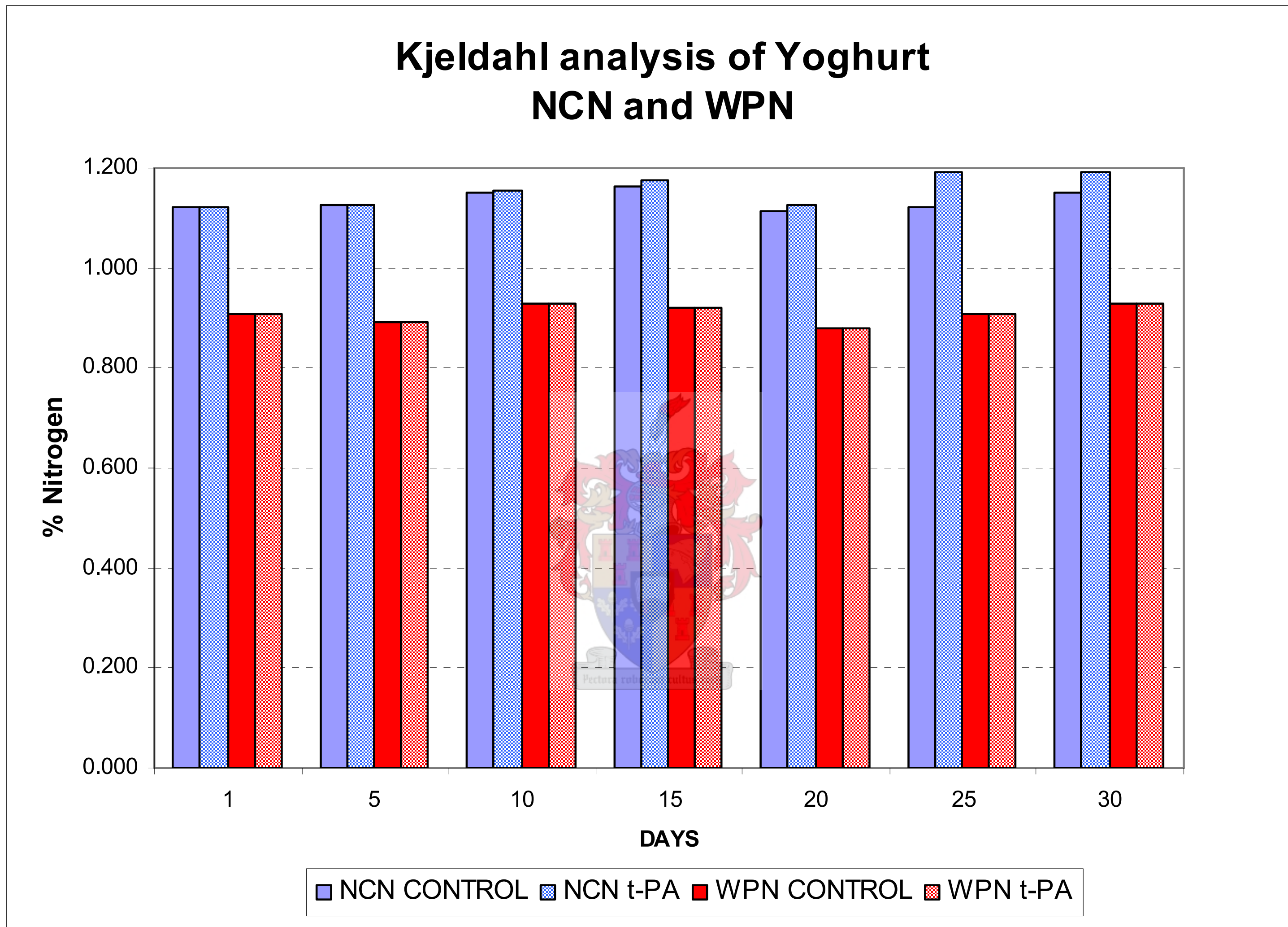

Figure 6.21: Kjeldahl analysis of NCN and WPN in yoghurt samples throughout 30 days shelf-life.

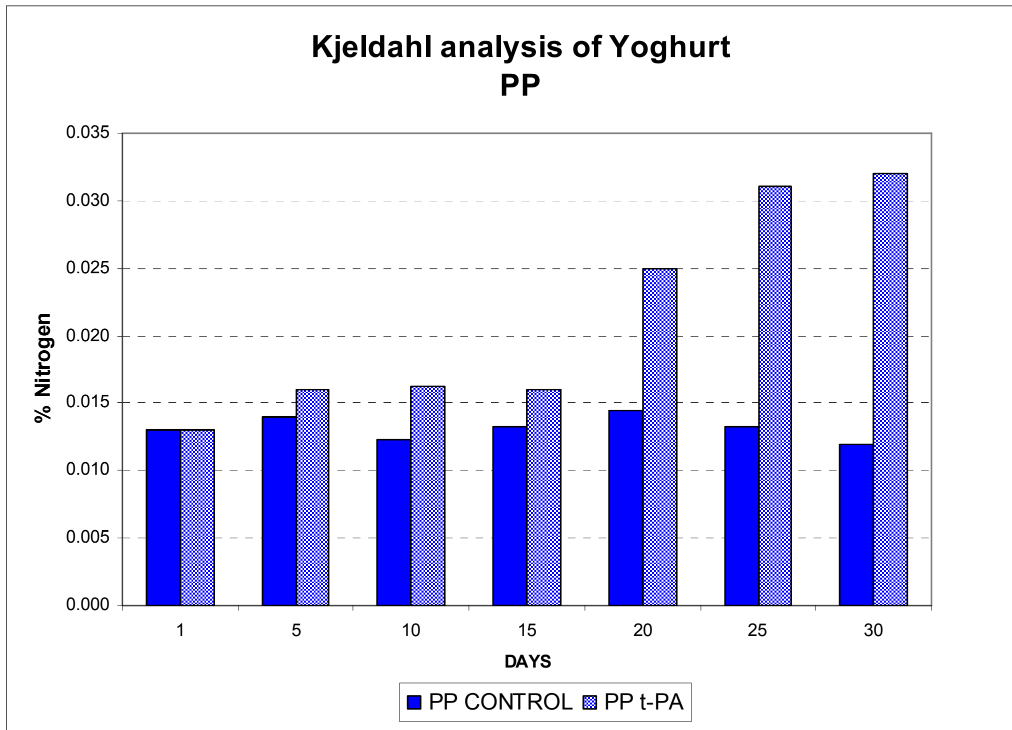

Figure 6.22: Proteose peptone (PP) analysis of the yoghurt samples throughout shelf-life.

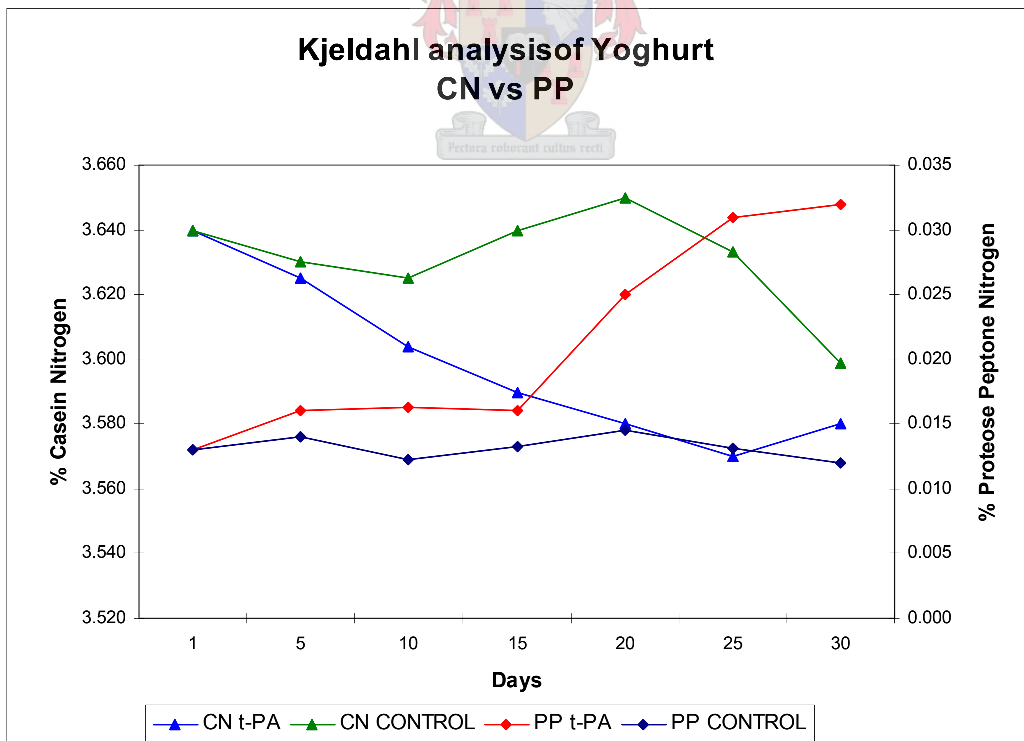

Figure 6.23: Kjeldahl results of casein nitrogen (CN) vs. proteose peptones (PP) of yoghurt samples throughout shelf-life.

Table 6.18: Absorbance values of whey isolated from yoghurt samples throughout shelf-life at $A_{280\ nm}$

ABSORBANCE YOGHURT WHEY 280nm						
Day	**Control 1**	**Control 2**	**Control AVE**	**t-PA 1**	**t-PA 2**	**t-PA AVE**
1	0.070	0.073	**0.072**	0.153	0.160	**0.157**
5	0.068	0.067	**0.067**	0.214	0.203	**0.208**
10	0.055	0.060	**0.058**	0.390	0.303	**0.346**
15	0.068	0.069	**0.068**	0.201	0.200	**0.201**
20	0.088	0.084	**0.086**	0.300	0.394	**0.347**
25	0.115	0.114	**0.115**	0.323	0.330	**0.327**
30	0.117	0.117	**0.117**	0.336	0.330	**0.333**

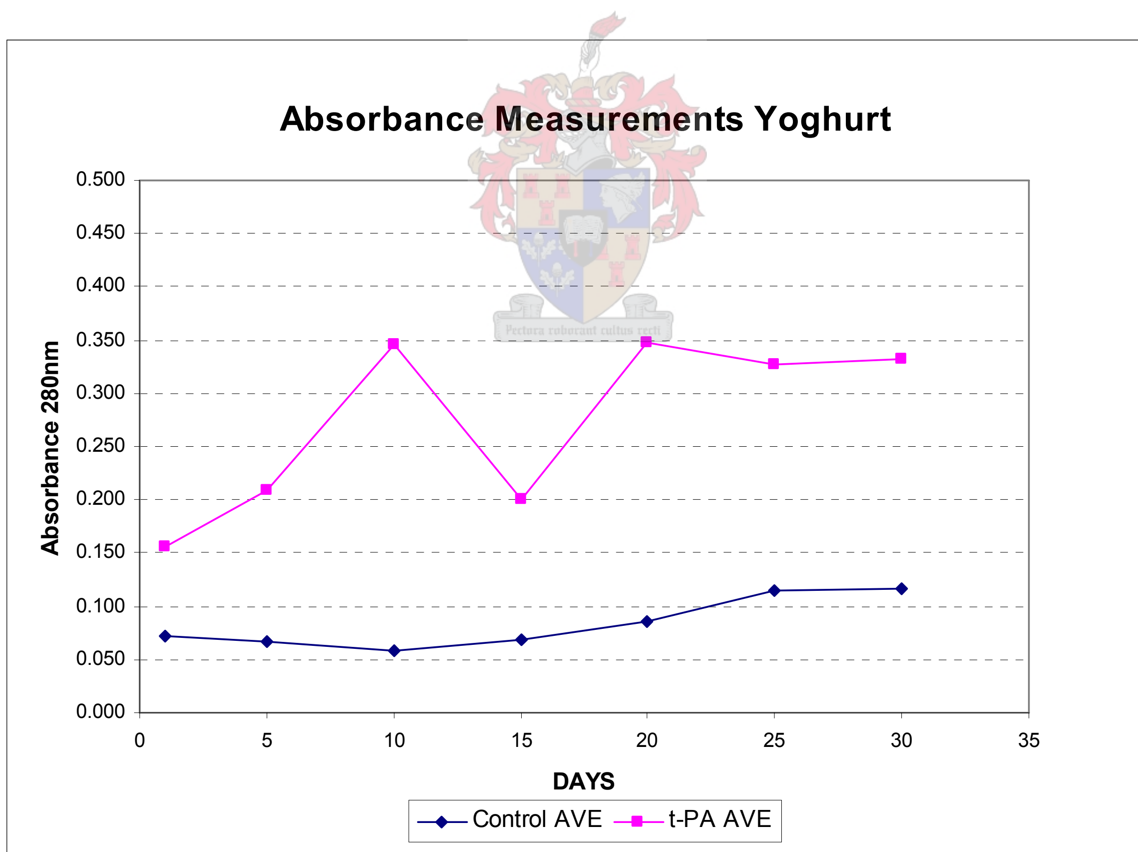

Figure 6.24: Measurements of yoghurt whey at $A_{280\ nm}$ throughout shelf-life to detect and quantify casein hydrolysates.

Table 6.19: pH measurement of yoghurts samples throughout 30 days shelf-life

pH (YOGHURT)						
Day	**Control 1**	**Control 2**	**Control AVE**	**t-PA 1**	**t-PA 2**	**t-PA AVE**
1	4.55	4.54	**4.55**	4.55	4.55	**4.55**
5	4.50	4.50	**4.50**	4.50	4.50	**4.50**
10	4.45	4.45	**4.45**	4.45	4.45	**4.45**
15	4.48	4.48	**4.48**	4.44	4.44	**4.44**
20	4.43	4.43	**4.43**	4.42	4.42	**4.42**
25	4.44	4.44	**4.44**	4.43	4.43	**4.43**
30	4.38	4.38	**4.38**	4.40	4.40	**4.40**

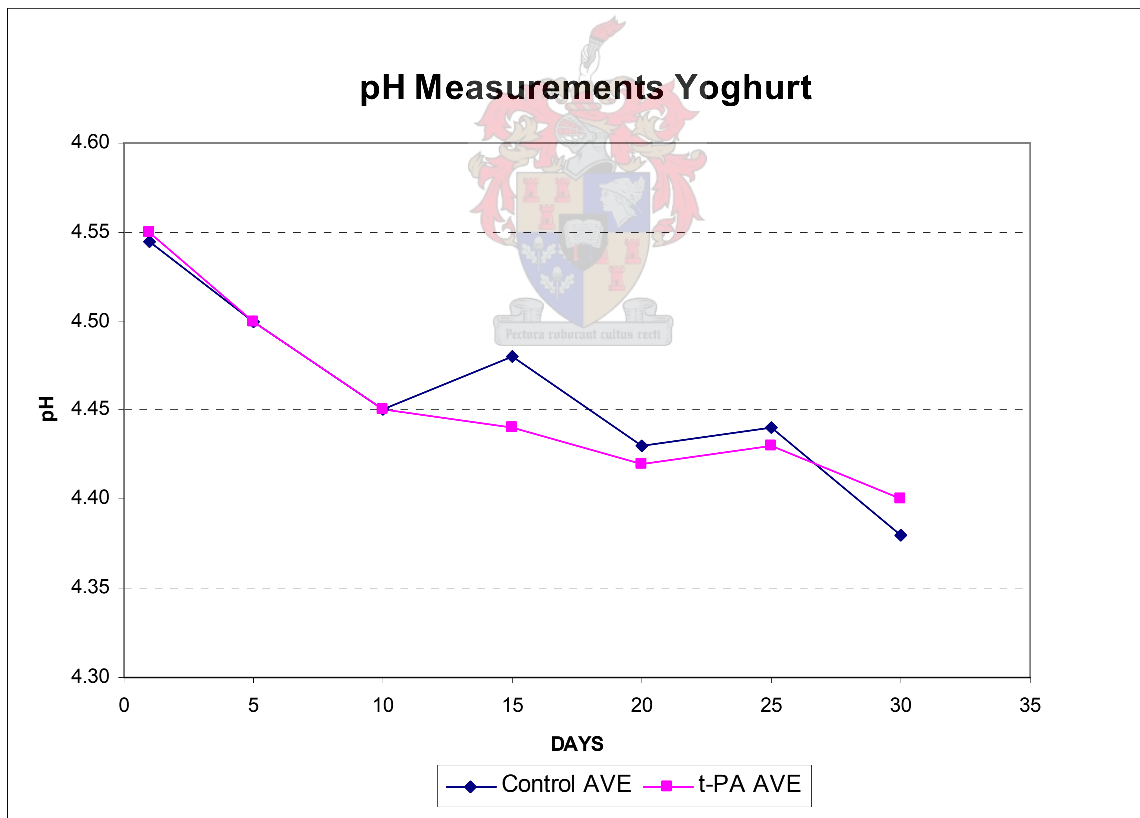

Figure 6.25: pH measurement of yoghurts samples throughout 30 days shelf-life.

The results of the sensory analysis indicated that the t-PA yoghurt lacked freshness and also had a flat flavour in comparison to the control sample. The panelists did note the white spec formation in the t-PA sample as a defect, which resulted in a slight grainy or sandy texture. As a result the control sample scored better marks overall for the final product. The decrease in viscosity was also noted, however, it was still within acceptable limits for yoghurt produced without any stabilizing agent. Free whey was noted after day 15 on the t-PA sample and only after day 25 in the control sample. The most common descriptors used by the panelists could be summarized as follows:

- Control Yoghurt: Neutral colour, good body and texture, glossy, creamy, clean flavour, mild acidity, slight diacetyl flavour. Preferred to t-PA sample.
- t-PA Yoghurt: Neutral colour, white specs, slight dry mouthfeel, slightly weaker body than control, grainy / sandy texture, wheying-off, mild acidity, lacks freshness, flat.

The results of the tasting panel is summarised in Table 6.20. Scores were allocated as follow: Maximum 2 points for appearance, maximum 7 points for body and texture, maximum 11 points for flavour to give a final total out of 20 points.

Table 6.20: Sensory scores of yoghurt during shelf-life

YOGHURT SENSORY ANALYSIS - Average Scores (n=5)				
DAY	**Appearance (2)**	**Body and texture (7)**	**Flavour (11)**	**Score (20)**
CONTROL				
1	1.00	4.50	9.50	15.00
5	0.80	4.30	9.60	14.70
10	1.20	4.45	9.56	15.21
15	1.23	4.78	9.21	15.22
20	0.80	4.92	8.97	14.69
25	0.80	4.28	8.88	13.96
30	0.80	4.00	8.60	13.40
Average	**0.95**	**4.46**	**9.19**	**14.60**
t-PA				
1	1.50	6.00	10.00	17.50
5	1.43	6.20	10.00	17.63
10	1.55	6.20	10.50	18.25
15	1.60	6.33	10.10	18.03
20	1.60	6.35	9.90	17.85
25	1.66	6.20	9.85	17.71
30	1.62	6.20	9.85	17.67
Average	**1.57**	**6.21**	**10.03**	**17.81**

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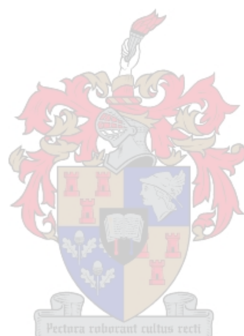
6.4.5. Discussion

Published information on the effect of plasmin and PAs on fermented milk products is limited. The results of this study confirmed that although plasmin activation had some effects on final product quality, the effect on chemical composition of the final product was limited and statistically not significant. This can be attributed to the fact that yoghurt is a shorter shelf-life product, and also due to the loss in plasminogen and plasmin activity as a result of the extensive holding time during heat treatment, normally associated with yoghurt manufacture.

Therefore the potential for plasmin activation is less, due to less plasminogen being available for activation by PAs. This correlates well with the D-values as measured by Alichanidis *et al.* [109] of 2 min at 85°C for plasmin and plasminogen as indicated in Table 6.1. The most noticeable difference between the control and t-PA yoghurt samples was that the t-PA sample was a less viscous product that also had visible white spec formation. Although the severity of the specs was mild, it is characterised as a negative product attribute and is as a result of protein destabilisation. During acid gel formation, denatured proteins associate with casein to form a co-precipitate, should casein breakdown occur this would negatively influence the potential for association of the denatured α-lactalbumin and β-lactoglobulin with casein.



Chapter 7

DISCUSSION

Plasminogen, plasmin, plasminogen activators, plasminogen activator inhibitors and plasmin inhibitors are key elements in the regulation of the fibrinolytic system in bovine blood. When inflammation (haemostasis and thrombosis) in the udder of the cow occurs, the natural defense mechanisms of the cow are activated to combat the infection, which leads to an increased blood supply to and concentration of white blood cells in the infected areas. Due to an increase in vascular permeability, plasmin and plasminogen activators can enter the milk supply through the blood, resulting in increased proteolytic activity in the milk.

Uncontrolled proteolysis in raw milk will affect the quality of the final products, as casein constitutes over 80% of the total protein content in milk. Furthermore, the unique properties of casein make it the main functional component in most of the secondary value added products produced by the dairy industry, such as rennet casein and acid casein gels. Increased proteolysis, induced by the increased activity of plasmin, will not only effect organoleptic properties, but also have a negative effect on the shelf-life and the yield of the final products. To validate this hypothesis, tissue type plasminogen activator (t-PA) was isolated and purified from mid to late lactation bovine milk. The purified t-PA was then added to 3 different dairy products to activate the zymogen, plasminogen, to the active form plasmin, subsequently promoting proteolysis. The products were then analysed throughout shelf-life to investigate and quantify the influence of added t-PA.

The study focused mainly on the isolation of t-PA from bovine milk, which is associated with casein fraction in the milk, rather than urokinase plasminogen activator (u-PA), which is associated with the somatic cell fraction of the milk.

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t-PA losses in secondary (processed) dairy products will theoretically be limited, unlike u-PA, where losses could occur as a part of the normal processing at dairy factories. Processing that will increase u-PA losses include clarification, centrifugation, micro-filtration, ultra-filtration and standardization of milk. These losses will subsequently diminish the effect of u-PA on dairy products.

The characteristics of t-PA isolated and purified from a mixed herd of Jersey and Friesland cows at Elsenburg Dairy Laboratory, Stellenbosch, correlated well with the characteristics of the bovine t-PA previously isolated and partially characterized [9,10,20,22,56]. A modified combination of treatments and methods were used to isolate the t-PA, including de-fatting of the milk, concentration of the casein by ultracentrifugation, salting out, and dissociation of the t-PA from the casein micelles. The t-PA in the resulting fraction, SUP3B, was purified by a factor of 1200 from the raw milk fraction, with a specific activity of 72 $U.g^{-1}$ protein and a yield of 79.61 %. Further purification of t-PA from SUP3B was obtained by a combination of gel filtration and metal chelate (affinity) chromatography. The t-PA obtained after zinc chelating chromatography (ZNF fraction) was purified by a factor of 6,000 from the raw milk fraction with a final specific activity of 398 $U.g^{-1}$ protein with a final yield of 73.46 %. The t-PA was subsequently characterized by SDS-PAGE, IEF, HPGPC and analyzed for amino acid composition. The Mr of t-PA, estimated by gel filtration, was approximately 75 kDa. This result correlated well with the Mr obtained from the SDS-PAGE anlyses, which was approximately 73 kDa, and HPGPC which yielded an Mr of 74,5 kDa. The IEF analyses yielded 3 distinct pI values for the purified t-PA of 6.60, 6.85 and 7.30 respectively, indicating the presence of three isoforms. The presence of three isoforms for bovine t-PA has not been unequivocally established and is an area for future investigation.

To determine the effect of increased proteolysis on dairy products, purified t-PA, obtained after Zinc chelating chromatography, was added aseptically to UHT milk

post packaging (20 U.l^{-1}), Gouda cheese milk (20 U.l^{-1}) prior to manufacture and yoghurt milk (20 U.l^{-1}) prior to the manufacture.

Gelation of the UHT milk occurred after 60 days of incubation with t-PA. In addition to the gelation, visual and sensory analyses of the samples revealed a severe bitter off-flavour in the coagulated milks. The PA activity in the t-PA added UHT milk was higher than that of the control milk, and remained constant throughout the 180 days of evaluation. It is also noteworthy that the casein breakdown metabolites of the t-PA inoculated UHT milk were approximately 9 fold higher than that of the control samples. These results are significant, as the normal shelf-life of UHT milk is from 9 – 12 months, while the severe detrimental effects of t-PA addition were evident after 1 month post t-PA inoculation.

The Gouda cheese, manufactured with added t-PA, exhibited a reduced yield of almost 1.0% in comparison to the control Gouda cheese. The t-PA Gouda exhibited a 3 fold increase in WSN:TN and a significant increase in free amino acids formed, as opposed to the control Gouda, indicating accelerated proteolysis in the t-PA Gouda cheese due to enhanced plasmin activity. The residual rennet activity and starter culture proteolysis should be seen as a constant in both sets of Gouda cheeses manufactured in this study. Hydrolysis of casein negatively affected the rheological properties of the rennet-induced milk gel in this experiment, increasing rennet coagulation time by 83%, moisture content in the final cheese by 10.48% and fat losses in the whey by 83%. The results obtained are important to secondary cheese producers and will have serious financial implications, as increased proteolysis will have a negative impact on the production efficiency of cheese and product viability. To illustrate this point, the loss in cheese yield and fat losses in the whey, based on the trial, was extrapolated to the production of cheese in a medium sized factory (100,000 kg of cheese milk per day). The financial loss in revenue could theoretically be calculated as follows (estimation based only on the loss of fat in the whey and reduced cheese yield):

CHEESE YIELD:

100,000 kg milk at yield of 10.83%	= 10, 830 kg cheese
100,000 kg milk at yield of 9.95%	= 9,950 kg cheese
Total loss in kg cheese per day per 100,000 kg milk	= 880 kg
Total loss in Rand value per day per 100,000 kg milk	= 880 kg X R26,00/kg
	= R 22,880 / day
	= R 686,400 / month

FAT LOSS IN WHEY:

90,050 kg whey at 0.29% fat loss of (0.63% -0.34%)	= 261.15 kg
Total loss in Rand value per day per 100,000 kg milk	= 261.15 X R38,00/kg
	= R 9,923 / day
	= R 297,705 / month

TOTAL LOSS INCURRED PER MONTH **= R 984,105 / month**

The financial effect of inferior cheese in the marketplace is difficult to estimate, but some indicators could be an increase in product returns, shorter shelf-life of products, less profit on sales and a decrease in consumer demand.

The main defects in the yoghurt produced with added t-PA were noted during the sensory analysis, which revealed that the t-PA yoghurt lacked freshness and also had a flat flavour in comparison to the control sample. The panelists also recorded white spec formation in the t-PA sample as a defect, which resulted in a slight grainy or sandy texture, as well as syneresis (wheying-off) after a shelf-life of only 12 days. The PA activity of the t-PA yoghurt was three times higher than the PA activity in the control sample, and no significant increase in plasmin activity was measured over the 30 day storage period, in both the t-PA and control samples. There was a slight increase in the NCN and PP fractions (3-

fold) of the t-PA yoghurt and a decrease in the CN in comparison to the control yoghurt.

This study shows that the complex biochemical cascades present in bovine milk, have a profound influence on the final dairy products derived from this essential foodstuff. It is therefore apparent that the elucidation of the role of the fibrinolytic system and its major components in bovine milk will lead to practical applications in the dairy industry; it will not only improve the quality of the products, but also the financial viability of the industry as a whole.

This field of research is receiving attention internationally. The Food Science Department of Purdue University is currently investigating the use of Fourier-transform infrared (FTIR) spectroscopy, in combination with statistical data analysis, to determine the plasminogen concentration in various dairy protein solutions. Preliminary results are promising, as they have already succeeded to differentiate between plasmin and plasminogen in different dairy protein solutions. It is clear that a physical technique such as FTIR spectroscopy has much to offer the dairy industry, as it provides the opportunity to investigate specific biochemical entities in a non-destructive manner. FTIR equipment is commercially available, easy to operate and is currently used for the rapid analysis of milk solids in milk and other dairy products.

Future research of the fibrinolytic system in bovine milk offers exciting possibilities. Knowledge of the fibrinolytic system and its components in bovine milk can lead to the manipulation of these enzymes, by changing specific parameters such as the severity of heat treatment and the pH of milk during fermentation. Manipulation of the fibrinolytic system could promote controlled proteolysis in cheese, which can shorten ripening times and enhance flavour development. In fluid milks and yoghurts, such knowledge can be used to prevent the negative effect that proteolysis has on the quality of the final products.

Chapter 8

EXPERIMENTAL

8.1. Origin of milk samples

Raw bulk milk samples of morning and evenings milk of a mixed herd of Holstein and Jersey cows were collected from the Elsenburg Dairy Research Centre of the Department of Agriculture Stellenbosch, South Africa. The average days in lactation of the cows were between 150 and 180 days (mid to late lactation).

All samples were kept refrigerated at a temperature below 4°C and were tested within 48 hours of collection, unless otherwise specified. The milk was analysed to confirm whether it conforms to the normal specifications for raw bulk milk. The following tests were conducted: Standard plate count (SPC), Coliform bacteria, somatic cell count (SCC), percentage fat, protein and lactose, freezing point, ethanol stability, pH and percentage titratable acidity (TA). An additional clot boiling test was also done to confirm protein stability of the raw bulk milk.

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8.1.1. Standard Plate Count

<u>Preparation of samples:</u>

1. Thoroughly mix samples of raw milk.
2. A 1:10 dilution (m/m) was prepared by adding 1 ml of the product to 9 ml of sterile diluent or 11 ml of the product to 99 ml of diluent.

Plating of samples:

1. 1 ml of each of the dilutions was transferred in duplicate to sterile Petri dishes, beginning with the highest concentration and ending with the lowest.
2. 10 ml of the standard plate count agar, which has been melted beforehand and cooled to 45°C +/- 1°C, were added to the petri dishes.
3. The contents of each dish was thoroughly mixed using horizontal rotational movement while the medium is still fluid.
4. Once the medium has set, the dishes were inverted and incubated at 30°C +/- 1°C for 72+/-2 hours.
5. At the end of the incubation the dishes were removed from the incubator and colony-forming units (CFU) were counted, with the aid of magnification, under uniform artificial illumination.

Table 9.1: Preparation of the phosphate buffer solution

Diluent – Phosphate Buffer Solution	
Potassium dihydrogen orthophosphate	5.08 g
Disodium hydrogen orthophosphate	13.63 g
Distilled Water	2 litres

Table 9.2: Preparation of standard plate count agar

Standard Plate Count Agar*	
Tryptone (pancreatic digestive product of casein)	5 g
Yeast Extract	2.5 g
Glucose	1 g
Agar (bacterial grade)	15 g
Distilled Water	1 litre
Final pH of sterilised medium	7.0 ± 0.1

**Sterilise for at least 15 minutes at 121°C.*

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8.1.2. Violet red bile (MUG) agar method for coliforms and Escherichia coli

Preparation of samples:

1. Thoroughly mix samples of raw milk.
2. A 1:10 dilution (m/m) was prepared by adding 1 ml of the product to 9 ml of sterile diluent or 11 ml of the product to 99 ml of diluent.

Table 9.3: Preparation of violet red bile agar

Violet Red Bile Agar	**$g.l^{-1}$**
Brain Heart Infusion	7.0
Peptone	4.0
Lactose	9.0
Bile Salts Number 3	1.5
Neutral Red	0.03
Crystal Violet	0.002
MUG (4-methylumbelliferyl B-D-glucuronide)	0.1
Sodium chloride	4.5
Disodium phosphate	1.0
Agar	13.0

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Plating of samples:

1. Dilutions were prepared to obtain plates with colony counts of more than 10 and fewer than 150. Micro-organisms in the test samples were distributed as evenly as possible by inverting the sample containers 25 times. When foam was formed, it was allowed to disperse. The interval between mixing and removing the test portion was not longer than three minutes. 1 ml of the test sample was removed with a sterile pipette and added to 9 ml of the diluent (or 10 ml of the test sample to 90 ml of the diluent or 11 ml of the test sample to 99 ml of the diluent). The primary dilution was shaken thoroughly. In this way, a 10^{-1} dilution was obtained.

2. Further dilutions were prepared by transferring 1 ml of the primary dilution to another test tube containing 9 ml of sterile diluent by using a sterile pipette and avoiding contact between the pipette and the diluent. A fresh pipette was used for each dilution. Alternatively, 10 ml of the primary dilution was transferred to a bottle, containing 90 ml of the sterile diluent, or 11 ml of the primary dilution to 99 ml of the sterile diluent. Samples were mixed thoroughly by mixing mechanically for 5 to 10 seconds to obtain the 10^{-2} dilution. When necessary, this procedure was repeated, using the 10^{-2} and further dilutions to obtain 10^{-3}, 10^{-4}, etc., dilutions until the appropriate number of micro-organisms has been obtained.
3. 15ml of the VRB MUG agar was poured at 45°C +/- 1°C into each Petri dish. The sample was mixed immediately after pouring by rotating the petri dish sufficiently to obtain evenly dispersed colonies after incubation. The samples were allowed to solidify on a cool horizontal surface. After complete solidification, about 4 ml of the VRB agar, at 45°C +/- 1°C, was poured onto the surface of the inoculated medium and allow to solidify.
4. Plates were incubated in an adverted position at 30°C +/- 1°C for 24°C +/- 2 hours.
5. Plates were examined under a 366 nm ultra violet light. All colonies, showing a blue fluorescence in the surrounding medium, were counted. Five or more fluorescent colonies are regarded as positive for *Escherichia coli*. Plates under normal light were examined and counted to obtain the coliform organisms. Dark red coloured colonies, with a diameter of at least 0.5 mm, are characteristic of coliform organisms.

8.1.3. Percentage Fat, Protein, Lactose

The % fat, protein and lactose in milk were detected using a Milkoscan 133B infra-red milk analyser from Foss Electric, Germany.

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8.1.4. *Somatic Cell Counts*

A Bentley Somacount 300 analyser of Bentley Industries, USA was used for the detection of SCC in the milk. The Somacount utilise laser based flow cytometry for detection; ethidium bromide is mixed with the samples (automatically) to stain the DNA in somatic cells. The laser based counting section uses the fluorescence characteristics of the dye to count the cells one by one.

8.1.5. *Freezing Point*

The % water added in milk was detected by freezing point depression with an automatic Fiske cryoscope (Model 4D3), Fiske Associates, USA.

8.1.6. *Ethanol Stability Test*

1. Mix one volume of 70% (v/v) aqueous ethanol with one volume of milk.
2. No signs of coagulation, the milk have passed the ethanol stability test.

8.1.7. *Titratable Acidity*

1. Pipette 9 ml of milk into a white dish.
2. Add either 10 drops or 0.5 ml of a 1.6% phenolphthalein indicator solution in 50% ethanol to the milk.
3. Titrate with 0.1 M NaOH solution until the first tinge of pink that persists for 30 seconds appears.
4. To express the titratable acidity of the milk, as the percentage of lactic acid, divide by 10 the number of milliliters of 0.1 M NaOH used in the test.

8.1.8. The clot-on-boiling test

1. Thoroughly mix the milk before sampling.
2. Pour 5ml of milk into a test tube.
3. Place the tube in boiling water.
4. Ensure that the level of the boiling water is higher than the milk level.
5. Stand the test tube of milk in the boiling water for five minutes.
6. Remove the test tube from the water and tilt the tube almost horizontally without shaking the milk inside.
7. Wait until a thin film is formed on the milk.
8. The result is positive if all the milk clots or if floccules are seen to be adhering to the sides of the tube when it is returned to the vertical position. Colostrum in milk will result in a positive clot-on-boiling test result. The heat stability of the milk is also affected by other factors.

8.2. Isolation and purification of PA from bovine milk

8.2.1. Centrifugation, ultracentrifugation and precipitation

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A combination of centrifugation and ultracentrifugation steps were used in order to partially purify the PA. The isolation and purification steps were modified from methods used by Politus *et al.* [74], Deharveng and Nielsen [22] and Yamauchi *et al.* [138]. All steps were conducted at 4°C unless otherwise specified.

Raw bulk milk collected, that conformed to standard raw milk specifications, was centrifuged at 4000 X g for 30 minutes with a Beckman model J21-B centrifuge. The fresh raw bulk milk was separated into a cream layer, a SCC pellet and the skim milk supernatant containing the casein and PA.

The skim milk fraction was centrifuged at 100,000 X g for 1 hour with a Beckman L5-75 ultracentrifuge to isolate the milk serum (supernatant) from

the casein (pellet). The SCC pellet (SP), containing PA, obtained after the initial centrifugation step of 4,000 X g for 20 minutes at 4°C, was washed three times with 0.01 M phosphate buffer containing 0.15 M NaCl (pH 7.2). The RSP1 pellet was then resuspended in 0.023 M phosphate buffer (pH 7.0) to yield at least 10^6 viable cells per millilitre. Tryphan blue (5 µl of a 2.5 $g.l^{-1}$ solution) was added to 20 µl of the SSP fraction. The proportion of cells, excluding the dye, was then counted and sonicated for 45 seconds. The sonicated cell extract was then ultracentrifuged at 100,000 X g for 1 hour to remove the entrapped casein (pellet). The SPP extracts (supernatant) was stored at –80°C for further investigation.

In order to dissociate the PA from the casein micelle structure the casein pellet (CP1) was reconstituted to the original volume (5 ml) with 0.023 mM phosphate buffer (pH 7.0) and stirred overnight at 4°C (RCP1). 1 M of NaCl was added to the reconstituted casein pellet fraction (RCP1) and incubated for 3 hours at 22°C and then ultracentrifuged at 100,000 X g for 60 min at 4°C. The resulting casein pellet (CP2a) was reconstituted to the original volume (5 ml) with 50 mM Tris-HCl (pH 8.0) and the supernatant fraction (SUP3a) was then dialysed for 24 hours against 1000 volumes of 0.023 M phosphate buffer solution (pH 7.0) containing 0.2 M of boric acid pH 7.0 (pH of boric acid adjusted to 7.0 with 5 M KOH) and 20% DMF with one change of the external medium. The function of the DMF is to dissociate any κ-casein still present in SUP2.

8.2.2. Gelfiltration

Sephadex G75 superfine gel was swollen in 20 mM of Tris-HCl buffer (pH 8.0), and packed in a column (length 950 mm and a diameter of 16 mm). The fractionation range for globular protein solutes are between the range of molecular weight of 3,000 to 80,000 Da.

The column was equilibrated at a flow rate of 7.0 ml per hour with 1 bed-volumes of 50 mM Tris-HCl buffer (pH 8.0) containing 0.50 M of NaCl. The column was calibrated by with molecular weight standards and the void (V_0 – 59.5 ml) and total volumes (V_e) were determined using a mixture of N-2,4-DNP-glycine and dextran blue at concentrations of 2 $mg.l^{-1}$. A graph of V_e / V_0 of the molecular weight standards were plotted against their log Mr values (figure 8.1). The PA containing fractions were loaded onto the column and were run at a flow rate of 6.0 $ml.h^{-1}$. The V_e of the PA was determined and subsequently the molecular mass of the PA could be derived from the graph. Absorbance was measured at 280 nm to determine elution of proteins.

Table 8.1: Determination of the molecular mass of t-PA with Sephadex G75 gelfiltration, V_0 measured at 59.5 ml

Protein	**Mr (Da)**	**Elution volume**	**V_e/V_0**	**Log Mr**
Molecular weight standards				
BSA	68,000	56.20	0.94	4.83
Ovalbumin	43,000	68.50	1.15	4.63
Chymotrypsinogen	25,000	88.30	1.48	4.39
Myoglobin	16,900	92.20	1.55	4.22
Lysozyme	14,300	104.80	1.76	4.16
PA containing samples				
t-PA	74,830	52.50	0.88	4.87
u-PA 1	47,600	64.50	1.08	4.68
u-PA 2	30,480	77.60	1.30	4.48

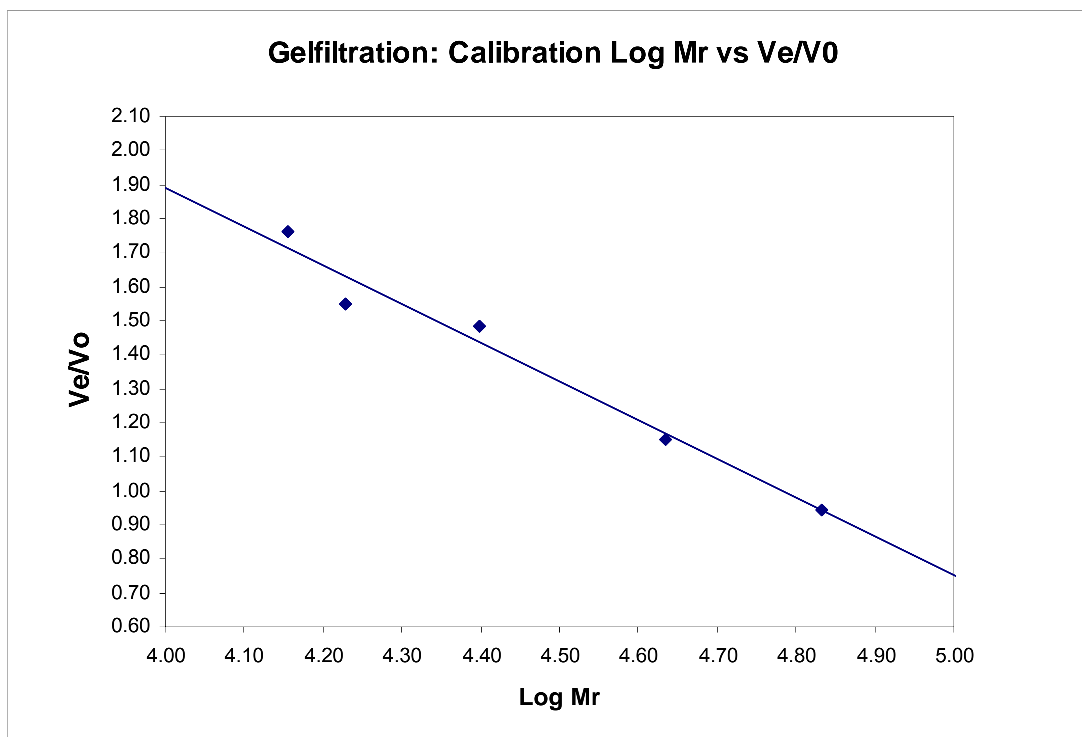

Figure 8.1: Log Mr versus the V_e/V_0 to determine molecular mass of the purified PA by Sephadex G75 gelfiltration. The protein standards used were BSA (66 kDa), Ovalbumin (43 kDa), Chymotrypsinogen (25 kDa), Myoglobin (16.9 kDa) and Lysozyme (14.3 kDa).

8.2.3. Metal chelate (affinity) chromatography

A Zn-chelating resin was prepared with chelating Sepharose 6B as per the manufacturer's instructions (Pharmacia Biotech). The resin was equilibrated with running buffer of 20 mM phosphate buffer containing 1 M NaCl, 0.01% (v/v) Tween 80 (pH 7.50). After the equilibration the resin was mixed with the PA containing fractions and mixed on a shaker plate at 4°C for 3 hours. The resin and protein mixture was then poured into the column (length 100 mm and a diameter of 25 mm).

The column was washed at a flow-rate of 40 $ml.h^{-1}$ with 4 volumes of running buffer. An imidazole gradient of 0 – 0.1 M was then applied to the column and 3 ml samples were collected. After the gradient was applied an additional 75 ml of 0.1 M imidazole buffer was used to wash the column to elute all the

remaining protein from the column, and 5 ml fractions were collected. All the fractions, containing PA activity, were then pooled, and dialysed against deionised distilled water overnight at 4 °C.

8.3. Colorimetric assay for PA activity

The PA assay was determined spectrophotometrically, according to the method of Zachos *et al.* [8], using the synthetic substrate, D-Val-Leu-Lys-ρ-nitroanalide (V0882, Sigma Aldrich; millimolar extinction coefficient of 5.57 $mM^{-1}.cm^{-1}$). Measurements were carried out on a Dynex Opsys MR microtiter plate reader. The reactions were measured by determining the change in absorbence at 405 nm due to the hydrolyses of the chromatogenic substrate D-Val-Leu-Lys-ρ-nitroanalide. The chromatogenic substrate was cleaved at the Lys residue which yielded the free chromophore ρ-nitroanalide.

Assays were performed in triplicate with 250 µl 100 mM Tris-HCl buffer (pH 8.0) containing 50 $\mu g.ml^{-1}$ plasminogen (P5661, Sigma Aldrich) and 0.6 mM D-Val-Leu-Lys-ρ-nitroanalide and 5 µl of a PA fraction. White *et al.* [55] reported no influence of casein on PA activity, therefore no correction for turbidity was done for the fractions with casein present in this study. The reaction mixtures were incubated at 37°C in a New Brunswick Scientific Co. G24 Environmental Shaker for 2 and 3 hours respectively, after which the absorbence was measured at 405 nm. A sample without added plasminogen served as a control.

The reaction was stopped by adding 100 µl of 30% (v/v) acetic acid to the samples. The samples were centrifuged at 8,000 X g for 5 minutes before reading the absorbance at 405 nm. A sample without added plasminogen served as a control. The detection of the indigenous plasmin was subtracted from total plasmin activity. The plasminogen activity used as substrate in the assay can be quantified as follows: one unit of plasminogen activity relates to a change of 0.1 in absorbency from α-casein at 275 nm in 20 minutes at 37°C, pH 7.5 where urokinase is used as activator.

There was also distinguished between t-PA and u-PA by adding 20 μg fibrin and 20 mM amiloride to the PA-containing fractions. In the presence of fibrin there will be an increase in t-PA activity and in the presence of amiloride a decrease in u-PA activity. Tissue type PA is not affected by amiloride and the activity of u-PA will not be enhanced by the addition of fibrin [139,140].

8.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

8.4.1. Preparation of running and stacking gels

The SDS-PAGE gel system of Life technologies was used for gel electrophoresis. The system contains a set of premixed solutions containing all the chemicals needed for the preparation of any size or percentage continuous, gradient or discontinuous separation of proteins according to molecular weight. The system consists of the following components: 40% (w/v) Acrylamide: Bisacrylamide (37.5: 1), Resolving gel buffer concentrate [1.5 M Tris-HCL, 0.4% (v/v), N, N, N, N-tetramethylethyldiamine (TEMED), 0.4% (m/v) Sodium dodecyl sulphate (SDS), pH 9.0] and a stacking gel buffer [0.14 M Tris-HCL, 0.11% (v/v), TEMED, 0.11% (m/v) SDS, pH 6.8].

The concentrated solutions were prepared according to the prescribed method to yield a 10% polyacrylamide resolving gel and a 3.9% polyacrylamide stacking gel solution (refer to table 8.2).

Freshly prepared ammoniumpersulphate solution (10% m/v) was added to the running gel mixture and directly thereafter the gel was poured. A small volume of distilled water was added on top of the mixture and was discarded after the gel had polymerised (approximately 30 minutes for polymerisation). The stacking gel mixture was prepared, the ammoniumpersulphate solution (10% m/v) was added and the gel was poured on top of the polymerised

running gel. A comb was inserted into the stacking gel to form the wells for sample application.

Table 8.2: SDS – Polyacrylamide gel formulations for the resolving and stacking gels

SDS-Polyacrylamide Gel Formulations						
Resolving Gel %(m/v)						**Stacking Gel % (m/v)**
Component, Volume (ml)	5.0%	7.5%	10%	12.5%	15.0%	3.9%
40% Acrylamide: bis	5.1	7.7	10.3	12.8	15.4	1.0
Resolving buffer	10.0	10.0	10.0	10.0	10.0	-
Stacking buffer	-	-	-	-	-	9.0
Distilled water	24.5	21.9	19.3	16.8	14.2	-
10% Ammoniumpersulphate	0.4	0.4	0.4	0.4	0.4	0.05
Total volume (ml)	40.0	40.0	40.0	40.0	40.0	10.0

8.4.2. Preparation of samples

The samples (20 µl) were diluted with equal volumes of the treatment buffer (67.5 mM Tris-HCl, pH 6.8, 2% (m/v) SDS, 10% (v/v) glycerine and 5% (v/v) 2-mercaptoethanol) and bromophenol (1 µg) as front marker. The samples (1 - 5 µg protein) were boiled for 2 minutes and allowed to cool to room temperature before they were loaded onto the gel.

8.4.3. Gel electrophoresis of PA

Electrophoresis buffer [25 mM Tris buffer, 0.25 M glycine, 0.1 % (m/v) SDS, (pH 8.3)] was added to the upper and lower chambers of the electrophoresis cell. The wells of the cells were rinsed with the electrophoresis buffer before the samples were loaded. After the samples were loaded a constant current of 18 mA was applied to the electrophoresis cell until the Bromophenol Blue reached the end of the running gel (approximately 90 minutes). The gel was

removed to be stained or to elute the PA proteins or to use in immunoblotting experiments.

8.4.4. Staining and destaining procedures

The gel was stained for a minimum of 1 hour in a Coomassie blue solution [0.125% (m/v) Coomassie blue R250, 50% (v/v) methanol and 10% (v/v) acetic acid]. The staining solution was removed and the gel was destained in the first solution (50% methanol, 10% acetic acid, and 40% distilled water) for a maximum of 1 hour and then destained in the second solution (5% methanol, 7% acetic acid, and 88% distilled water) overnight.

8.4.5. Elution of PA from preparative SDS-PAGE-gels

For localisation of PA activity the electrophoretic lanes were cut into slices of approximately 5 mm and incubated overnight at 4°C in 0.1 M Tris-HCl (pH 8.0) buffer containing 5.5 g Triton X100 per litre to remove the SDS. After 2 hours the buffer was discarded and the slices soaked in 0.5 ml fresh 0.1 M Tris-HCl (pH 8.0) buffer for 24 hours to elute the proteins. The buffer containing t-PA protein (0.1 M Tris-HCl, pH 8.0 buffer) was dialysed against deionised distilled water for 24 hours at 4 °C. The PA fraction was then lyophilised.

8.4.6. Determination of molecular mass of purified bovine PA by SDS-PAGE

A SDS-PAGE gel was prepared as described in paragraphs 8.4.1 - 8.4.3. the PA fractions obtained from the centrifugation and ultracentrifugation steps, the Sephadex G75 column and the Zn chelating column were loaded on the gel together with 6 high molecular weight protein standards, namely phosphorylase B (94,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da),

carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da) and a-lactalbumin (14,400 Da). A constant current of 18 mA was applied to the electrophoresis cell until the Bromophenol Blue reached the end of the running gel (approximately 90 minutes). The gel was removed, stained and destained as described in paragraph 8.3.4. The Rf values of the seven protein markers were calculated and a graph of the Rf values against the log Mr was drawn, from which the approximate molecular mass of the PA could be calculated (refer to table 8.3 and figure 8.2).

Table 8.3: Determination of the molecular mass of PA with SDS-PAGE

Protein	**Front (mm)**	**Rf**	**Log Mr**	**Mr (Da)**
Standard Curve				
Phosphorylase B	10.0	0.127	4.97	94,000
Albumin	22.0	0.278	4.83	67,000
Ovalbumin	34.0	0.430	4.63	43,000
Carbonic anhydrase	40.0	0.506	4.48	30,000
Trypsin inhibitor	55.0	0.696	4.30	20,100
α-lactalbumin	65.0	0.823	4.16	14,400
Samples				
Plasmin	15.0	0.190	4.93	85,345
t-PA	17.0	0.215	4.87	73,282
u-PA1	31.0	0.392	4.70	50,119
u-PA2	40.0	0.506	4.52	33,343

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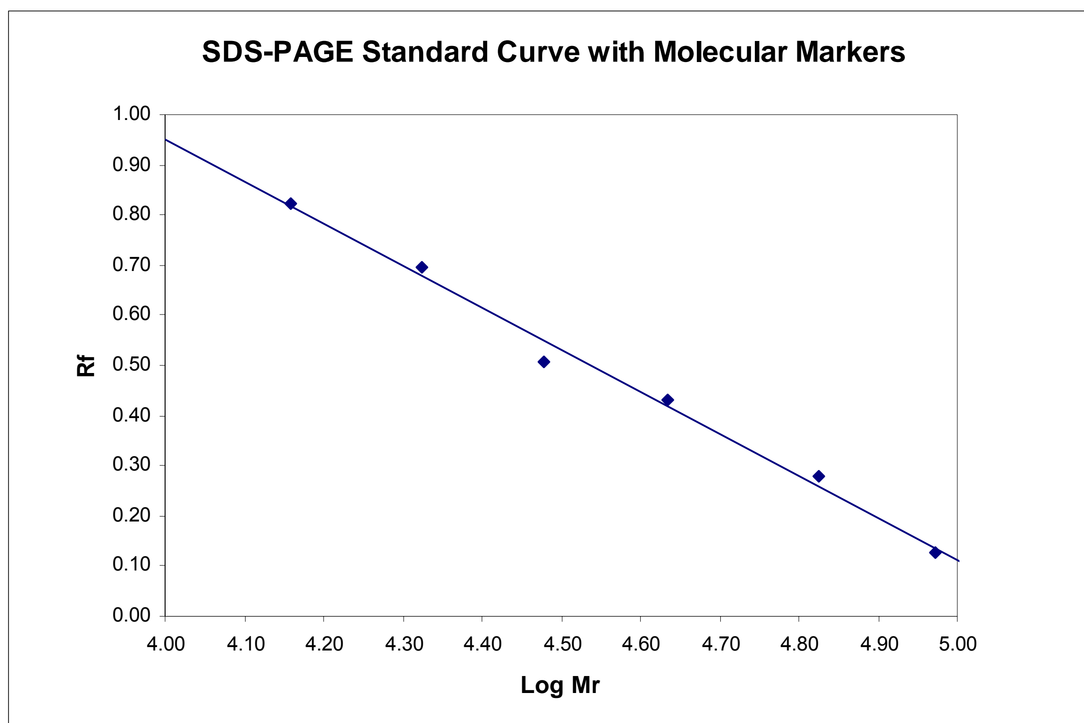

Figure 8.2: Determination of the molecular mass of purified PA with SDS-PAGE.

8.5. Isoelectric focusing (IEF)

Isoelectric focusing electrophoresis was performed using a PhastGel IEF kit (Amersham Biosciences), pH 3-10. PhastGel IEF media are homogenous polyacrylamide gels (5% T, 3% C) containing Pharmalyte carrier ampholyte. Pharmalyte generates stable, linear pH gradients in the gels during the run. Proteins migrate under an electric field, essentially unhindered by the porous gel, to a point in the pH gradient that corresponds to their pI (isoelectric point). After the run the gel was stained using the silver staining method to enable visualisation of the different protein fractions. The calibration kit (pI 5.2-10.3) for pI determination consisted of β-lactoglobulin A (pI 5.20), carbonic anhydrase B (bovine) (pI 5.85), carbonic anhydrase B (human) (pI 6.55), myoglobin (acidic) (pI 6.85), myoglobin (basic) (pI 7.35), lentil lectin (acidic) (pI 8.15), lentil lectin (middle) (pI 8.45), lentil lectin (basic) (pI 8.65), trypsinogen (pI 9.30) and cytochrome C (pI 10.25).

The prepared gel was placed on the separation bed. The sample well-stamp was covered with parafilm and 5 ml of sample pipetted into the well depressions, which is drawn into the applicator capillaries by capillary action. The sample applicators were then loaded near the cathode on the sample applicator arm. Total running time was approximately 30 minutes for the gel.

8.6. HPGPC analysis

8.6.1. Molecular mass analyses of t-PA

High performance gel permeation chromatography (HPGPC) of t-PA and standard protein markers was performed on a 0·75 × 60 cm prepacked TSK G 3000 SW column (Sigma Aldrich). The TSK G 3000 SW column contain silica-based, hydrophilic bonded phase packings that minimise interaction with proteins, thus the reason for being selected. Purified t-PA (200 µg) was analysed by HPGPC on the column equilibrated with 50 mM Tris-HCl, 100 mM NaCl (pH 7.2). The same buffer was used to elute the protein at a flow rate of 0·4 $ml.min^{-1}$. Absorbance was monitored at 280 nm.

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8.6.2. Amino acid analysis of t-PA

A 100 µg lyophilised t-PA protein was added to 1 ml of 6 M HCl and sealed in an evacuated ampoule. The acidified mixture was flushed with nitrogen while in an ultrasonic water bath, flame-sealed in a 5 ml ampoule, and transferred into a pre-heated oven at 110°C for 24 h. Twenty µl of filtrate was transferred to a test tube and dried by blowing gently with nitrogen gas. Each dried sample was hydrated with 250 µl of 0.1 M HCl dilution buffer spiked with 7.5 mM L-norvaline (Sigma Aldrich), filtered through a HV 0.45 µm pore size filter (Millipore), and loaded into an amino acid analyzer sample cartridge. Amino acids were derivatised with *o*-phthaldialdehyde and 2-mercaptoethanol (MCE). The OPA reagent was prepared by combining 25 ml of 100 mM

sodium borate, 2.5 ml 20% (w/w) SDS, 40 mg OPA dissolved in 1 ml methanol, and 100 µl MCE, then adjusting the volume to 50 ml with water. A tetrahydrofuran/methanol solvent gradient [solvent A, tetrahydrofuran / methanol / 0.1 M sodium acetate, pH 7.2 (5:95:900, by vol.); solvent B, methanol] was used for the separation of amino acids with a flow rate of 2.2 ml/min. Samples were analysed by HPLC on a reverse phase C^{18} column. Effluent was monitored by fluorescence at an emission wavelength of 330 nm with excitation at 450 nm.

Amino acid standard samples (AA-S-18 Sigma Aldrich) were prepared by mixing 80 µL of the 45 µmol / ml amino acid standard mixture with 20 µL of 10 mM norvaline and analyzed directly by RP-HPLC, within 24 h from preparation.

8.7. Brookfield viscosity

Gel formation and apparent viscosity were measured with a Brookfield viscometer at 20°C (spindle 1 at a speed of 60 rpm) for UHT milk and at 4°C (spindle 3 at a speed of 60 rpm) for yoghurt. Triplicate readings were taken directly in centipoise when the spindle had been rotating for 30 s.

8.8. Kjeldahl analysis

Protein analyses of the samples were done according to standard IDF procedure (IDF, 1964) by Kjeldahl analysis of the total protein (TP), non-protein nitrogen (NPN) and non-casein nitrogen (NCN). Casein nitrogen (CN) was calculated as the difference between NCN and NPN. All nitrogen results were expressed as protein equivalent using a conversion factor of 6.38 and were analysed in triplicate. The basic analytical procedure was as follows:

Total nitrogen (TN): Sample is digested in 95-98% H_2SO_4, using $CuSO_4.5H_20$ as catalyst with K_2SO_4 as boiling point elevator, to release nitrogen from protein and retain nitrogen as ammonium salt. Concentrated 50% (w/w) nitrate-free NaOH is added to release NH_3, which is distilled, collected in 40g.l^{-1} H_3BO_3 solution, and titrated.

Total protein nitrogen (TPN) and non-protein nitrogen (NPN): Protein is precipitated from milk by trichloroacetic acid (TCA) solution. Precipitation must be done in Kjeldahl flask or tube. Final concentration of TCA in mixture is 12%. The 12% TCA solution, which contains NPN components of a sample, is separated from protein precipitate by filtration. Nitrogen content of protein precipitate and NPN filtrate is determined as in the TN method.

The water soluble N (WSN) extracts were analysed according to the method of Kuchroo and Fox [163]. The N content of the extracts was determined by the Kjeldahl method; WSN was expressed as a percentage of TN.

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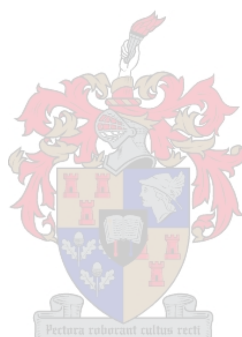
8.9. Spectrophotometric analysis at 280 and 507 nm

In UHT and fermented milks, absorption of milk or whey serum, isolated from the samples at 280 nm, was tested, in order to detect the aromatic amino acid side chains of short peptides of hydrolysed casein molecules, according to the method of Richardson and Te Whaiti (1978) [105]. An increase in absorbence would indicate the presence of the liberated breakdown products (proteose peptones) of casein molecules in the serum.

In Gouda cheese, total free amino acids were determined by a modified Cd-ninhydrin reactive (CdN) method. The cadmium-ninhydrin method of Folkertsma and Fox [164] was used to quantify free amino acids produced during Cheddar cheese ripening. Lyophilized WSN extracts were reconstituted in distilled water (10%, w/v), and a 50 µl sample was diluted to 1 ml with distilled water. The 1 ml sample was then added to 2 ml of cadmium-ninhydrin reagent (0.8 g ninhydrin (Sigma Aldrich) in 80 ml of 95% (v/v)

ethanol and 10 ml of glacial acetic acid, to which 1 g of $CdCl_{2,}$ (previously dissolved in 1 ml of distilled water), is added. The mixture was heated at 84°C for 5 min, cooled, and the absorbance was measured at 507 nm was. The concentration of free amino groups in the mixture was expressed as mM Leucine equivalent.l^{-1} (mM Leucine equivalent.l^{-1}) using a standard curve of D-L-Leucine (Sigma Aldrich).



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