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Tamarilllin - the purification and properties of a novel serine protease from tamarillo fruit (Cyphomandra betacea (Cav.)), and the characterisation of its milk-clotting activity

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Food Science by

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2017
Abstract of thesis

In this thesis, a protease extract was isolated from the fruit of two tamarillo (Cyphomandra betacea) cultivars. The purified protease from the Laird’s Large cultivar, with a molecular weight of approximately 70 kDa, exhibited its highest proteolytic activity at pH 11 and 60°C. This activity was inhibited by phenylmethylsulfonyl fluoride (PMSF), indicating that the enzyme was a serine protease. It was stable in the presence of organic solvents and most metal ions, but its activity was reduced by Hg^{2+}, suggesting that it contained cysteine residues near its active site. Further, de novo sequencing analysis indicated that the protease was likely to be a subtilisin-like protease.

The tamarillo protease exhibited rennet-like milk-clotting properties. When added to milk as either a crude or a purified extract, the milk aggregated and then gelled. The tamarillo protease crude extracts showed faster milk gelation kinetics, compared to rennet. The milk aggregation time was also shorter when the temperature was increased or when the pH is decreased. Particularly, at 20°C rennet did not coagulate milk even after 3 hrs, while the tamarillo protease coagulated milk within 2 hrs. Ultra-small angle neutron scattering (USANS) showed that the large-scale structure of the milk gels induced by both the tamarillo protease and rennet were similar. However, confocal microscopy showed that the milk gels obtained by the addition of the tamarillo protease were more porous than those induced by rennet. This was confirmed by Small-angle X-ray scattering (SAXS) which revealed that the milk gel made with rennet were denser. The difference in the microstructure of the milk gels explained their large-deformation rheological behaviour, where the tamarillo protease-induced milk gels were found to be less brittle than those made with rennet.

The differences in the aggregation kinetics and structures of the milk gels made by the addition of the tamarillo protease and rennet, were presumed to be due to the broad
caseinolytic activity of the tamarillo protease. While both proteases preferably hydrolysed κ-casein, the tamarillo protease also displayed a high proteolytic activity towards both α- and β-casein. The major peptide generated from κ-casein under the action of the tamarillo protease had a molecular weight of 14,290 Da, and the κ-casein cleavage site was confirmed to be at Asn_{123}-Thr_{124} by in-gel tryptic digestion and time-to-flight mass spectrometry. Finally, “tamarillin” was suggested as an appropriate name to identify this tamarillo protease.
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First of all, I would like to acknowledge my supervisor Associate Professor Yacine Hemar who provided me with an opportunity to work in his lab. Without his patient supervision, motivation, overall plan, immense knowledge and dexterous guidance, it would have been impossible for me to complete this thesis. During difficult times, he always supported me and gave me the help needed to carry on. I have benefited from his guidance all the time during both the research and writing phases of this thesis. In addition, his logical thought and broad knowledge in aspects of science, history, literature and politics have been beneficial in my learning journey.

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Thirdly, I have been fortunate to carry out some research work at ANSTO, to perform USANS experiments with the expert support of Dr. Christine Rehm and Dr. Liliana de Campo. I am also grateful for the help of Dr. Guang Mo at the Beijing Synchrotron and Dr. Na Li and Dr. Feng Tian at the Shanghai Synchrotron for their help with the SAXS experiments.

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<tbody>
<tr>
<td>α-CN</td>
<td>α-casein</td>
</tr>
<tr>
<td>β-CN</td>
<td>β-casein</td>
</tr>
<tr>
<td>κ-CN</td>
<td>κ-casein</td>
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<tr>
<td>α-Lac</td>
<td>α-lactalbumin</td>
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<tr>
<td>β-Lg</td>
<td>β-lactoglobulin</td>
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<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>ANSTO</td>
<td>Australia National Science and Technology Organisation</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCP</td>
<td>Colloidal calcium phosphate</td>
</tr>
<tr>
<td>CM</td>
<td>Carboxymethyl</td>
</tr>
<tr>
<td>CSLM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethanol</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>$G'$</td>
<td>Storage (elastic) modulus</td>
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<td>$G''$</td>
<td>Loss (viscous) modulus</td>
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<td>$G^*$</td>
<td>Complex modulus</td>
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<tr>
<td>GDL</td>
<td>Gluconolactone</td>
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GMP  Glycomacropeptide
HCD  Higher energy collision
HPLC  High performance liquid chromatography
Ig  Immunoglobulins
LC-MS  Liquid chromatography-mass spectrometry
LF  Lactoferrin
LVR  Linear viscoelasticity region
Met  Methionine
PAGE  Polyacrylamide gel electrophoresis
PCMB  \( p \)-chloromercuribenzoic acid
Phe  Phenylalanine
PMSF  Phenylmethlysulphonyl fluoride
SAXS  Small-angle X-ray scattering
SDS  Sodium dodecyl sulphate
SMP  Skim milk powder
SSRF  Shanghai Synchrotron Radiation Facility
\( t_{ag} \)  Aggregation time
TCA  Trichloroacetic acid
TEMED  Tetramethylethylenediamine
TFA  Trifluoroacetic acid
Thr  Threonine
TOF  Time-of-Flight
USANS  Ultra-small-angle neutron scattering
Co-authorship Chapter 4

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Chapter 4: unpublished

 purification and characterization of a protease (tamarillin) from tamarillo

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Chapter 5: unpublished
Protease activity of enzyme extracts from tamarillo fruit and its specific hydrolysis of bovine casein

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Last updated: 19 October 2015
Co-authorship Chapter 7
Chapter 1

1. Introduction and overview
1.1 Introduction

Cheese is a dairy product rich in nutrients, such as protein, fat, carbohydrate, minerals and vitamins. Generally, cheese manufacture involves aggregation of milk to form a milk gel, dehydration of the milk gel to form a curd, and curd ageing. Milk-clotting is a basic step in cheesemaking, which can be achieved either enzymatically, or by lactic acid fermentation, or a combination of both (Belitz et al., 2009; Guinee and Wilkinson, 1992). Enzyme-induced milk-clotting is achieved primary through the cleavage of κ-casein, which results in the destabilization of casein micelles and their aggregation to form a gel (Silva et al., 2003). Chymosin (EC 3.4.23.4), which is present in calf rennet, is the most widely used clotting enzyme in cheese making (Ahmed et al., 2010). Approximately 75% of all cheeses are made with calf rennet (Ahmed et al., 2009b). As the oldest and most used enzyme in the cheese making industry, calf rennet has high specificity to cleave the Phe105-Met106 peptide bond of κ-casein (Pires et al., 1994; Vishwanatha et al., 2010). However, in the last 10 years the demand for rennet has markedly increased, due mainly to the increase in the world population and the subsequent demand for increasing amounts of cheese (Elsamani et al., 2014; Tajalsir et al., 2014). A consequence of this increased demand was that the cost of calf rennet increased, while its quality decreased (Ahmed et al., 2010). In addition, the application of calf rennet is limited for religious (for example, Judaism and Islam), diet (vegetarianism), and safety (bovine spongiform encephalopathy) reasons, or also as some people are against the use of recombinant engineered food (Germany and Netherlands) (Badgujar and Mahajan 2014; Roseiro et al., 2003). Due to the reasons mentioned above, new sources of proteases with rennet-like properties have attracted a lot of interest, and proteases from different sources such as animal, microbial and plant have been investigated (Jacob et al., 2011). However, most of the milk-clotting proteases exhibit broad specificity of hydrolysis on caseins resulting in bitter flavour, or low milk-clotting activity, leading to a low yield of cheese (Shah et al., 2014; Anusha et al., 2014).

Proteases from plant sources have been shown to have a high potential as rennet substitutes and have been used in cheese-making since ancient times (Huang et al., 2011; Katsaros et al., 2010). For example, cheeses made with plant extracts are produced in Mediterranean, West African and southern European countries (Shah et al., 2014). These extracts are obtained from the flowers of cardoon (*Cynara cardunculus*), and also from Sodom apples (*Calotropis procera*). However, the excessive proteolytic nature of most vegetable coagulants has also
limited their use in cheese manufacture due to lower cheese yield, and their detrimental effects on flavour and texture (Lo Piero et al., 2002). Compared to rennet, plant proteases are less specific and result in more peptide bonds in casein molecules being degraded, resulting in the cleavage of $\alpha_{s1}$- and $\beta$-casein into low molecular weight peptides, which can impart a bitter taste to the cheese, due to their high hydrophobicity. In addition, some peptides resulting from the hydrolysis of $\alpha_{s1}$- and $\beta$-caseins, with molecular weights of approximately 2000-4000 Da, are lost in the whey, leading to reduced cheese yield (Roseiro et al., 2003). Therefore, further research for new potential plant proteases for milk-clotting and cheese making is needed.

Tamarillo, or tree tomato (Cyphomandra betacea Cav.), is a small and fast growing tree, which is cultivated for its edible fruit (Prohens and Nues, 2001). The tamarillo tree was originally from South America (Bohs, 1989a; Bohs, 1994), and was introduced from India to New Zealand in the late 19th century (Bohs, 1989b), where it is now commercially planted. Every year, approximately 2000 tons of tamarillo are produced in New Zealand, of which approximately 85% is exported to the US and Europe (Richardson and Patterson, 1993; Eagles et al., 1994). Tamarillo fruit, a juicy fruit with a distinctive acidic taste, are elliptical with a 4 to 8 cm length and a 3 to 5 cm width (Prohens and Nuez, 2001). Tamarillo fruit are low in calories and rich in vitamins A and C (Prohens and Nues, 2001). Although tamarillo has a number of varieties, there are only a few cultivars, such as red, yellow and dark red, which are planted commercially. In New Zealand, mostly fruits from the red tamarillo cultivar are exported (Richardson and Patterson, 1993).

While tamarillo has been studied for its chemical composition (Acosta-Quezada et al., 2015), its pH stability, its volatile constituents (Torrado et al., 1995), and the antioxidant activity of anthocyanin rutinosides isolated from its fruit (Hurtado et al., 2009), to the best of our knowledge extracts from tamarillo fruits have never been considered as a milk-clotting or cheese making substitute for rennet.

1.2 Objective of this project

The main aim of this thesis is to purify and characterise a protease from tamarillo fruit, and develop a fundamental understanding of the coagulation of milk by this protease. To do so, the physical and chemical properties of skim milk gels obtained by the addition of crude and
purified extracts from tamarillo fruits are compared to those obtained by rennet addition. The effect of these extracts on the hydrolysis of individual casein molecules is also considered. More specifically, the different objectives of the thesis are:

1. Extract and purify the protease from tamarillo fruit, and determine its caseinolytic activity under different environmental factors such as pH, temperature, with inhibitors, metal ion and organic solvents. In addition, the identification of this protease is performed using peptide fragments from this protease and a database of the known protein sequences.

2. Investigate the hydrolysis of the individual caseins by rennet, crude, and purified tamarillo protease-containing extract. While all the caseins are considered, an emphasis is put on κ-casein, which is believed to be at the surface of the casein micelle, and when cleaved results in milk coagulation.

3. Investigate the effect of the tamarillo fruit, in comparison to rennet, on the coagulation of reconstituted skim milks. This is performed due to the importance of milk coagulation as the first step in cheesemaking. Coagulation of milk by the tamarillo extracts (both crude and purified) is investigated under different pH, concentration, and temperature condition in order to (i) determine the optimal conditions under which milk coagulation occur; (ii) select the best conditions allowing to obtain a kinetic of milk coagulation similar to that obtained using rennet. In addition to the kinetics of coagulation, the microstructural properties of the milk gels made by the tamarillo extracts and rennet are investigated, since the microscopic properties influence the macroscopic behaviour of the milk gels.

Please note that since this protease has never been studied before, we wish to name it *tamarillin*.

### 1.3 Thesis structure

*Chapter 2* is a literature review on milk constituents, casein micelles, cheese manufacture, and coagulation of milk by rennet and other proteases for cheese manufacture.

---

1 While from a fundamental view-point it is more suitable to work with purified extracts; with cost saving in mind, the use of crude extracts is more relevant to industrial applications.
Chapter 3 reports the materials and methods, and introduces sample preparations, and the methods used for the physical and the chemical analysis.

In Chapter 4 a protease present in the crude extract of Laird Large tamarillo fruit is further purified to homogeneity and named as “tamarillin”. Caseinolytic activity of this purified extract is studied for its stability and activity under different environmental factors such as pH, temperature, inhibitors, metal ion and organic solvent. In addition, the purified tamarillo protease is further characterised using de novo peptide sequencing.

In Chapter 5 an isolation method of crude extracts from Laird Large and Amber tamarillo fruits is reported. The hydrolysis of bovine milk (10% w/w) by these extracts is compared to rennet. In addition, the effect of pH and temperature on the gelation kinetics of reconstituted milk by the tamarillo extracts is compared to rennet. The kinetic of milk gelation is investigated by small-amplitude oscillatory rheology (SAOR), and the microstructure of the milk's gel is observed by confocal laser scanning microscopy (CLSM).

Chapter 6 reports on the hydrolysis of the different caseins by rennet and purified tamarillo protease. The hydrolysis of κ-casein was thoroughly investigated by mass spectrometry, since this casein is believed to be the first affected by rennet or tamarillo protease hydrolysis.

Chapter 7 investigates the milk gelation under the action of rennet and purified tamarillo (Laird Large) enzyme. The milks were reconstituted in deuterium oxide (D₂O) to improve Ultra-Small Neutron Scattering (USANS) measurements. USANS measurements were performed in order to investigate the structure of the milk gels. Small Angle X-Ray Scattering (SAXS) measurements were also performed on these samples, in order to investigate the internal structure of the protein network. These measurements were also completed by rheological measurements and CLSM observations. In this Chapter, the milk concentration was varied, in order to understand the effect of milk concentration on the structure of the milks treated by rennet and tamarillo protease.

Chapter 8 summarises the main findings of the thesis and proposes some future work.
Chapter 2

2. Literature review on Milk and Plant proteases
2.1 Introduction

Milk is a colloidal dispersion of butterfat globules and proteins in a continous phase of mainly water, minerals, vitamins and lactose (Jost, 2002; Jensen et al., 1991, 1995). Milk and its derived dairy products have been a part of the human diet for thousands of years. Specifically, cheese has been produced as food for the past 7500 years (Hallen, 2008). Milk and dairy products contain a large number of essential nutrients which are beneficial for the growth and development of all age groups. For instance, minerals present in the whey and caseins promote the rapid growth for the young (Mølgaard et al., 2011). Linoleic acid and α-linoleic fatty acids play an important role in the nervous system development (Michaelsen et al., 2007). Lipids, lactose and protein in milk are a principal source of energy for the human daily requirement, and the milk proteins provide essential amino acids and amino groups. In addition, essential fatty acids, vitamins and inorganic elements and water also play beneficial roles in human nutrition (Fox, 1997; Gautheron and Lepouze, 2012). While humans consume milk and their dairy products from the principal animal species such as cow, buffalo, sheep, and goat (Fox and McSweeny, 1998), this literature review focuses mainly on bovine milk.

Milk-clotting is one of the basic steps in cheesemaking. Coagulants, such as rennet, have been applied in cheesemaking for thousands of years and are reported to be the oldest recorded application of enzymes (Harboe et al., 2010). Historically, most enzyme preparations applied for cheesemaking have been isolated from the fourth stomach of ruminants, mostly calves (Harboe et al., 2010). Stomach-extracted rennet contains two proteolytic enzymes: chymosin and pepsin. Generally, there is around 50% to 95% chymosin in rennet, depending on the age and the previous diet of the animals (Jacob et al., 2011). Chymosin plays a key role in milk-clotting as it has a high specificity for hydrolysing the Phe$_{105}$-Met$_{106}$ peptide bond of κ-casein resulting in aggregation of the casein micelles followed by milk-clotting (Hyslop, 2003; Crabbe, 2004). Approximately 75% of all cheeses are produced using calf rennet (Ahmed et al., 2009b). However, in the last 10 years the demand for rennet has increased concomitantly with the increase in both the world population and global cheese consumption (Elsamani et al., 2014; Tajalsir et al., 2014). Consequently, the cost of calf rennet has increased while the quantity of available rennet has decreased (Ahmed et al., 2010). Chymosin/milk-clotting enzymes are now also derived from microbial and plant sources and from genetically engineered rennet as substitutes for calf rennet.
Microbial sources, such as bacteria and fungi, have been considered as potential rennet substitutes (Rao, 1984). Mohanty et al. (1999) reported that microbial rennet is used in more than half of the cheese produced in the world. Proteases from *Rhizomucor miehei*, *Rhizomucor pusillus* and *Cryphonectria parasitica* have been widely applied in commercial cheese production (Phelan, 1985; Van Den Berg, 1992). However, as animal chymosin substitutes, microbial renin still present some problems during cheesemaking. Animal chymosin has a milk-clotting activity of approximately 1.5 times higher than microbial chymosin. In addition, when using microbial chymosin it is difficult to form a hard curd, due to significant fat and protein loss (Mohanty et al., 1999). Although improvements in yield, off flavours and bitter taste resulting from the use of microbial chymosin, have been achieved through modified enzyme technology or mixing microbial rennin with other proteases, microbial chymosin still cannot totally replace animal rennet in the manufacture of cheeses at industrial scale (Rao, 1984). In the early 1990s, fermentation-derived chymosin (FDC) was the first enzyme approved by the American Food and Drug Administration (FDA) to be used in cheesemaking. Not only in America, but also many other parts of the world also applied recombinant DNA biotechnology to produce chymosin by fermentation. FDC currently represents approximately 80% of the global market for coagulants (Johnson and Lucy, 2006). Compared to rennet, the use of FDC in cheesemaking improves the cheese yield (Barbano and Rasmussen, 1992). FDC was also reported to improve the thermal stability, the control of proteolysis and the maintenance of curd pliability, which in turn contributed to better cheese quality and yield in Swiss and Mozzarella cheese (Johnson and Lucy, 2006).

The application of calf rennet can be limited for religious (for example, Judaism and Islam), dietary (vegetarianism), and safety (bovine spongiform encephalopathy) reasons, and also when consumers do not accept recombinant engineered food (Germany and Netherlands) (Badgujar and Mahajan, 2014; Roseiro et al., 2003). In these situations, milk-clotting proteases with rennet-like properties that are from plant sources have been investigated and applied in the cheesemaking industry. A number of plant sources have been shown to have high potential to be considered as rennet substitutes and have already been regularly used in the cheesemaking process since ancient times (Huang et al., 2011; Katsaros et al., 2010). Plant enzyme induced cheeses are mainly produced in Mediterranean, West African and southern European countries (Shah et al., 2014). This research project will mainly focus on the first stage of cheese making, which is enzyme induced milk-clotting. The review will describe milk and its components, and different proteases and their application in cheesemaking.
2.2 Milk constituents

The composition of bovine milk is approximately 87% water, 4.6% lactose, 3.4% protein, 4.2% fat, 0.8% minerals and 0.1% vitamins (Lindmark Månsson, 2008). The content of these components in milk vary as a result of species, breed, health, nutritional status, lactation period, climate factors, age, feeding and milking intervals (Fox and McSweeney, 1998). The principal constituents in milk are shown in Table 2.1.

Table 2.1 Principal composition of milk$^{a}$ (Walstra et al., 2006)

<table>
<thead>
<tr>
<th>Component</th>
<th>Average content in milk (% w/w)</th>
<th>Range$^{b}$ (% w/w)</th>
<th>Average content in dry matter (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87.1</td>
<td>85.3 - 88.7</td>
<td>—</td>
</tr>
<tr>
<td>Solids-not-fat</td>
<td>8.9</td>
<td>7.9 – 10.0</td>
<td>—</td>
</tr>
<tr>
<td>Fat in dry matter</td>
<td>31.0</td>
<td>22.0 – 38.0</td>
<td>—</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.6</td>
<td>3.8 – 5.3</td>
<td>36</td>
</tr>
<tr>
<td>Fat</td>
<td>4.0</td>
<td>2.5 – 5.5</td>
<td>31</td>
</tr>
<tr>
<td>Protein$^{c}$</td>
<td>3.3</td>
<td>2.3 – 4.4</td>
<td>25</td>
</tr>
<tr>
<td>casein</td>
<td>2.6</td>
<td>1.7 – 3.5</td>
<td>20</td>
</tr>
<tr>
<td>Mineral substances</td>
<td>0.7</td>
<td>0.57 – 0.83</td>
<td>5.4</td>
</tr>
<tr>
<td>Organic acids</td>
<td>0.17</td>
<td>0.12 – 0.21</td>
<td>1.3</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>0.15</td>
<td>—</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^{a}$ General milk of lowland breeds.

$^{b}$ These values will rarely be exceeded, e.g., in 1 to 2% of samples of separate milkings of healthy individual cows, excluding colostrum and milk drew shortly before parturition.

$^{c}$ non-protein nitrogen compounds are not included.

2.2.1 Lipids

The lipids exist as microscopic globules in bovine milk similar to an oil-in-water emulsion (MacGibbon and Taylor, 2006). They consist of approximately 98% triglycerides, about 1% diacylglycerol, less than 0.5% cholesterol, around 1% phospholipids, 0.1% free fatty acids (FFA) and trace amounts of ether lipid, hydrocarbons, fat-soluble vitamins, flavour compounds and compounds introduced by the feed (Jensen and Newburg, 1995; Fox and McSweeney, 1998; Parodi, 2004). The principal constituents are shown in Table 2.2.
Milk lipids are present as droplets containing a lipid core enveloped by a membrane. These fat droplets have diameters ranging from 2 to 5 μm. The fat droplets in milk are assembled in the endoplasmic reticulum in the epithelial cells of the alveoli (Lindmark Måansson, 2008). When fat droplets move toward the apical cell membrane and are extruded into the alveolar lumen, the globule is coated with cell membrane (consisting of proteins and polar lipids), known as the milk fat globule membrane (MFGM) (Buchheim, 1986; Keenan and Dylewski, 1985; Mulder and Walstra, 1974). The MFGM acts as a barrier between the serum and the lipids to protect the globules from coalescence and enzymatic degradation (El-Loly, 2011). According to Walstra (2006), the constituents of the MFGM include protein, phospholipids, cerebrosides, cholesterol, monoglycerides, water and trace amounts of minerals and vitamins. The predominantly lipids in the MFGM consist of 35% phosphatidyl choline (Ph. choline), 30% phosphatidyl ethanolamine (Ph. Ethanolamine), 25% sphingomyelin, 5% phosphatidyl inositide (Ph. inositide) and 3% glucosylceramide (Danthine et al., 2000; Deeth, 1997). While the major proteins in the MFGM include mucin 1, xanthine dehydrogenase/oxidase, butyrophilin and adidophilin (Pallesen et al., 2001; Berglund et al., 1996; Spitsberg and Gorewit, 1998; Nielsen et al., 1999).

The primary nutritional function of fat in milk is to provide energy. In addition, milk lipids also provide essential fatty acids (EFA), such as linoleic and linolenic acid, and it is a solvent for the fat soluble vitamins A, D and E (Walstra et al., 2006). In some dairy products, milk fat plays an important role in their flavour and texture (Fox and McSweeney, 1998).
Table 2.2 Composition and concentrations of lipids in fresh whole bovine milk (Walstra et al., 2006).

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Alcohol residue</th>
<th>Other constituent</th>
<th>Molecular weight</th>
<th>Milk Fat (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral glycerides</td>
<td></td>
<td></td>
<td>98.70</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>Glycerol</td>
<td></td>
<td>728</td>
<td>98.30</td>
</tr>
<tr>
<td>Diglycerides</td>
<td>Glycerol</td>
<td></td>
<td>536</td>
<td>0.30</td>
</tr>
<tr>
<td>Monoglycerides</td>
<td>Glycerol</td>
<td></td>
<td>314</td>
<td>0.03</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td></td>
<td></td>
<td>253</td>
<td>0.1</td>
</tr>
<tr>
<td>Phospholipids (Ph.)¹</td>
<td></td>
<td>Phospho group</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Ph. Choline (lecithin)</td>
<td>Glycerol</td>
<td>Choline</td>
<td>764</td>
<td>0.27</td>
</tr>
<tr>
<td>Ph. Ethanolamine²</td>
<td>Glycerol</td>
<td>Ethanolamine</td>
<td>742</td>
<td>0.26</td>
</tr>
<tr>
<td>Ph. Serine²</td>
<td>Glycerol</td>
<td>Serine</td>
<td>784</td>
<td>0.03</td>
</tr>
<tr>
<td>Ph. Inositol³</td>
<td>Glycerol</td>
<td>Inositol</td>
<td>855</td>
<td>0.04</td>
</tr>
<tr>
<td>Sphingomyelin⁴</td>
<td>Sphingosine</td>
<td>Choline</td>
<td>770</td>
<td>0.20</td>
</tr>
<tr>
<td>Cerebrosides³,⁴</td>
<td>Sphingosine</td>
<td>Hexose</td>
<td>770</td>
<td>0.10</td>
</tr>
<tr>
<td>Gangliosides³,⁴</td>
<td>Sphingosine</td>
<td>Hexose⁵</td>
<td>~1600</td>
<td>0.01</td>
</tr>
<tr>
<td>Sterols</td>
<td></td>
<td></td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td>387</td>
<td>0.30</td>
</tr>
<tr>
<td>Cholesteryl esters</td>
<td>Cholesterol</td>
<td></td>
<td>642</td>
<td>0.02</td>
</tr>
<tr>
<td>Carotenoids and Vitamin A</td>
<td></td>
<td></td>
<td>-</td>
<td>0.002</td>
</tr>
</tbody>
</table>

¹ Approximately 1% is present as lysophosphatides.

² Phosphatidylethanolamine + phosphatidylserine = cephalin.

³ Glycolipids.

⁴ Sphingolipids.

⁵ Also neuraminic acid.

### 2.2.2 Carbohydrates

Lactose (0-4-D-galactopyranosyl-(1,4)-glucopyranose) is the main carbohydrate in milk, but bovine milk also contains a trace amount of other carbohydrates, such as glucose, fructose, galactose, and oligosaccharides (Fox and McSweeney, 1998). Lactose is a disaccharide composed of D-glucose and D-galactose, that are connected by the aldehyde group of the
galactose with the C-4 group of the glucose through a β-1,4-glycosidic bond (Walstra et al., 2006; Guetouache et al., 2014). The structure of lactose is shown in Figure 2.1. The principal function of lactose in milk is to provide energy to the young offspring. It also contributes to the sweet flavour of milk. In the production of fermented dairy products (yoghurt and cheese), lactose is a substrate for lactic acid bacteria, especially *Lactobacillus* and *Lactococcus* during the acidification processes (Guetouache et al., 2014; Spreer, 1998; Fox and McSweeney, 1998).

![Structure of lactose](image)

**Figure 2.1** Structure of lactose.

### 2.2.3 Proteins

Proteins account for approximately 3.0-3.5% of normal bovine milk. Their nutritional function is to provide essential amino acids for muscular development and other protein-related tissues (Phadungath, 2005). Moreover, milk protein also supplies biological active proteins such as immunoglobulins, vitamin-binding and metal-binding proteins (Fox and McSweeney, 1998). In milk proteins consist of approximately 80% (w/w) caseins and 20% (w/w) whey proteins. The casein in bovine milk is comprised of α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, while the whey proteins include β-lactoglobulin (β-Lg), α-lactalbumin (α-Lac), bovine serum albumin (BSA), lactoferrin, immunoglobulins, enzymes and growth factors (McGregor and Poppitt, 2013). The approximate protein content in bovine milk is shown in Table 2.3.
Table 2.3 Approximate protein content in milk (Walstra et al., 2006).

<table>
<thead>
<tr>
<th>Name of protein</th>
<th>Concentration in milk (% w/w)</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>2.60</td>
<td></td>
</tr>
<tr>
<td>α_{s1}-Casein</td>
<td>1.07</td>
<td>23600</td>
</tr>
<tr>
<td>α_{s2}-Casein</td>
<td>0.28</td>
<td>25200</td>
</tr>
<tr>
<td>β-Casein</td>
<td>0.86</td>
<td>23983</td>
</tr>
<tr>
<td>κ-Casein</td>
<td>0.31</td>
<td>19550</td>
</tr>
<tr>
<td>γ-Casein</td>
<td>0.08</td>
<td>20500</td>
</tr>
<tr>
<td>Serum protein</td>
<td>0.63</td>
<td>-</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>0.32</td>
<td>18283</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>0.12</td>
<td>14176</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>0.04</td>
<td>66267</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>0.08</td>
<td>4000-40000</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>0.08</td>
<td>-</td>
</tr>
<tr>
<td>IgG1, IgG2</td>
<td>0.065</td>
<td>150000</td>
</tr>
<tr>
<td>IgA</td>
<td>0.014</td>
<td>385000</td>
</tr>
<tr>
<td>IgM</td>
<td>0.005</td>
<td>900000</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>0.09</td>
<td>-</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.001</td>
<td>86000</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.0001</td>
<td>76000</td>
</tr>
<tr>
<td>Membrane protein</td>
<td>0.07</td>
<td>-</td>
</tr>
<tr>
<td>Enzymes</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.2.3.1 Caseins

Caseins make up approximately 80% of the total protein of milk proteins (Brunner, 1997). Caseins are relatively small proteins with molecular masses ranging from approximate 19 to 25 kDa (Vincent et al., 2016). The genus of caseins in milk was defined by the American Dairy Science Association Committee (Jenness et al., 1956) based on the properties of those phosphoproteins precipitated by reducing the pH of raw milk to 4.6 at 20°C. According to DNA sequences analysis, the caseins consist of four types, α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein (Walstra et al., 1999). Trace amounts of γ-casein are also present in milk naturally, and
are generated from β-casein under the action of plasmin (Swaisgood, 1992). The properties of the caseins in bovine milk are presented in Table 2.4.

Apolar amino acids, such as Val, Leu, Ile, Phe, Tyr and Pro, account for approximately 35-45% of the total amino acids of all the caseins, resulting in the low solubility of caseins in an aqueous solution. κ-Casein contains high level of phosphate groups and carbohydrate and low amounts of the sulphur-containing amino acids (Gennadios, 2002). Genetic variants of the major caseins, together with variability in the number of phosphoseryl residues, have been reported. Specifically, αs2-casein contains variable numbers of phosphoseryl residues, while there is only one phosphorylated phosphoseryl residue in κ-casein (Phadungath, 2005). In addition, caseins contain high numbers of proline residues, specifically there are 17, 10, 35 and 20 proline residues/mole of αs1-casein, αs2-casein, β-casein and κ-casein, respectively. The large amount of proline residues inhibits the formation of α-helical and β-sheet, which results in a lack of secondary structure in caseins (Swaisgood, 1992). Therefore, caseins are more sensitive to proteolysis due to their lack of high level structure (Fox and McSweeney, 1998). As most caseins are composed principally of primary structure, caseins exhibit high stability against denaturation because there is little structure to unfold. Walstra (2006) also reported that caseins have little tertiary structure because they contain a high level of proline residues and few cysteine residues. According to Fox and McSweeney (1998), αs2-casein and κ-casein contain only two cysteine residues/mole and there are no sulphur amino acids in αs1-casein and β-casein. As the high amount of apolar residues are not uniformly distributed on the primary structures of the caseins, this results in the occurrence of hydrophobic and hydrophilic regions, giving caseins emulsifying and foaming properties.

There are 8-10 seryl phosphate groups in αs-casein while there are 5 phosphoserine residues in β-casein, making β-casein more hydrophobic than αs-casein (Phadungath, 2005). There is 5% of carbohydrate (N-acetyleneuraminic acid, galactose and N-acetylgalactosamine) associated with κ-casein, and these carbohydrates are mainly connected through an O-threonyl bond to Thr131, which is located at the C-terminal group of κ-casein. Therefore, the C-terminal of κ-casein tends to be more hydrophilic due to the high amount of carbohydrate and the trace amount of apolar and aromatic residues, while the N-terminal group of κ-casein is quite hydrophobic (Fox and McSweeney, 1998).

The influence of Ca2+ on the individual caseins is well documented, with both αs-casein and β-casein susceptible to precipitation when Ca2+ is present in excess. Compared to αs-casein and
β-casein, κ-casein is more stable to the presence of Ca\(^{2+}\) because it contains carbohydrates and only one phosphoserine group (Horne, 2002). The genetic variants of α\(_{s1}\)-caseins were insoluble when Ca\(^{2+}\) was present. Both α\(_{s1}\)-casein B and C precipitate when the concentration of Ca\(^{2+}\) is higher than 4 mM, while α\(_{s1}\)-casein A is soluble in up to 0.4 M Ca\(^{2+}\) but its solubility is dependent on the temperature. The solubility of α\(_{s2}\)-casein is very low when concentration of Ca\(^{2+}\) is above approximately 4 mM. Compared with α\(_{s}\)-casein, β-casein is soluble at low temperatures (below 18°C) even with 0.4 M Ca\(^{2+}\). However, when the temperature is increased to more than 18°C, β-casein becomes insoluble even with a low concentration of Ca\(^{2+}\) (4 mM). In addition, κ-casein is soluble in the presence of Ca\(^{2+}\), and plays an important role in the stability of the casein micelles against aggregation and precipitation (Whitney, 1998; Walstra et al., 1999).
Table 2.4 Chemical properties of the major caseins in bovine milk (Eigel et al., 1984; Fox and Mulvihill, 1983; Farrell et al., 2004)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Percentage of total casein (%)</th>
<th>Genetic variants&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Molecular mass&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Isoionic point</th>
<th>Phosphate residues (mol/mol protein)</th>
<th>Proline residues (mol/mol protein)</th>
<th>Hydrophobic regions</th>
<th>Sulphhydryl groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>α&lt;sub&gt;s1&lt;/sub&gt;-Casein</td>
<td>44-46</td>
<td>B</td>
<td>23,615</td>
<td>4.92-5.05</td>
<td>8-10</td>
<td>17</td>
<td>1-44, 90-113, 132-199</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>23,542</td>
<td>5.00-5.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α&lt;sub&gt;s2&lt;/sub&gt;-Casein</td>
<td>12</td>
<td>A</td>
<td>25,226</td>
<td>-</td>
<td>10-13</td>
<td>10</td>
<td>90-120, 160-207</td>
<td>2</td>
</tr>
<tr>
<td>β-Casein</td>
<td>32-35</td>
<td>A&lt;sup&gt;1&lt;/sup&gt;</td>
<td>24,023</td>
<td>5.41</td>
<td>4-5</td>
<td>35</td>
<td>2/3 of C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>23,983</td>
<td>5.30</td>
<td></td>
<td></td>
<td></td>
<td>terminal end</td>
</tr>
<tr>
<td>κ-Casein</td>
<td>8-12</td>
<td>A</td>
<td>19,037</td>
<td>5.53</td>
<td>1</td>
<td>20</td>
<td>5-65, 105-115</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>19,006</td>
<td>5.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Major variants

<sup>2</sup> Molecular mass was calculated as formula weight from composition. All acidic groups are protonated; all basic groups are not protonated. The major disulphide linkages are taken into account; there are no disulphides in κ-casein, but its N-terminal pyroglutamic is included.
2.2.3.1.1 $\alpha_{s1}$-Casein

$\alpha_{s1}$-Casein makes up to 40% of the bovine casein in milk and is comprised of one major and one minor component identified as $\alpha_{s1}$-casein and $\alpha_{s0}$-casein, respectively (Whitney, 1988). These proteins have the same amino acid sequence but differ in their degree of phosphorylation, as $\alpha_{s1}$-casein has one more phosphorylated serine residue at position 41 (Eigel et al., 1984). $\alpha_{s1}$-Casein consists of 199 amino acids, with a molecular weight ranging from 22,068 to 23,724 Da due to the existence of a number of genetic variants. The primary structure of $\alpha_{s1}$-casein is given in Figure 2.2. $\alpha_{s1}$-Casein contains a high amount of negative charge and phosphate content (Goff, 2014). There are 8 phosphoserine residues present in the amino acid sequence of $\alpha_{s1}$-casein, and 7 of them are located in positions from 43 to 80. In addition, there are 12 carboxyl groups present in this sequence region (Belitz et al., 2009; Whitney, 1988). This hydrophilic region is also acidic and can bind Ca$^{2+}$ ions. The most susceptible bond in bovine $\alpha_{s1}$-casein to the action of rennet is Phe$^{23}$-Phe$^{24}$ resulting in the generation of $\alpha_{s1}$-fraction 1-23 and fraction 24-199 (McSweeney et al., 1993).

\[
\begin{align*}
1 & \quad H-RPKHIPKHQG \quad LPQEVLNENL \quad LRFFVAPFPE \quad VFGKEKVNEL \\
41 & \quad SKDIGSESTE \quad DQAMEDIKQM \quad EAESISSSEE \quad IVPNSVEQKH \\
81 & \quad IQKEDVPSES \quad YLGYLEQLLR \quad LKKYKVQPLE \quad IVPNSAEERL \\
121 & \quad HSMKEGIAHQ \quad QKEPMIGVNO \quad ELAYFYPELF \quad RQFYQLDAYP \\
161 & \quad SGAWYYVPLG \quad TQYTDAPSFS \quad DIPNPIGSEN \quad SEKTMTPLW-OH \\
\end{align*}
\]

Figure 2.2 Primary structure of $\alpha_{s1}$-casein (Farrell et al., 2004). The sequence was obtained from ExPASy, file name P02662 (CASA1_BOVIN).

2.2.3.1.2 $\alpha_{s2}$-Casein

$\alpha_{s2}$-Casein constitutes approximately 10% of the total caseins in bovine milk and has A, B, C and D genetic variants. It is comprised of 207 amino acid residues with a molecular weight of 25,226 Da for its variant A (Farrell et al., 2004). The primary structure of $\alpha_{s2}$-casein is shown in Figure 2.3. At the neutral pH of milk (pH ~6.7), $\alpha_{s2}$-casein is present as a dipolar structure.
with anionic groups located at the N-terminus (which consists of phosphoseryl and glutamyl residues), and cationic groups (which contain 47 residues with positive net charge) present in the C-terminus (Belitz et al., 2009; Swaisgood, 1992). Hence, α_s2-casein is the most hydrophilic of all the caseins. There are 11 phosphoserines and 2 cysteine residues and no carbohydrate present in α_s2-casein. The two cysteine residues are able to form a α_s2-casein dimer through a disulphide linkage. The presence of 11 phosphoserine residues means α_s2-casein is very susceptible to Ca\(^{2+}\) and can form a calcium-binding precipitate. α_s2-casein is also sensitive to hydrolysis by proteases, with the regions of chymosin activity of 88 to 98 and 164 to 180, and a cleavage site on the α_s2-casein primary structure occurring at Phe\(^88\)–Tyr\(^89\) (McSweeney et al., 1994), although the cleavage levels are considerably lower than for those observed for the action of chymosin on κ-casein.

![Figure 2.3](image)

**Figure 2.3** Primary structure of α_s2-casein (Farrell et al., 2004). The sequence was obtained from ExPASy, with the file name P02663 (CASA2_BOVIN).

### 2.2.3.1.3 β-casein

β-Casein accounts for approximately 45% of the total bovine caseins. It consists of 209 amino acid residues with molecular weights between 23,944 to 24,902 Da due to a number of genetic variants. The primary amino acid sequence of β-casein is shown in Figure 2.4. β-casein is the most hydrophobic casein as it contains a large amount of proline residues. It also contains 5 phosphoserine residues that are located in the hydrophilic N-terminus in positions 1-40. In addition, apolar residues are present in the 136-209 positions of the C-terminal. Belitz (2009)
stated that β-casein does not contain cysteine residues. β-casein also forms precipitates in the presence of Ca$^{2+}$ at the level found in milk. However, at a temperature lower than 1°C, the calcium bound β-casein becomes soluble. The activity of the protease plasmin on β-casein generates 29-209, 106-209 and 108-209 fragments which are known as γ1-casein, γ2-casein and γ3-casein, respectively (Farrell et al., 2004). Farrell (2011) reported that sites 189-190 or 192-193 are particularly susceptible to the action of chymosin, although at cleavage levels considerably lower than for those observed for the action of chymosin on κ-casein.

![Primary structure of β-casein](image)

Figure 2.4 Primary structure of β-casein (Farrell et al., 2004). The sequence was obtained from ExPASy, file name P02666 (CASB_BOVIN).

### 2.2.3.1.4 κ-Casein

In bovine milk, κ-casein makes up 10-12% of the caseins. It contains 169 amino acid residues, with molecular weights ranging from 19,006 to 19,037 Da due to a number of genetic variants. The primary structure of κ-casein is shown in Figure 2.5. Generally, κ-casein contains 1 phosphoserine and 2 cysteine residues. With the presence of cysteine residues, the majority of κ-casein exists as a trimer or higher oligomer due to the formation of disulphide bonds (Belitz et al., 2009). Carbohydrates, which consist of approximately 1% galactose, 1.2% galactosamine and 2.4% N-acetyl neuramic acid, are bound to κ-casein through the amino acid residues Thr$^{131}$, Thr$^{133}$, Thr$^{135}$ and Thr$^{136}$. In the presence of Ca$^{2+}$, at the levels found in milk, κ-casein is the only casein that can remain soluble. κ-casein can also prevent both αs1-casein and β-casein
from aggregating in the presence of Ca\(^{2+}\) in milk. This function of κ-casein is essential in maintaining the stability of the caseins and casein micelles in milk. Chymosin has very high specific cleavage activity on the Phe\(_{105}\)-Met\(_{106}\) peptide bond in κ-casein. One peptide generated, para-κ-casein (amino acids 1-105), precipitates in the presence of Ca\(^{2+}\) while the other, glycomacropeptide (GMP, amino acids 106-169), remains soluble. The protective effect of κ-casein on the casein micelle stability is lost after hydrolysis of κ-casein by chymosin resulting in the coagulation of casein and casein micelles (Jollès et al., 1963).

**Figure 2.5** The primary structure of κ-casein (Farrell et al., 2004). The sequence was obtained from ExPASy with the file name P02668 (CASK_BOVIN).

### 2.2.3.2 Whey proteins

Milk whey proteins include β-lactoglobulin (β-Lg), α-lactalbumin (α-Lac), bovine serum albumin (BSA), lactoferrin, immunoglobulins (Ig) and other trace amount components (Madureira et al., 2007). Whey proteins remain soluble in milk serum through the isoelectric precipitation of the caseins by acidification or rennet coagulation. Acid, basic, hydrophobic and hydrophilic amino acids are uniformly distributed along the polypeptide chains of whey proteins which are globular molecules with large amounts of α-helix motifs (Evans, 1982). The chemical and physiochemical properties of the major whey proteins found in bovine milk are presented in Table 2.5. The pH of whey protein is approximately 5.1 when obtained from acidification of milk and 5.6 when obtained from rennet coagulation of milk (Pintado et al., 2001). Whey proteins have tight hydrophobic folded peptide chains. A significant difference
to caseins is that the whey proteins contain relatively high amounts of secondary and tertiary structure (α-helix and β-sheet).

Table 2.5 Chemical and physiochemical properties of the major whey proteins in bovine milk (Madureira et al., 2007; Farrell et al., 2004)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration in skim milk (g/L)</th>
<th>Molecular weight (Da)</th>
<th>Number of amino acids residues</th>
<th>Isoelectric point</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactoglobulin</td>
<td>1.3</td>
<td>18,277</td>
<td>162</td>
<td>5.35</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>1.2</td>
<td>14,175</td>
<td>123</td>
<td>5.10</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.4</td>
<td>66,267</td>
<td>582</td>
<td>5.13</td>
</tr>
<tr>
<td>Immunoglobulins (A, M and G)</td>
<td>0.7</td>
<td>25,000 - light chain, 50-70,000 - heavy chain</td>
<td>-</td>
<td>5.80-7.30</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.1</td>
<td>80,000</td>
<td>700</td>
<td>8.95</td>
</tr>
</tbody>
</table>

1 De Wit (1998).
2 Eigel et al. (1984).
3 Brew et al. (1970)
4 Korhonen (1995)
5 Conrado et al. (2005)

2.2.3.2.1 β-Lactoglobulin

β-Lactoglobulin (β-LG) is the major whey protein and accounts for 58% of the total whey proteins. It consists of 162 amino acid residues with molecular weights of 18,277 and 18,366 Da for the B and A genetic variants, respectively (Eigel et al., 1984). β-LG is composed of a high amount of β-sheet and also contains 2 disulphide bonds and 1 free sulfhydryl group. The structure of β-LG is generally dependent on the pH, it exists as a stable dimer with a molecular weight of approximately 36,700 Da at pH values between 5.2, and 7, i.e. the natural pH of milk (Walstra et al., 2006). When the pH is between 5.2 and 3.5, β-LG exists as an octamer with a molecular weight of approximately 140,000 Da. However, at pH 3 or above pH 8, β-LG exists
as a monomer (De Wit, 1989). According to Walstra et al. (2006), β-LG changes its tertiary structure into a monomer when the reactive sulphydryl groups are exposed. The denaturation of β-LG was reported by Belitz et al. (2009) to occur when the pH was above 8.6, or when a high temperature or a high concentration of Ca\textsuperscript{2+} was applied.

2.2.3.2.2 α-Lactalbumin

The second major whey protein is α-lactalbumin (α-Lac), which accounts for 20% (w/w) of the total whey protein. α-Lac is synthesized in the mammary gland and contributes to the biosynthesis of lactose as a coenzyme (De Wit, 1998; Walstra et al., 2006). It consists of 123 amino acid residues, including 8 cysteines, with a molecular weight of 14,175 Da (Brew et al., 1970). Four disulphide bonds in α-Lac contribute to the stabilization of the globular structure over a pH range of 5.4-9.0 (Evans, 1982). There is a non-exposed Ca\textsuperscript{2+} binding site in α-Lac that also contributes to maintaining the stability of its conformation by tightly binding Ca\textsuperscript{2+}. Walstra et al. (2006) stated that the Ca\textsuperscript{2+} ion is lost from its binding site when the pH is lower than 4, resulting in the partial unfolding of α-Lac. Therefore, irreversible heat denaturation of partially unfolding of α-Lac can be performed even at low temperatures.

2.2.3.2.3 Bovine serum albumin

Bovine serum albumin (BSA) occupies approximately 1.5% of the total bovine milk protein and approximately 8% of the total whey protein (Farrell et al., 2004). BSA contains 582 amino acid residues, has a molecular weight of 66,276 Da, with 17 intermolecular disulphide bonds and one thiol group at residue 34 (Fox, 1989). It is a large molecule in the form of an elongated shape made up of three globular domains. Kinsella and whitehead (1989) indicated that BSA can complex with free fatty acids such as lipids and flavour compounds due its high level of structure and size. The characteristic of BSA is its resistance to denaturation by heating at pH 6.5 due to the interaction between the thiol group and an intermolecular disulphide bond.

2.2.3.2.4 Immunoglobulins

Immunoglobulins (Igs) represent approximately 6% of total whey protein and 1% of total milk protein. Each Ig molecule consists of two light chains with molecular weights of approximately 25,000 Da and two heavy polypeptide chains with molecular weights ranging from 50,000 to 70,000 Da (Mulvihill and Donovan, 1987). The various basic classes of Ig present in milk include G (gamma globulins), A and M (macroglobulins). IgG has the highest concentration of
the Igs (up to 80%). Chen and Chang (1998) reported that IgG (0.02 mg/mL) was susceptible to denaturation at pH values lower than 4 or higher than 10, or when heated at temperatures higher that 75°C. In addition, Devi et al. (2011) stated that bovine IgG (25 mg/mL) exhibited its highest thermal stability at pH 6 and 7. IgM comprises two types of lactenin, L₁ and L₃, which act as inhibitors of Gram-positive bacteria in milk. L₃ especially has high specificity against some strains of *Lactococcus lactis* (Walstra et al., 2006). Each IgM molecule contains at least one cryoglobulin molecule that is related to the flocculation of milk fat globules. Bacteria could therefore be bound to fat globules, accumulate in the cream layer and can be easily removed, thus inhibiting the growth of the bacteria.

2.2.3.2.5 Lactoferrin

Lactoferrin (LF) is an iron-binding monomeric glycoprotein, which contains 689 amino acid residues with a molecular weight of approximately 80,000 Da and two carbohydrate groups (De Wit, 1989; Lonnerdal and Iyer, 1995). The concentration of lactoferrin in bovine milk is low (approximately 0.02 to 0.35 mg/mL) and it can act as a bacteria inhibitor, especially for *Bacillus stearothermophilus* and *Bacillus subtilis*. The antibacterial action is related to the removal of the iron from the milk serum by lactoferrin (Walstra et al., 2006). Elagamy (2000) found that LF was denatured and lost its biological activity after heating bovine milk at 85°C for 30 min. At pH 7.0, the thermal denaturation temperature of bovine LF was 70 ± 1°C (Sreedhara, 2011). The thermal stability of bovine LF decreases with a decrease in pH from 7.0 to 3.0, while LF aggregates at pH 2.0.

2.2.4 Casein micelles

Most of the casein proteins (approximately 95%) are present in milk in the form of large colloidal particles, named casein micelles (Fox and McSweeney, 1998). On a dry basis, casein micelles are composed of 94% protein and 6% low molecular colloidal calcium phosphate (CCP). CCP consist of calcium, phosphate, and small amounts of magnesium and citrate (Fox and McSweeney, 1998). In addition, casein micelles are highly hydrated and bind around 2 to 4 g H₂O per g protein (Walstra, 1979; Jeurnink and De Kruif, 1993). The diameter of casein micelles ranges from 80 to 400 nm, and most of the casein micelles in bovine milk are approximately 200 nm (De Kruif, 1998; Udabage et al., 2003). The molecular weight of casein micelles varies between 10⁶ and 10⁹ Da (De Kruif, 1998; Fox, 2003).
As mentioned above, in bovine milk, both α- and β-caseins are rich in phosphorylated serine residues (phosphoserine, SerP) and contain the amino acid sequence SerP-SerPSerP-X-SerP. This large amount of phosphorylation contributes to the sequestration of Ca\(^{2+}\) ions, resulting in the precipitation of these caseins (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981). Calcium phosphate is bound to the casein in the casein micelle through phosphoseryl residues. κ-casein is the only phosphorylated casein that does not precipitate under high Ca\(^{2+}\) (Swaisgood, 2003). The hydrophobic region (1-105) of κ-casein is located inside the casein micelles (De Kruif and Holt, 2003). The glycomacropeptide portion (GMP, amino acid sequence 106-169) is the most hydrophilic part of the κ-casein, and interacts with water and extends 5-10 nm from the surface of the casein micelle to form a “hairy layer” (Horne, 1986; Horne and Davidson, 1986; De Kruif and Zhulina, 1996), contributing to the stability of the casein micelle.

In general, there are two methods to destabilise the casein micelle. One, where the GMP layer is removed by enzymatic (e.g. chymosin and pepsin) hydrolysis (Horne, 1986; Walstra et al., 1981; De Kruif, 1999). The other method is through acidification to decrease the pH from 6.7 to 5.5, resulting in a reduction of the charge on the GMP and the loss of the inter-chain repulsions (De Kruif and Zhulina, 1996; Donato et al., 2007; Horne, 2003). Aggregation of casein micelles is achieved when the pH of the milk approaches the isoelectric point at approximately pH 4.6.

### 2.2.4.1 Casein micelle structure

Although a number of models describing the casein micelle structure have been extensively studied, the exact structure of the casein micelle has not been confirmed so far and is still under debate. Three proposed models, the submicelle (or subunit) model (Slattery and Evard, 1973; Schmidt, 1982; Walstra, 1999), the dual-binding model (Horne, 1998) and the nanocluster model (Holt, 1992; De Kruif and Holt, 2003) are currently the most favoured. These three models are discussed below.

### 2.2.4.2 Submicelle (or subunit) model

The submicelle model was proposed by Slattery and Evard (1973) and amended by Schmidt (1980) (Figure 2.6). Submicelles are made of interacting casein monomers. Rollema (1992) stated that the most acceptable submicelle model was established by Walstra and Jenness
(1984). Each submicelle contains approximately 20-25 casein molecules, and the size of the submicelles vary between 12-15 nm in diameter. The interior of the casein micelles are made of submicelles which consist mainly of $\alpha_s$- and $\beta$-caseins (these submicelles containing little $\kappa$-casein are believed to be hydrophobic), while the exterior of the casein micelles are made up of submicelles which contain $\alpha_s$, $\beta$- and $\kappa$-caseins. Schmidt (1980) suggested that the connections between each submicelle are only through the colloidal calcium phosphate. Later, hydrophobic interactions between proteins were also suggested, in addition to calcium phosphate, for the connection between the submicelles (Rollema, 1992). Most of the $\kappa$-caseins are located at the surface of the casein micelles to provide electrostatic and steric stability (Walstra, 1999; Walstra et al., 1999). The size of casein micellar particles is limited by their full coverage by the hydrophilic $\kappa$-casein region. Therefore, $\kappa$-casein is responsible for the stabilisation and size distribution of the casein micelles (Brunner, 1977; Wong, 1988; Rollema, 1992).

![Submicelle model](image)

**Figure 2.6** Submicelle model (adapted from Walstra, 1999).

### 2.2.4.3 Nanocluster model

The nanocluster model was proposed by Holt (Holt, 1992; De Kruif and Holt, 2003) to describe the structure of casein micelles (Figure 2.7). Holt stated that the structure of casein micelles is made of interlaced network-like casein forming a gel-like structure. The flexible casein network is formed by cross-linking caseins through CCP located at casein phosphate centres. Both $\alpha_s1$- and $\alpha_s2$-casein contain at least two phosphoseryl clusters, which can form crosslinks by interacting with nanoclusters into 3-dimensional network structures (Horne, 2006). In addition, the C-terminal region of $\kappa$-casein protrudes from the surface of micelles to form a hairy layer. This hair layer has a zeta potential of approximately -20 mV at pH 6.7 and contributes to stabilising the casein micelle through steric repulsions (Holt, 1994; Holt and

![Figure 2.7](image-url) The schematic structure of casein in nanocluster model (adapted from Hristoy et al., 2016; De Kruif and Holt, 2003).

### 2.2.4.4 Dual-binding model

One of the most recent models is the dual-binding model (Figure 2.8) proposed by Horne (1998). In the dual-binding model, casein molecules are stuck together through attractive hydrophobic and electrostatic interactions. The crosslinks are formed by hydrophobic interactions between hydrophobic groups on the different casein molecules. For instance, it is assumed that the $\alpha_s^1$-casein molecule, which forms a train-loop-train structure with anchor points at both sides of the molecule, and the $\beta$-casein molecule, which forms a tail-train structure when they are adsorbed at hydrophobic interfaces (Dickinson et al., 1997), can associate through hydrophobic interactions and polymerise. In addition, the CCP nanoclusters present between phosphoseryl clusters on $\alpha_s^1$, $\alpha_s^2$ and $\beta$-casein also serve as crosslinks (Lucey, 2004). CCP acts as a bridge between the phosphoserine residues of the casein molecules to neutralise the negative charge by binding to those residues. This neutralisation reaction attracts more casein molecules resulting in more associations of casein molecules. However, $\kappa$-casein, which does not have phosphoseryl clusters, can only link to other casein molecules via the hydrophobic region on the N-terminal. Therefore, the hydrophilic C-terminal of $\kappa$-casein is stuck out to form an extended hairy layer on the outside of the casein micelle, providing electrostatic repulsion (Horne, 1998). $\kappa$-casein is therefore responsible of controlling the growth of the casein micelle.
2.2.5 Minerals

Minerals play an important role in milk, especially in the structure and stability of casein micelles (Gaucheron, 2004; Holt, 1997; Walstra and Jenness, 1984). Milk contains a small amount of mineral (approximately 8-9 g/l), including cations such as Ca$^{2+}$, Mg$^{2+}$, Na$^{+}$ and K$^{+}$ and anions such as Cl$^{-}$, CO$_3^{2-}$, SO$_4^{2-}$ and PO$_4^{3-}$. However, only part of the mineral salts are in the dissolved form, while the undissolved salts are contained in the casein micelles, especially the counter ions (such as Ca$^{2+}$, Mg$^{2+}$, Na$^{+}$ and K$^{+}$) of the negatively charged caseins (Walstra et al., 2006). The mineral content in milk varies depending on parameters such as breed, seasonal variation, and stage of lactation. The composition of minerals in milk is reported in Table 2.6.
Table 2.6 Composition of minerals in milk and their distribution in the serum and the casein micelle (Adapted from Walstra et al., 2006)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar Mass (Da)</th>
<th>Concentration in bovine milk(a) (mmol/kg)</th>
<th>Fraction in serum (mmol/g dry casein)</th>
<th>Fraction in micelles (mmol/g dry casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>23</td>
<td>17-28</td>
<td>0.95</td>
<td>0.04</td>
</tr>
<tr>
<td>K</td>
<td>39.1</td>
<td>31-43</td>
<td>0.94</td>
<td>0.08</td>
</tr>
<tr>
<td>Ca</td>
<td>40.1</td>
<td>26-32</td>
<td>0.32</td>
<td>0.77</td>
</tr>
<tr>
<td>Mg</td>
<td>24.3</td>
<td>4-6</td>
<td>0.66</td>
<td>0.06</td>
</tr>
<tr>
<td>Anions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl</td>
<td>35.5</td>
<td>22-34</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>CO(_3^2)</td>
<td>60</td>
<td>~2</td>
<td>~1</td>
<td>-</td>
</tr>
<tr>
<td>SO(_4^{2-})</td>
<td>96.1</td>
<td>~1</td>
<td>~1</td>
<td>-</td>
</tr>
<tr>
<td>PO(_4^{3-})</td>
<td>95</td>
<td>19-23</td>
<td>1</td>
<td>0.39</td>
</tr>
<tr>
<td>Citrate(c)</td>
<td>189</td>
<td>7-11</td>
<td>0.92</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(a\) Gaucheron, (2005).

\(b\) Inorganic only.

\(c\) \((\text{CH}_2 - \text{COO}^-) - (\text{COH} - \text{COO}^-) - (\text{CH}_2 - \text{COO}^-)\).

2.2.5.1 Minerals in solution

Holt (1981) calculated the free salt ion concentrations in the aqueous phase (Table 2.7), where calcium was present in three different forms: free ions as ionic calcium, complexed with citrate (mainly with trivalent citrate), and bound to inorganic phosphate (including H\(_2\)PO\(_4^-\) and HPO\(_4^{2-}\)) (Holt et al., 1981; Gaucheron, 2005). Other cations such as Na\(^+\) and K\(^+\) are present in the serum mainly as free ions and there is only a small amount of Na\(^+\) and K\(^+\) associated with Cl\(^-\), PO\(_4^{3-}\) and citrate.
Table 2.7 Concentration of free and complexed ions (mM) in the aqueous phase of milk (Holt et al., 1981).

<table>
<thead>
<tr>
<th>Anion ion</th>
<th>Free ion</th>
<th>Cation complex ion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca$^{2+}$</td>
</tr>
<tr>
<td>H$_2$Cit$^-$</td>
<td>+$^a$</td>
<td>+</td>
</tr>
<tr>
<td>HCit$^{2-}$</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>Cit$^{3-}$</td>
<td>0.26</td>
<td>6.96</td>
</tr>
<tr>
<td>H$_2$PO$_4^-$</td>
<td>7.50</td>
<td>0.07</td>
</tr>
<tr>
<td>HPO$_4^{2-}$</td>
<td>2.65</td>
<td>0.59</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>+</td>
<td>0.01</td>
</tr>
<tr>
<td>Glc 1-PH$^+$</td>
<td>0.50</td>
<td>+</td>
</tr>
<tr>
<td>Glc 1-P$^{2-}$</td>
<td>1.59</td>
<td>0.17</td>
</tr>
<tr>
<td>H$_2$CO$_3$</td>
<td>0.11</td>
<td>-</td>
</tr>
<tr>
<td>HCO$_3^-$</td>
<td>0.32</td>
<td>0.01</td>
</tr>
<tr>
<td>CO$_3^{2-}$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>30.90</td>
<td>0.26</td>
</tr>
<tr>
<td>HSO$_4^-$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>0.96</td>
<td>0.07</td>
</tr>
<tr>
<td>RCOOH</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>RCOO$^-$</td>
<td>2.98</td>
<td>0.03</td>
</tr>
<tr>
<td>Free ion</td>
<td>2.00</td>
<td>0.81</td>
</tr>
</tbody>
</table>

$^a$ Concentration detected at less than 0.005 mM.

- Not detected.

2.2.5.2 Colloidal calcium phosphate

Part of the undissolved mineral in milk exists in the casein micelles in the form of colloidal particles. On a dry basis, casein contains approximately 0.07 % (w/w) colloidal calcium phosphate, and also includes other salts such as K$^+$, Na$^+$, Mg$^{2+}$ and citrate (Table 2.8). These cation salts counter the negative charges on the phosphate group of the phosphoserine residues of the caseins by binding to the cation site on the casein (Gaucheron, 2005). According to Gaucheron (2005), the colloidal calcium in milk consists of a calcium caseinate which contains
organic phosphate and calcium phosphate (inorganic phosphate). There are relatively large amounts of calcium phosphate and calcium citrate present in the casein micelles. As stated in Section 2.2.4, Schmidt (1980) proposed that the CCP crosslinks internal submicelles, while Holt (1992) stated that the CCP crosslinks entangled the casein networks to form a gel-like structure. Horne (1997) suggested that the CCP in the dual-binding model links the phosphoseryl clusters of αs1-, αs2- and β-casein.

**Table 2.8 Composition of the minerals in the casein micelle (Belitz et al., 2009)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>93.2</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>2.9</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>0.1</td>
</tr>
<tr>
<td>K(^{+})</td>
<td>0.3</td>
</tr>
<tr>
<td>Na(^{+})</td>
<td>0.1</td>
</tr>
<tr>
<td>PO(_4^{3-}) (inorganic)</td>
<td>2.9</td>
</tr>
<tr>
<td>PO(_4^{3-}) (organic)</td>
<td>2.3</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.4</td>
</tr>
</tbody>
</table>

### 2.2.6 Enzymes

Indigenous enzymes exist in different parts of milk (Table 2.9). Some of these enzyme are associated with the fat globule membrane, while the rest are present in the milk serum (Walstra et al., 2006). Most of the enzymes in milk have no biological function or nutrition benefits, and have little influence on the milk, despite some of these enzymes having high concentrations.
Table 2.9 Approximate composition of enzymes in milk (Walstra et al., 2006)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme commission (EC) number</th>
<th>Optimum pH</th>
<th>Temperature (°C)</th>
<th>Location in milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine oxidase</td>
<td>1.1.3.22</td>
<td>~8</td>
<td>37</td>
<td>Fat globule membrane</td>
</tr>
<tr>
<td>Sulfhydryl oxidase</td>
<td>1.8.3.2</td>
<td>~7</td>
<td>~45</td>
<td>Plasma</td>
</tr>
<tr>
<td>Catalase</td>
<td>1.11.1.6</td>
<td>7</td>
<td>~37</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>1.11.1.7</td>
<td>6.5</td>
<td>20</td>
<td>Serum</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>1.15.1.1</td>
<td>-</td>
<td>~37</td>
<td>Plasma</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>3.1.1.34</td>
<td>~9</td>
<td>33</td>
<td>Casein micelles</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>3.1.3.1</td>
<td>~9</td>
<td>37</td>
<td>Fat globule membrane</td>
</tr>
<tr>
<td>Ribounclease</td>
<td>3.1.27.5</td>
<td>7.5</td>
<td>37</td>
<td>Serum</td>
</tr>
<tr>
<td>Plasmin</td>
<td>3.4.21.7</td>
<td>8</td>
<td>37</td>
<td>Casein micelles</td>
</tr>
</tbody>
</table>

One biological function of some of the enzymes in milk is antibacterial activity. Lactoperoxidase (approximately 0.4 μM - a relatively high concentration) can catalyse the oxidation of thiocyanate to inhibit most bacteria. Lysozyme is another antibacterial enzyme with an optimal activity at pH 6.35. Lysozymes are highly resistant to heat treatment and relatively stable at acid pH values between 3-4. In milk, lysozyme inhibits bacteria by hydrolysing the β (1-4)-linkage between the muramic acid and N-acetylglucosamine of the mucopolysaccharide bacterial cell walls (Fox and McSweeny, 1998). However, the activity of lysozyme in milk is relatively weak.

Plasmin is a serine protease in milk, which has a large range of pH and temperature stability. Generally, plasmin is isolated from milk at pH 3.5, and its optimal pH and temperature are at around 8 and 37°C, respectively (Fox and McSweeney, 1998). Plasmin is relatively stable under heating over the pH range of 4 to 9. The function of plasmin in milk is to hydrolyse proteins to generate large degradation peptide products. β-casein is very sensitive to the action of plasmin and yields γ-casein (Swaisgood, 1992). In addition, the primary proteolysis of casein by plasmin contributes to the rennet-induced coagulation of milk in cheesemaking (Kethireddipalli and Hill, 2015).
The main oxidoreductases in milk include xanthine oxidase and sulfhydryl oxidase. Xanthine oxidase has an optimal pH of approximately 8.5. This enzyme can convert nitrate into nitrite. Nitrite can inhibit the detrimental butyric acid bacteria which lead to texture and flavour defects in cheese (Walstra et al., 2006). Sulfhydryl oxidase exhibits an optimum pH between 6.8 and 7 (Janolino and Swaisgood, 1975). Sulfhydryl oxidase in milk has the capability of oxidizing the sulfhydryl groups of cysteine, glutathione and proteins into disulphides (Walstra et al., 2006). In processed milk products, the partial activity of sulfhydryl oxidase is inhibited by pasteurisation (Chandan and Kilara, 2010). However, the residual activity of the enzyme can reduce the cooked flavour caused by –SH compounds (Walstra et al., 2006).

Lipoprotein lipase, a serine lipase with a concentration of 10 to 20 nmol/L, is the principal lipolytic enzyme in bovine milk (Fox et al., 1967). Its optimal pH and temperature are approximately pH 8.5-9 and 33°C, respectively. This enzyme hydrolyses fatty acid, including tri- and diglycerides at the fat droplet interface (Walstra et al., 2006). Generally, there is not much hydrolysis occurring in the milk because most of the lipoprotein lipase (around 90%) is associated with the casein micelles, and the triglyceride is protected by the milk fat globule membrane (Fox and McSweeny, 1998).

2.3 Cheese

Cheese is one of the many processed milk products that retains some of milks essential nutrients, such as protein, fat and minerals. Milk coagulation is the basic step during cheese manufacture; after dehydration of the gel to form a curd, protein and fat in cheese can be concentrated up to 6-10 times higher than in the raw milk (Omotosho et al., 2011). Animal rennin (or rennet), especially calf rennet, has been applied in cheese making for a long time. The rennet is obtained from the fourth stomach (also known as the true stomach) of baby calves for commercial applications, and only 5 to 10 g of rennet can be obtained from each calf (Mahajan et al., 2014). Rennet has a high specificity to cleave the \text{Phe}^{105} \text{-Met}^{106} peptide linkage in \text{\kappa-casein} which destabilises the surface of the casein micelles, leading to milk-clotting (Pires et al., 1994; Vishwanatha et al., 2010).

Since 1961 the production of cheese has increased approximately 3.5 times, resulting in a universal shortage of rennet supply due to the limitation in the availability of suckling calves stomachs (Jacob et al., 2011). In addition, the usage of rennet has been restricted in some sectors for religious and diet (vegetarianism) reasons. For example, in India and Israel the use
of animal products may not be accepted for religious reasons. Vegetable enzymes, such as fig latex, are suitable to be applied in cheese manufacture as a rennet substitute (Whitaker, 1959; Gordin and Rosenthal, 1978). Also some countries, like Germany and Netherlands, prohibit the use of genetically engineered foods including recombinant calf rennet. The high price and usage limitations of calf rennet have encouraged the search for alternative milk-clotting enzymes from other sources (Roseiro et al., 2003).

To meet the requirement of increasing demand in the milk coagulant market due to the decrease in the natural rennet supply, new sources of proteases with rennet-like properties have been studied. Rennet substitutes, including microbial, recombinant and plant enzymes, have been studied for their potential in cheesemaking. Although enzyme sourced from microorganisms and genetically engineered microorganisms are suitable substitutes for animal rennet, there is a growing interest in the use of plant-based coagulants (Shah et al., 2014).

Plant sources have been used in cheesemaking since ancient times (Huang et al., 2011; Katsaros et al., 2010). In Mediterranean, West African, and southern European countries plant clotting enzymes are still widely used. In the Iberian Peninsula dried cardoon flowers of *Cynara cardunculus* L. and *Cynara humillis* L. have been used for centuries to prepare certain varieties of cheeses with a creamy soft-texture and exquisite flavour (Roseiro et al., 2003). Cheeses, such as the Portuguese Serra and Serpa cheese (Macedo et al., 1993), the Spanish Los Pedroches, La Serena (Roa et al., 1999), Torta del Casar cheeses made using ewes’s milk, Los Ibores cheese made using goat milk and a cheese made with a mixture of ewe and cow milk called Flor de Guía (Fernández-Salguero et al., 1991; Fernández-Salguero and Sanjuán, 1999; Sanjuán et al., 2002), are all made using plant coagulants. According to Roseiro (2003), protease extracted from *Calptropis procera* (Sodom apple) has been used in traditional cheesemaking in Nigeria and the Republic of Benin and some other West African countries. Plant coagulants, such as actinidin from kiwifruit, with a high specific activity for milk-clotting, have been identified as a potential rennet substitutes (Mazorra-Manzano et al., 2013). Unfortunately, most proteases extracted from plants, such as papain, ficin and bromelain, exhibit low milk-clotting activity due to their broad proteolytic activity and nonspecific hydrolysis of caseins, which consequently affects the texture, flavour and yield of cheese (Garg and Johri, 1994; Jacob et al., 2011; Lo Piero et al., 2002). Therefore, studies on new plant coagulants, as potential rennet substitutes, are being undertaken to meet the increased global requirement of high-quality cheese production (Hashim et al., 2011).
2.3.1 Cheese manufacture

Cheese manufacture involves aggregation of milk to form a milk gel, dehydration of the milk gel to form a curd, and curd ageing. The ageing periods of curd varies from 1 week for fresh cheeses or 2 weeks to 2 years for ripened cheeses (Fuquay et al., 2011). Although cheese manufacture involves different processing methods, some of the essential process steps are given below:

Clotting of the milk: Raw milk or pasteurized milk is mixed with a starter culture at 18-50°C in a vat (Belitz et al., 2009). The starter culture can include propionic acid or lactic acid bacteria, molds (such as Penicillium camemberti) and red or yellow smearing cultures. Generally acidification is caused by the addition of lactic acid bacteria culture which convert lactose into lactic acid. Fox et al. (2000) stated that it takes around 30-60 min after adding starter to cheese before rennet addition. Clotting of milk is achieved either enzymatically (at pH 6.6-6.3), by lactic acid fermentation (to pH 4.9-4.6) or by a combination of both (Belitz et al., 2009; Guinee and Wilkinson, 1992). In terms of enzymatic coagulation, the glycomacropeptide sequence is cleaved from κ-casein by the action of the enzyme, which results in casein micelles aggregation. This is the basic step in cheese manufacture by rennet (e.g. Cheddar, Gouda, Emmental and Parmesan), which accounts for approximately 77% of total cheese production (Guinee and Wilkinson, 1992). Kwak et al., (2002) reported on this milk-clotting step for Cheddar cheese manufacture. Milk was pasteurised at 72°C for 17 s, then a mesophilic lactic starter culture was added. After 40 min of ripening, rennet (0.019%) was added and it took approximately 40 to 50 min to form the curd. The formed curd was cooked at 34°C for 40 min and a whey acidity of approximately 0.18-0.19% was developed. Under acidic conditions, the dissolution of colloidal calcium phosphate in the micelles results in neutralising the electric charge on the casein particles. According to Belitz et al., (2009), the duration times of acidification are dependent on the cheese variety, with 5-6 h for Cheddar and cottage cheese and 10-12 h for Dutch and Swiss cheese types. In addition, the optimal curd pH for most hard cheeses is approximately 5.0-5.3 while it is 4.6 for the acid-coagulated soft cheeses such as cottage and cream cheeses. Both acidic and enzymatic aggregations form a protein network, which encloses the milk serum and fat globules (Walstra et al., 2006).

Removal of whey protein: The formed gel then undergoes spontaneous syneresis. Whey expulsion is generally enhanced by cutting the gel into pieces and stirring the curd-whey mixture. The curd obtained makes up 10 to 30% of the original volume of milk (Walstra et al.,
The drier the curd, the firmer and the more durable the cheese will be. The decrease of the pH of cheese curd influences parameters such as syneresis, consistency and ripening of the cheese. According to Fox et al. (2000), there are different methods to separate the curd from the whey after the degree of syneresis and desired pH has been achieved. Again the methods used depend on the variety of cheese: e.g., for the common soft cheeses such as Camembert, the curd-whey is transferred into perforated moulds to remove the whey. In Gouda and Emmental cheese manufacture, the whey is removed by sucking it off from the vat after the curds have settled. For Cheddar and Mozzarella cheese, whey is drained from the curds using perforated screens. Finally, the curd of Parmigiano-Reggiano cheese is scooped from the vat using cloths and placed in moulds.

**Salting:** Normally, cheese contains 1-4% added sodium chloride. Salt affects the durability, flavour and consistency of the cheese. In addition, salt also has an influence on the cheese ripening. According to (Fuquay et al., 2011), most cheeses are salted at the end of curd manufacture. There are a variety of salting methods, such as mixing curd chips with dry salt in Cheddar cheese, rubbing dry salt on the surface of pressed cheese (blue cheese) and submersing curd into salt brine (Gouda, Emmental and Camembert cheese).

**Fusion of curd grains into a coherent loaf:** Rind forms on the surface of the traditional aged cheese (Wolfe et al., 2014). The rinds are formed through the fusion of curd particles and then enhanced by local moisture loss around the cheese loaf (Walstra et al., 2006). The presence of a rind protects the interior of the cheese from further microbial contamination.

**Curing:** Ripening plays a key role in the flavour and texture of the cheese. The ripening time depends on the cheese variety (Fox et al., 2004; Sorrentino et al., 2013). Generally, the ripening periods of the rennet-induced cheeses range from approximately 2 weeks (Mozzarella cheese) to 2 or more years (Parmigiano-Reggiano cheese and extra-mature Cheddar cheese) (McSweeney, 2004). Different cheese varieties can be achieved by keeping the cheese under suitable ripening conditions. For example, in the industrial scale manufacture of pasta filata cheeses (Scamorza), the ripening of the cheeses is conducted at approximately 12-16°C with 85% relative humidity. However, in small scale production, the ripening of traditional pasta filata cheeses is performed at around 15°C in refrigerated chambers without controlled airflow and at 50% relative humidity (Niro et al., 2012). The storage conditions also depend on the type of cheese.
2.3.2 Enzymes used in cheese manufacture

2.3.2.1 Rennet and chymosin

Historically, most enzyme preparations used for cheese manufacture have been extracted from the fourth stomach of ruminants, mostly calves. The stomach-extracted rennet contains two proteolytic enzymes, chymosin and pepsin. Chymosin is a neonatal, gastric, aspartic protease. Generally, rennet is composed of between 50% to 95% chymosin, depending on the age and previous diet of the animals (Jacob et al., 2011). Mature animal rennet extracts consist of 90-94% pepsin and only 6-10% chymosin (Broome and Limsowtin, 1998). In general, rennet extracted from milk-fed calves that are 3 weeks-old contains over 90% chymosin, and the rest is pepsin. Chymosin plays a key role in milk-clotting, as it has a high specificity for hydrolysing the Phe\textsubscript{105}-Met\textsubscript{106} bond of \( \kappa \)-casein resulting in milk-clotting (Hyslop, 2003; Crabbe, 2004). The pepsin protein cleavage sites are at Phe, Tyr, Leu or Val residues (Agudelo et al., 2004; Papoff et al., 2004).

Chymosin belongs to the aspartic group of proteases (EC 3.4.23.4), and its molecular weight is approximately 40 kDa (Andren, 2003; Foltmann, 1993). As an aspartic protease, chymosin is an endopeptidase, which means that it can split proteins into relatively large fragments. Chymosin A (Asp in position 254) and chymosin B (Gly in position 254), the two major forms of calf chymosin that have been characterised, are allelic variants of a single gene locus (Donnelly et al., 1984). Compared with chymosin B, chymosin A expresses a higher hydrolysis activity, but chymosin A also shows a higher autocatalytic activity (Rampilli et al., 1992; Lilla et al., 2005). Chymosin C has also been found and identified (Foltmann, 1964; Foltmann et al., 1997). Chymosin C is believed to be either the product of the autolytic degradation of chymosin A (Danley and Geoghegan, 1988) or a distinct genetic product (Donnelly et al., 1986; Rampilli et al., 2005; Wislinski and Popielarz, 1994). Chymosin C has been reported as the most suitable chymosin for cheesemaking, as it has nearly 20% higher milk clotting activity than chymosin B. However, only trace amounts of chymosin C exist in rennet (Rampilli et al., 2005).

The coagulation of milk by rennet occurs in two sequential stages (Alais, 1956; Waugh and van Hippel, 1956). In the primary stage \( \kappa \)-casein is hydrolysed to yield para-\( \kappa \)-casein and GMP, resulting in a reduction of the electrostatic and the steric repulsions between the casein micelles. In the secondary stage, casein micelles aggregate to form a gel when the temperature is higher than 20°C. To summarise, milk coagulation is achieved through limited hydrolysis of caseins
(specifically κ-casein) followed by casein micelles aggregation (Fox and McSweeney, 1998). When approximately 70% of κ-casein is hydrolysed, the stability of micelles is sufficiently weakened so that the aggregation will start (Walstra et al., 2006). The resulting three-dimensional protein network is formed through hydrophobic bonds and calcium cross-links. Factors like low pH, high temperature and increasing calcium can enhance gel coagulation.

2.3.2.2 Milk coagulation by plant enzymes

Although microbial chymosin and recombinant chymosin have been proven to be suitable rennet substitutes at the industrial scale, increasing interest has been shown to natural and low-cost milk-clotting enzyme extracted from plants. Domsalla and Melzig (2008) reported that the plant proteases investigated so far have originated from the latex of plant families such as Asteraceae, Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae and Euphorbiaceae.

On the basis of their catalytic mechanism, proteases have been divided into serine, aspartate, metallo and cysteine proteases (Bah et al., 2006). Plant coagulants mainly belong to serine, aspartate and cysteine groups, and there has been no published research on the effect of metalloproteases on milk-clotting (Shah et al., 2014). Most of the plant enzymes studied are aspartic proteases, but cysteine and serine proteases have also been studied for their milk-clotting properties (Shah et al., 2014). The nucleophile of the catalytic site in serine and cysteine proteases is part of an amino acid, while the nucleophile of the catalytic site in aspartic and metalloproteases is an activated water molecule (Bruno et al., 2006). Enzymes isolated from plants are considered as potential proteases since they can show milk-clotting activities over a wide range of temperature and pH values (Uhlig, 1998). Apart from milk-clotting activity, characterisation of plant coagulants properties, such as optimal pH and thermostability, also need to be considered as these properties might be essential in cheesemaking.

2.3.2.2.1 Aspartic proteases

Aspartic proteases are extensively present in vertebrates, plants, yeasts, nematodes, parasites, fungi and viruses (Davies, 1990). There are two aspartic residues located at the catalytic site in aspartic proteases and these proteases show preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues (Domingos et al., 2000). The optimum pH for aspartic proteases is acidic. Most of the plant aspartic proteases identified so far are synthesised with a prepro-domain and subsequently converted to mature two-chain enzymes (Simoes and
Faro, 2004). To date all aspartic proteases applied commercially in milk coagulation have optimal activity under acidic pH conditions (Silva and Malcata, 2005). Some plant aspartic proteases exhibited similar characteristics to calf rennet, and have been used as additives to improve flavour and texture in cheese manufacture (Nai-Wan et al., 2014).

Three aspartic proteases (cynarases 1, 2 and 3), with the same optimal pH values, were isolated and separated from the dried flowers of *Cynara cardunculus* (Heimgartner et al., 1990). Cynarases 1 and 2 were similar to each other and are denominated jointly as cardosin A, while cynarase 3 is denominated as cardosin B (Faro et al., 1992). Both cardosins A and B showed similar hydrolysis activities and specificities to chymosin and pepsin (Esteves, 1995). The crude extract from *C. cardunculus* has been applied in traditional cheesemaking in Portugal and Spain for years (Barros et al., 2003). Domingos et al. (2000) studied a cenprosin from the flowers of *Centaurea calcitrapa*, a thistle related to cardoon. *C. calcitrapa* was suggested to be used in a similar way as the *Cynara* species in making traditional artisanal cheese (Heimgartner et al., 1990; Veríssimo et al., 1996; Tavaria et al., 1997). Vairo-Cavalli et al. (2005) reported a new source of vegetable rennet from the flowers of *silybum marianum*, which exhibited rennet-like properties on bovine casein. It could be considered as an addition to calf rennet for cheese ripening acceleration to save both time and costs on cheese storage. Compared to bovine casein, the enzyme extract from *silybum marianum* flower displayed less degradation of ovine and caprine caseins (Vairo-Cavalli et al., 2008). An aspartic protease derived from *Rhizopus oryzae*, peptidase R, was characterised by Hsiao et al. (2014). The aspartic protease from *Streblus asper* has been isolated and partially characterised by Senthilkumar et al. (2006). The sunflower (*Helianthus annuus*) enzymes show high milk-clotting activity with low proteolytic activity compared with other plant enzymes. The sunflower enzyme was reported to have a higher cheese yield than rennet (Nasr et al., 2016). Protein extract from the ripe berries of *Solanum elaeagnifolium* has been reported to have the capability to form milk gels under acidic conditions. This coagulant showed lower milk-clotting activities compared to calf rennet, and could be more suitable for soft cheese manufacture (Néstor et al., 2012).

Typical aspartic proteases in plants share some common characteristics, such as the presence of two aspartic acid residues Asp-Thr/Ser-Gly (DT/SG) in the active site responsible for their catalytic reaction at acidic pH (Lufrano et al., 2012). Aspartic proteases are also sensitive to pepstatin A. Some of the characterised plant aspartic proteases are reported in Table 2.10.
Table 2.10 Aspartic proteases derived from different plants.

<table>
<thead>
<tr>
<th>Name of Protease</th>
<th>Source</th>
<th>Optimal pH</th>
<th>Optimal Temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cynarases 1, 2 and 3 Cynara cardunculus</td>
<td>5.1</td>
<td>NR</td>
<td>Heimgartner et al., 1990</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract Silybum marianum</td>
<td>3.8</td>
<td>NR</td>
<td>Vairo-Cavalla et al., 2005</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract Centaurea calcitrapa</td>
<td>4.0-5.0</td>
<td>NR</td>
<td>Domingos et al., 1998</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract Cynara scolymus</td>
<td>5.0</td>
<td>NR</td>
<td>Llorente et al., 2004</td>
<td></td>
</tr>
<tr>
<td>Cynarase A from flower Cynara scolymus</td>
<td>5.0</td>
<td>70</td>
<td>Sidrach et al., 2005</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract from fruit pulp Balanites aegyptiaca</td>
<td>5.0</td>
<td>50</td>
<td>Beka et al., 2014</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract from seeds Fagopyrum esculentum Moench</td>
<td>3.1</td>
<td>NR</td>
<td>Timotijevic et al., 2003</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract Cirsium vulgare</td>
<td>4</td>
<td>30-37</td>
<td>Lufrano et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Cardosin A and B Cynara cardunculus</td>
<td>5.5 and 5.0</td>
<td>NR</td>
<td>Faro et al., 1995</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract from twigs Streblus asper</td>
<td>5.5</td>
<td>NR</td>
<td>Senthilkumar et al., 2006</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract from cell suspensions Centaurea calcitrapa</td>
<td>5.1</td>
<td>52</td>
<td>Raposo and Domingos, 2008</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract from seed Centaurea calcitrapa</td>
<td>3.5</td>
<td>52</td>
<td>Salvador et al., 2006</td>
<td></td>
</tr>
<tr>
<td>Onopordosin Onopordum acanthium</td>
<td>2.5</td>
<td>NR</td>
<td>Brutti et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Oryzasin Oryza sativa</td>
<td>3</td>
<td>50</td>
<td>Asakura et al., 1997</td>
<td></td>
</tr>
<tr>
<td>Ficin from latex Ficus racemosa</td>
<td>4.5-6.5</td>
<td>60</td>
<td>Devaraj et al., 2008</td>
<td></td>
</tr>
</tbody>
</table>

NR: Not reported.
2.3.2.2 Cysteine Protease

Cysteine proteases, also known as thiol proteases, share a common catalytic mechanism that involves a cysteine group in the active site (Gonzalez-Rabade et al., 2011). Cysteine proteases are widely present in plants, such as papaya, pineapple, fig and kiwifruit (Nam et al., 2016). Because of the properties of cysteine proteases, which are active over a wide range of temperature and pH values, these proteases have been considered for having great potential in the food, biotechnology and medical industries (Shah et al., 2014). As cysteine proteases naturally exist in different plant tissues, they offer abundant sources for the production of cysteine proteases (Gonzalez-Rabade et al., 2011).

Plant cysteine proteases with milk-clotting capacity have been reported (Table 2.11). The ability of actinidin extracted from the kiwifruit to hydrolyse casein was investigated, even in the presence of up to 5% fat content, indicating its potential application in cheese manufacture (Puglisi et al., 2012). Lo Piero (2011) reported actinidin hydrolysates caseins in the order β-casein followed by κ-casein; this latest being hydrolysed into a few large fragments. Enzymes extracted from the sunflower (Helianthus annuus) and albizia (albizzia lebbeck) seeds were reported to have milk-clotting activity, and the extract from albizza seed exhibited good milk-clotting and hydrolysis activity on casein (Egito et al., 2007). The extract from the unripe fruit of Bromelia balansae Mez has also shown milk-clotting activity (Pardo et al., 2001). The papain isolated from the latex of Carica papaya as a conventional milk-clotting enzyme displayed a high optimal temperature (60°C) (Badgujar and Mahajan, 2012). The capparin from the capsules of caper (Capparis spinosa) was suggested to be used in cheese or other food products by Demir et al. (2008). The enzyme extract from the root latex of Jacaratia corumbensis O. kuntze, characterised by Duarte (2009), was reported as a new source for milk-clotting activity. Badgujar and Mahajan (2012) compared different cysteine proteases, namely Calotropis procera, Euphorbia nivulia, Ficus carica and Carica papaya, and reported that Euphorbia nivulia showed relatively high milk-clotting activity, with a straightforward and economical purification process, opening up a great possibility for large-scale preparation. Cysteine proteases from the flower part of Euphorbia microsciadia (Rezanejad et al., 2015) as well as from ginger rhizome (Zingiber officinale) (Nafi et al., 2014) were also reported as new sources of milk coagulants.
Table 2.11 Cysteine protease from different plants.

<table>
<thead>
<tr>
<th>Name of Protease</th>
<th>Source</th>
<th>Optimal pH</th>
<th>Optimal Temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract from flower</td>
<td><em>Euphorbia microisciadia</em></td>
<td>4.5</td>
<td>45</td>
<td>Rezanejad et al., 2015</td>
</tr>
<tr>
<td>Extract from capsules of caper</td>
<td><em>Capparis spinosa</em></td>
<td>5.0</td>
<td>60</td>
<td>Demir et al., 2008</td>
</tr>
<tr>
<td>Extract from root latex</td>
<td><em>Jacaratia corumbensis</em></td>
<td>6.5-7.0</td>
<td>55</td>
<td>Duarte et al., 2009</td>
</tr>
<tr>
<td>Actinidin</td>
<td><em>Actinidia</em></td>
<td>6.5</td>
<td>55</td>
<td>Lo Piero et al., 2011</td>
</tr>
<tr>
<td>Extract from rhizome</td>
<td><em>Zingiber officinale</em></td>
<td>6.0-8.0</td>
<td>60</td>
<td>Nafi et al., 2014</td>
</tr>
<tr>
<td>Papain</td>
<td><em>Carica papaya</em></td>
<td>6.6</td>
<td>60</td>
<td>Badgujar and Mahajan, 2012</td>
</tr>
<tr>
<td>Extract from latex</td>
<td><em>Calotropis procear</em></td>
<td>7.0-9.0</td>
<td>55-60</td>
<td>Dubey and Jagannadham, 2003</td>
</tr>
<tr>
<td>Protease</td>
<td><em>Calotropis procear</em></td>
<td>6.3</td>
<td>55</td>
<td>Badgujar and Mahajan, 2012</td>
</tr>
<tr>
<td>Extract from latex</td>
<td><em>Ficus carica</em></td>
<td>5</td>
<td>NR</td>
<td>Nouani et al., 2009</td>
</tr>
<tr>
<td>Extract from latex</td>
<td><em>Ficus carica</em></td>
<td>6.8</td>
<td>50</td>
<td>Badgujar and Mahajan, 2012</td>
</tr>
<tr>
<td>Extract from latex</td>
<td><em>Euphorbia nivulia</em></td>
<td>6.3</td>
<td>45</td>
<td>Badgujar and Mahajan, 2012</td>
</tr>
</tbody>
</table>

NR: Not reported.

2.3.2.2.3 Serine protease

Serine proteases, or serine endopeptidases, contain a nucleophilic serine residue in their active site (Hedstrom, 2002). They undertake a nucleophilic attack on the carbonyl group of the peptide bond (Dunn, 2001). Based on their structure, serine proteases have been divided into two broad categories: chymotrypsin-like (trypsin-like) and subtilisin-like proteases (Madala et al., 2010). Serine proteases account for approximately one-third of all proteases, one of the largest groups of proteolytic enzyme that exists in all kingdoms of life, which play essential roles in physiological pathways (Tripathi and Sowdhamini, 2006). Serine proteases were once
thought to be rare in plants, but in recent years, more serine proteases have been purified and characterised from different parts of various plants, ranging from seeds, latex, to fruits (Antão and Malcata, 2005). Rawlings and Barrett (2004) reported that the serine proteases from cucurbits, cereals and trees are usually classified together. While serine proteases are the largest class of proteases in plants, so far the identification on their physiological substrates are still unknown and the physiological roles of serine proteases in plant cells needs further research (Antão and Malcata, 2005). Generally, the molecular weights of plant serine proteases are between 19 and 110 KDa, with the majority having a molecular weight between 60 and 80 kDa. The optimum pH for their activity is alkaline ranging from pH 7 to 11 (Antão and Malcata, 2005). Compared to other proteases, serine proteases isolated from plants exhibited a good stability over a wide range of pH and temperature, even under oxidising agents or surfactants, imparting them a potential use in industrial applications (Tomar et al., 2008).

The milk-clotting activity of plant serine proteases from different plant parts, such as latex, seeds, flowers, stems, leaves and roots, has been investigated. Chymotrypsin-like serine proteases, including dubiumin from the seeds of Solanum dubium Fresen with high stability against autodigestion under various condition, has been isolated and characterised by Ahmed (2009a and 2009b). Ahmed (2010) reported that the high milk proteolytic activity of this protease has value as an additive to, or to be a substitute for, calf rennet. Neriifolin, a serine protease isolated from the latex of Euphorbia neriifolia Linn, was also reported to display high pH and temperature stability (Yadav et al., 2011). Subtilisin-like serine proteases with milk-clotting activities, including cucumisin isolated from melon fruit (Cucumis melo), were found to be stable at pH 7.1 and at 37°C for 24 h. Cucumisin displayed the same milk-clotting activity as papain, which in turn had half the milk-clotting activity of ficin (Uchikoba and Kaneda, 1996). A number of the unidentified serine proteases were reported by Kaneda et al. (1975) who isolated protease from the sarcocarp of melon fruit (Cucumis Melo L.) with a maximum activity at alkaline pH and high temperature (70 °C) against casein as a substrate. Lettucine from the lettuce leaves (Lactuca sativa L.) had a high milk-clotting activity, but the activity was affected by the fat content in milk (Lo Piero, 2002). A dimeric serine protease, with high pH, temperature, organic solvents, and autodigestion stability, from the latex of Euphorbia neriifolia has been isolated and purified by Yadav (2012). Streblin, purified from the latex of Streblus asper, also exhibited high stability against autodigestion (Tripathi et al., 2011). Three serine proteases extracted from the latex of Ficus religiosa, religiosin (Kumari et al., 2010),
religiosin B (Kumari et al., 2012) and religiosin C (Sharma et al., 2012) have also been reported (Table 2.12).

### Table 2.12 Serine protease from different plants.

<table>
<thead>
<tr>
<th>Name of Protease</th>
<th>Source</th>
<th>Optimal pH</th>
<th>Optimal Temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roxburghi</td>
<td>Cucumis trigonus</td>
<td>11.0</td>
<td>70</td>
<td>Asif-Ullah et al., 2006</td>
</tr>
<tr>
<td>Milin</td>
<td>Euphorbia milii</td>
<td>8.0</td>
<td>60</td>
<td>Yadav et al., 2006</td>
</tr>
<tr>
<td>Taraxulin</td>
<td>Taraxacum officinale</td>
<td>8.0</td>
<td>40</td>
<td>Rudenskaya et al., 1998</td>
</tr>
<tr>
<td>Dubiumin</td>
<td>Solanum dubium</td>
<td>11.0</td>
<td>70</td>
<td>Ahmed et al., 2009a</td>
</tr>
<tr>
<td>Cucumisin</td>
<td>Cucumis melo</td>
<td>7.1</td>
<td>70</td>
<td>Kaneda et al., 1975</td>
</tr>
<tr>
<td>Macluralisin</td>
<td>Maclura pomifera</td>
<td>8.5</td>
<td>58</td>
<td>Rudenskaya et al., 1995</td>
</tr>
<tr>
<td>Camein</td>
<td>Ipomoea camea</td>
<td>6.5</td>
<td>65</td>
<td>Patel et al., 2007</td>
</tr>
<tr>
<td>Religiosin</td>
<td>Ficus religiosa</td>
<td>8.0-8.5</td>
<td>50</td>
<td>Kumari et al., 2010</td>
</tr>
<tr>
<td>Religiosin B</td>
<td>Ficus religiosa</td>
<td>8.0-8.5</td>
<td>55</td>
<td>Kumari et al., 2012</td>
</tr>
<tr>
<td>Religiosin C</td>
<td>Ficus religiosa</td>
<td>6.0-8.0</td>
<td>45-50</td>
<td>Sharma et al., 2012</td>
</tr>
<tr>
<td>Lettucine</td>
<td>Lactuca sativa</td>
<td>6.5</td>
<td>50</td>
<td>Lo Piero et al., 2002</td>
</tr>
<tr>
<td>Neriifolin</td>
<td>Euphorbia neriifotia</td>
<td>8.5</td>
<td>55</td>
<td>Yadav et al., 2011</td>
</tr>
<tr>
<td>Extract from latex</td>
<td>Euphorbia neriifotia</td>
<td>9.5</td>
<td>45</td>
<td>Yadav et al., 2012</td>
</tr>
<tr>
<td>Streblin</td>
<td>Streblus asper</td>
<td>9.0</td>
<td>65</td>
<td>Tripathi et al., 2011</td>
</tr>
</tbody>
</table>

#### 2.3.3 Plant enzymes in cheesemaking

Cheese products made with plant coagulants are produced in Mediterranean, West African and southern European countries. Rennet substitutes derived from plants have been applied to manufacture cheese on an artisanal level, especially on a farm or small dairy level (Roseiro et al., 2003). The application of plant proteases in cheesemaking gives a significant economic contribution to the local agriculture. In addition, it also solves the problem for people who are opposed to using animal rennet (Gupta and Eskin, 1977). A large number of proteases from plant sources were reported for their capacity to clot milk (Domingos et al., 1998; Llorente et
However, although the application of vegetable rennet in cheesemaking industries was performed in the 1960s due to the shortage of calf rennet supplies, studies showed some limitations in the use of plant coagulants (Guinee and Wilkinson, 1992). Specifically, the major problem of plant coagulants in cheesemaking is their broad proteolytic effect on caseins, which results in producing bitter flavours, lower cheese yield and excessive acidity (Roseiro et al., 2003). Therefore, most proteases from plants were considered not suitable in cheese manufacture due to their low ratio of milk-clotting/proteolytic activities (Anusha et al., 2014; Shah et al., 2014).

The most important property of chymosin in cheesemaking is its milk-clotting activity, resulting from its specific hydrolysis on Phe$_{105}$-Met$_{106}$ of κ-casein (Jacob et al., 2011). Most calf rennet-like proteases, with chymosin-like behaviour, cleave the Phe$_{105}$-Met$_{106}$ peptide bond on κ-casein, although Endothia parasitica protease has been reported to cleave the Ser$_{104}$-Phe$_{105}$ peptide bond of κ-casein without affecting milk-clotting (Drohse and Foltermann, 1989). However, research on the specificity of plant coagulants on caseins remains scarce. The leucine from the leaves of Lactuca sativa was assumed to cleave Arg$_{97}$-His$_{98}$, Lys$_{111}$-Lys$_{112}$ or Lys$_{112}$-Asn$_{113}$ on κ-casein, in addition to Phe$_{105}$-Met$_{106}$ (Lo Piero et al., 2002). Proteases from cardoon exhibited a high specific hydrolysis to Phe$_{105}$-Met$_{106}$ of bovine and ovine κ-casein, and the Lys$_{160}$-Thr$_{117}$ bond was its preferred target in caprine κ-casein (Sousa and Malcata, 1997). Egito et al. (2007) stated that the preferred cleavage site of the extract from Albizia seed and sunflower seed was at Lys$_{116}$-Thr$_{117}$ and Phe$_{105}$-Met$_{106}$, respectively.

The requirement for good proteases in cheese manufacture is to have a high milk-clotting activity and a low proteolytic activity. Therefore, these two activities are essential to be tested when plant proteases are studied. However, it is hard to compare either milk-clotting activity or proteolytic activity reported for different plant proteases because the different investigations were conducted under different conditions. Most of the studies compared the properties of plant coagulants to the activity of rennet when estimating their potential as rennet substitutes.

### 2.3.3.1 Plant proteases applied in traditional cheesemaking

In the Iberian Peninsula, the vegetable coagulant extract from Cynara cardunculus (cardoon) flowers has been traditionally used in the on farm manufacture of ovine and caprine cheese for centuries (Pires et al., 1994; Trujillo et al., 1994; De Sa and Barbosa, 1972). Serra da Estrela
cheese in Portugal and La Serena cheese in Spain can be made only from raw ovine milk using an extract of the cardoon flowers as a coagulant (Vioque et al., 2000). Studies revealed that an extract from *Cynara Species* was not suitable for making bovine milk cheeses. This was because the high proteolytic activity of the extract on bovine caseins lead to the loss of cheese yield and impacted on the cheese texture and flavour (Roseiro et al., 2003). Agboola (2009) stated that compared to bovine cheese, cardoon extract is more suitable for sheep milk cheese due to the difference in protein profile of ovine and bovine milk (especially β-casein).

*Cynara cardunculus* and *Cynara humilis* are the two main species of *Cynara* which have been used as coagulants in cheesemaking (Del Pozo et al., 1988a; Del Pozo et al., 1988b; Fernández-Salguero and Sanjuán, 1999; Carmona et al., 1999; Tavaria et al., 1997), particularly in Portugal and Spain. Bovine milk cheese which was made by cardosin extract from the flowers of *Cynara cardunculus* had a bitter taste, because cardosin extract from *Cynara cardunculus* exhibited a larger proteolytic activity on αs- and β-casein (Macedo et al., 1996). With ovine milk cheese, there was little difference between the use of extracts from *Cynara cardunculus* and *Cynara cardunculus* on the chemical components, water activity, flavour and aroma during cheese ripening (Vioque et al., 2000). Therefore, it was suggested that the use of *Cynara humilis* extracts alone, or in combination with *Cynara cardunculus*, could be a good alternative in cheesemaking (Vioque et al., 2000; Tavaria et al., 1997; Fernández-Salguero and Sanjuán, 1999).

Cardosins have been reported to have a similar specific activity on κ-casein (i.e. only one cleavage site on κ-casein at Phe105-Met106) to chymosin (Macedo et al., 1993; Veríssimo et al., 1995, 1996; Ramalho-Santos et al., 1997). However, these extracts also showed a non-specificity to produce other peptides from the other caseins (Campos et al., 1990; Silva and Malcata, 1998; Silva and Malcata, 1999). Compared with rennet, more bonds were cleaved from αs1- and β-casein while there were also two peptides hydrolysed from αs2-casein. Some low molecular weight peptides cleaved from αs1- and β-casein resulted in a bitter taste in cheese due to their high hydrophobicity (Roseiro et al., 2003). Peptides hydrolysed from the C-terminal of αs1- and β-caseins (with molecular weight of approximately 2000-4000 Da) were lost in the whey leading to reduced cheese yields. Specifically, a preferable cleavage site on the bovine β-caseins which were hydrolysed by chymosin and cardosins are located at Leu192-Tyr193 and Ala189-Phe190 (Macedo, 1993; Sousa, 1993). Visser and Slangen (1977) reported that, in 0.05 M sodium acetate buffer (pH 5.4), there are seven sites on β-casein that can be cleaved
by chymosin. They are (in decreasing order of rate of attack) Leu$_{192}$-Tyr$_{193}$, Ala$_{189}$-Phe$_{190}$, Leu$_{165}$-Ser$_{166}$, Gln$_{167}$-Ser$_{168}$, Leu$_{163}$-Ser$_{164}$, Leu$_{139}$-Leu$_{140}$ and Leu$_{127}$-Thr$_{128}$. However, when $\beta$-casein was hydrolysed in 50 mM sodium citrate buffer (pH 6.2) at 30°C, (Charles and Ribadeau-Dumas, 1984), only the Ala$_{189}$-Phe$_{190}$ and Leu$_{192}$-Tyr$_{193}$ peptide bonds of bovine $\beta$-casein are cleaved by chymosin. Cardosins have been reported to cleave six bonds on bovine $\beta$-casein; these are Leu$_{192}$-Tyr$_{193}$, Leu$_{191}$-Leu$_{192}$, Leu$_{165}$-Ser$_{166}$, Phe$_{190}$-Leu$_{191}$, Ala$_{189}$-Phe$_{190}$ and Leu$_{127}$-Thr$_{128}$, in decreasing order of attack. For bovine $\alpha$-casein, the Phe$_{23}$-Phe$_{24}$ bond was reported to be the most susceptible bond on $\alpha_s$-casein under the action of chymosin and cardosins. However, Grappin (1994) stated that bovine $\alpha_s$-casein cannot be hydrolyzed by chymosin. In contrast, McSweeney (1994) reported that the bonds of Phe$_{88}$-Tyr$_{89}$ and Tyr$_{95}$-Leu$_{96}$ on $\alpha_s$-casein can be cleaved by chymosin. Cardosin B was more similar to pepsin in specificity and activity.

Extracts of Sodom apple leaf ($Caltrops procera$) have been applied in traditional cheesemaking in West African countries, such as Nigeria and the Republic of Benin (Aworth and Muller, 1987). In Nigeria, West African soft cheese (Warankasi, Wara) is a local dairy product, made from the surplus of fresh milk with the addition of sodom apple extract (Adetunji and Salawu, 2008). This soft cheese has a 2 to 3 days shelf life when stored in the whey (Belewu et al., 2005). Adetunji and Salawu (2008) compared the nutritional values in Wara cheese made from the extract of $Caltrops procera$ with that made from the extract from $Caltrops papaya$. The results revealed that the cheese made with $Caltrops procera$ extract was more nutritious, while the cheese made with $Caltrops papaya$ extract contained more minerals such as Mn and Fe (Adetunji and Salawu. 2008).

In northern Cameroon, a water extract from $Balanites aegyptiaca$ fruit was used to make a thick gruel with zebu milk and flour. Beka et al. (2014) isolated and characterised the proteases from $Balanites aegyptiaca$ to investigate their milk-clotting activities. The extract contained two proteases, one was an aspartic protease with an optimum pH at 5.0, while the other was a serine protease with an optimum pH at 8.0. The extract from $Balanites aegyptiaca$ also had a similar temperature optimum to chymosin (approximately 50°C). However, at a high temperature, the inactivation kinetics of the extract was twice slower than chymosin (Demerdash and Abd-El-Ghany, 1997; Turk et al., 1990). Beka et al. (2014) suggested that the extract from $Balanites aegyptiaca$ fruit had potential not only in local zebu cheesemaking, but also as a substitute for calf rennet.
An extract from the seed of Moringa oleifera (Calotropis procera) has been used to produce cottage cheese in Africa and Asia periurban areas (O’Connor, 1993). Mahami et al. (2012) demonstrated that increasing the amount of moringa seed extract could contribute to an increased protein content in cheese; 0.5, 1, 1.5 and 2% moringa seed extracts resulted in cheese yields of 14.69, 16.01, 18.31 to 18.50%, respectively. The mineral content in the cottage cheese also increased as the amount of moringa seed extract added was increased. Therefore, this enzyme extract has the potential to improve both the yield and the quality of cottage cheese. Moreover, the cost of the moringa seed extract was lower than other coagulants, enhancing its potential use on an industrial scale (Mahajan and Chaudhari, 2014).

In some parts of Iran, the latex of Euphorbia microsciadia is used by the local dairy farmers to make goat and sheep milk cheese with a white colour and soft texture (Rezanejad et al., 2015). A cysteine protease named microsciadin from the latex of Euphorbia microsciadia was partially purified and studied by Rezanejad et al. (2015). The protease had a relatively high ratio (20.1) of milk-clotting/proteolytic activity, compared to calf rennet (26.4) (Mazorra-Manzano et al., 2013). In addition, this enzyme displayed high stability to strong denaturants, organic solvents and metallic salts, and could only be strongly inhibited by Hg$^{2+}$ due to a thiol group present in or near its activity site.

Extracts from the berries of Withania coagulants was reported as a suitable coagulant in cheesemaking (Dinakar et al., 1989). It has been suggested to be an alternative for calf rennet in cottage and Cheddar cheese production (Naz et al., 2009). In Pakistan, this plant was also widely used as a coagulant in paneer cheese preparation. The suitable concentration for making cheese curd was 15 µL/mL to achieve an optimal clotting time and less bitterness (Naz et al., 2009). As an aspartic protease, this extract exhibited typical optimum pH (4.25) and temperature (40°C) values. The Ca$^{2+}$ concentration had an impact on the activity of the crude enzyme extract. The more Ca$^{2+}$ present in milk, the shorter the clotting time to form the cheese curd.

### 2.3.3.2 Plant Proteases applied in model cheeses

Extracts with milk-clotting activity from the seeds of sunflower (Helianthus annuus L) were partially purified using increasing salt strength in buffer (Nasr et al., 2016). The partially purified proteases contained two activity bands with molecular weights of approximately 120
and 62 kDa. Compared with other plant coagulants (dubiumin and papain), partially purified enzymes from sunflower seeds revealed a higher ratio of milk-clotting/proteolytic activity. The enzyme was evaluated on bovine and ovine milk cheese. The results showed that there was no significant difference in coagulation time between bovine and ovine cheese, but bovine milk cheese had a higher yield due to its high total solids and protein content. However, partially purified sunflower milk-clotting enzyme displayed a higher cheese yield and longer coagulation time compared to soft white cheese made with calf rennet. As a cheap milk-clotting agent for cheesemaking, partially purified enzyme from seeds was easy to prepare, and had a high ratio of milk coagulation specificity and curd formation ability compared to commercial rennet, suggesting its potential in cheese manufacture.

A serine protease named dubiumin was first isolated and purified from the seeds of Solanum dubium by Ahmed et al. (2009a and 2009b). The molecular weight of dubiumin is approximately 66 kDa with a pI value of 9.3. This enzyme has an optimal pH of 11, pH stability ranging from 3 to 12, and an optimal temperature of 70°C. In addition, dubiumin has a high stability under organic solvents condition and against autolysis at low concentrations. When its milk-clotting/proteolytic activity was compared with Endothia parasitica rennet (2590 U/OD), dubiumin showed a similar ratio of activity (2490 U/OD), indicating that this protease could be used as a coagulant in cheesemaking (Arima et al., 1970). Ahmed (2010) reported that dubiumin preferably hydrolysed both κ-casein and β-casein to α-casein. A 16 kDa peptide, assumed to be para-κ-casein, was obtained after 5 min incubation at 30°C. In contrast, bovine β-caseins was more hydrolysed, which indicates that dubiumin could be suitable for making soft-flavoured cheeses (Ahmed et al., 2010).

Bruno et al. (2010) applied a hieronymain extract from the fruit of Bromelia hieronymi and rennet in the production of miniature cheeses. Similar physical and chemical properties, including pH, moisture and NaCl contents, were obtained for the cheeses made by either hieronymain or rennet. However, the protein content in hieronymain treated cheese was a little less than that of the cheese made with rennet. In addition, the cheese made by rennet obtained a slightly higher score of sensory evaluation for appearance, body and texture and flavor. This is because the cheese made with hieronymain extract has a weak, smooth body and texture. There were no bitterness and off-flavour in the cheese made with the hieronymain extracts, but a little pungent taste was noted by the sensory evaluation panelists. Bruno et al. (2010)
suggested that hieronymain could be applied for cheesemaking as a substitute to or in combination with rennet.

2.4 Summary

This review of the literature reports on the composition of milk, with an emphasis on casein and casein micelle models. The details on casein and casein micelle might contribute to understanding the cleavage action of different proteases. The casein hydrolysis plays an important role in the aggregation of the casein micelle in cheesemaking. The most commonly used milk-coagulant is the calf rennet, which has high milk-clotting activity and high specific hydrolysis on Phe_{105}-Met_{106} of κ-casein. Three main groups of proteases from plant were reviewed and their properties were compared to rennet. Overall, the aspartic proteases were similar to calf rennet, but cysteine and serine protease have wider ranges of pH and temperature stability. As a milk coagulant source, plant proteases attract research investigations in the field of cheese making, despite that some of these plant proteases have been used in cheesemaking as rennet substitutes for a long time.
Chapter 3

3. Materials and methods
3.1 Materials

Low heat skim milk powder (SMP) was supplied by Synlait Milk Ltd., Rakaia, New Zealand. Natural calf rennet [Strength, 280 International Milk-clotting Units (IMCU) mL⁻¹] was obtained from RENCO New Zealand Laboratory (RENSO New Zealand, Eltham, New Zealand). Tamarillo fruit, including the red cultivar Laird’s Large tamarillo and the yellow cultivar Amber tamarillo, were purchased from a farm in Maungatapere, New Zealand.

All chemicals used in this thesis were of analytical grade, while the acetonitrile and trifluoroacetic acid were of HPLC grade.

3.2 Milk sample preparation

3.2.1 Preparation of reconstituted milk samples

Reconstituted skim milk was obtained by mixing skim milk powder with Milli-Q water to obtain a desired total solid concentration (e.g. 10%, 11.25% and 20% (w/w)). The mixtures were stirred using a magnetic stirring for at least 2 h at room temperature to ensure dispersion. The mixtures were kept in a 4°C fridge overnight to ensure full hydration. Prior to use, the reconstituted milks were left at room temperature for at least 4 h to ensure temperature equilibration to room temperature.

3.2.2 pH adjustment

pH adjustment of the reconstituted milk samples was achieved by the addition of glucono-δ-lactone (GDL) known to achieve accurate acification. GDL was mixed with the milk samples by magnetic stirring for 2 min to ensure full dissolution. Table 3.1 reports the amounts of GDL added to 10% (w/w) reconstituted skim milk to achieve the desired pH values.
Table 3.1 Amounts of GDL added to 10% (w/w) skim milk to achieve different pH values.

<table>
<thead>
<tr>
<th>pH</th>
<th>Amount of GDL (%) added (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.67</td>
<td>0.00</td>
</tr>
<tr>
<td>6.50</td>
<td>0.04</td>
</tr>
<tr>
<td>6.25</td>
<td>0.12</td>
</tr>
<tr>
<td>6.00</td>
<td>0.24</td>
</tr>
<tr>
<td>5.83</td>
<td>0.35</td>
</tr>
<tr>
<td>5.41</td>
<td>0.65</td>
</tr>
<tr>
<td>5.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

3.2.3 Preparation of milk sample for ultra-small-angle neutron scattering and small-angle X-ray scattering.

Milk samples for ultra-small-angle neutron scattering (USANS) and small-angle X-ray scattering (SAXS) were prepared by mixing skim milk powder with deuterium oxide (D$_2$O). D$_2$O instead of H$_2$O was used because it allowed a better contrast for USANS measurements. The mixtures were stirred using magnetic stirring for at least 2 h at room temperature. The mixing was performed in a container that was sealed by para-film to minimise D$_2$O degradation. The mixtures were kept at 4°C overnight to ensure completely hydration. The mixtures were equilibrated to room temperature for at least 4 h before use.

3.3 Sodium caseinate preparation

The method for the preparation of sodium caseinate was adapted from Lucey et al. (2000). Sodium caseinate was obtained from 10% (w/w) reconstituted skim milk as prepared in Section 3.2.1. The milk was acidified to pH 4.6 by adding 2 M HCl at room temperature. The formed curd was separated from the whey using two layers of cheesecloth. The filtered curd was washed five times with Milli-Q water and dewatered using two layers of cheesecloth. The washed curd was re-dissolved with Milli-Q water in a 1:1 mixture, and the pH of the mixture was adjusted to approximately 6.8 by 2 M NaOH with gentle magnetic stirring. Sodium caseinate solution was lyophilized using a freeze-drier (Labconco Corporation, Missouri, USA).
3.4 Preparation of crude protease extract from tamarillo fruit

3.4.1 Homogenization

Laird’s Large or Amber tamarillo fruit (300 g) were homogenized to break the plant cells and release the protein into solution using a Polytron PCU2 laboratory homogeniser (Brinkmann Instruments, Luzern, Switzerland). The filtrate (~85 mL) was filtered through two layers of cheesecloth to remove the insoluble material, and then mixed with 80 mL 0.05 M sodium citrate buffer (pH 5.5) to electrically charge the proteins. The mixture was centrifuged at 15,000 × g at 4°C for 20 min in a Sorvall LYNX superspeed centrifuge (Thermo Fisher Scientific, USA) with a Fiberlite rotor (F12-6x500 LEX, Thermo Fisher Scientific, USA). The supernatant was collected and the precipitate was discarded.

3.4.2 Ammonium sulphate precipitation and dialysis

Ammonium sulphate was used to salt out the proteins because of its relatively high solubility in water. According to the method of Burgess et al. (2009), 61.69 g of ammonium sulphate was slowly added to 155 mL of the supernatant to make a 65% saturated ammonium sulphate solution using a magnetic stirrer to mix gently for 20 min. The solution was held in an ice bath overnight to allow the proteins to precipitate. The precipitated proteins were collected by centrifuging at 15,000 × g at 4°C for 20 min and then re-dissolved in 30 mL 0.05 M citrate buffer pH 5.5. One volume of protein solution was dialysed against at least five volumes of citrate buffer using a cellulose dialysis tube (molecular weight cut-off of 12,000 Da) to remove ammonium sulphate salts at 4°C for 24 h with at least three changes of the buffer solution. Tamarillo crude extract solution was stored in a -80°C freezer.

3.5 Purification of tamarillo protease

The method of purification of the tamarillo protease was based on the purification of actinidin from kiwifruit (McDowall, 1970; Aminlari et al., 2009). Ion-exchange chromatography, both cation and anion, is frequently used in the separation and purification of proteins and polypeptides. For anion ion-exchange chromatography, the gel matrix beads are linked to diethylaminoethanol (DEAE) groups, imparting positive charges to the resin. This allows proteins or peptides with negative charges to adsorb to the resin. Conversely, the resin of cation exchange chromatography can be derivatised with carboxymethyl (CM), which contains
negative charge groups, and can therefore attract positively charged proteins (as shown in Figure 3.1).

DEAE-Sepharose fast flow (GE Healthcare Life Sciences, New Zealand) was used to purification the tamarillo proteases. The resin was packed into a 22 × 5 cm column and equilibrated overnight with 0.05 M sodium citrate buffer pH 5.5. Tamarillo crude extract (40 mL), with a protein concentration of 1.17 ± 0.03 mg/mL, was applied to the column and the flow rate was set at 1 mL/min. The separation of proteins was achieved using a 200 mL 0.0 - 1.0 M linear gradient of sodium chloride in citrate buffer. Proteins with the weakest negative charges were eluted first from the column at low concentrations of NaCl. A higher concentration of salt was required to elute proteins which have a stronger ionic interaction with the resin (Figure 3.1). 3 mL fractions of eluate solution were collected in 15 mL centrifuge tubes. The absorbance of each fraction was measured at 280 nm using an UVmini-1240 spectrophotometer (Shimadzu Corporation, Australia). The protease activity was assayed as in Section 3.8.1. Fractions corresponding to high activities were pooled and dialysed against Milli-Q water for 24 h at 4°C with at least three changes of the dialysis solution. The purified enzymes were lyophilised by freeze-drying and stored at -80°C until further use.

*Figure 3.1 Principle of ion exchange chromatography (Adapted from Berg et al., 2002)*
3.6 Determination of protein content

The concentration of protein was determined by the Bradford method (Bradford, 1976). The method relied on the binding of the Coomassie brilliant blue G-250 to the proteins, and the measurement of the dye-protein complex at 595 nm by an UVmini-1240 spectrophotometer (Shimadzu Corporation, Australia). Protein solution (0.1 mL) was added to test tubes containing 3 mL of Bradford reagent (Sigma-Aldrich, New Zealand) then vortexed for 30 seconds. The mixture was incubated at room temperature for 20 min. A blank was prepared by mixing 0.1 mL Milli-Q water with 3 mL Bradford reagent. Bovine serum albumin (BSA) was used to obtain a calibration curve with concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mg/mL protein.

3.7 Determination of bovine milk hydrolysed by rennet and tamarillo extract

Bovine milk sample preparation for electrophoresis was adapted from Jin and Park (1996) with minor modifications. Calf rennet (6 μL, 10 times diluted) and tamarillo crude extract (0.7 mL of a 1.17 ± 0.03 mg/mL protein extract) were added to 10% (w/w) skim milk (6 g) and 11.25% (w/w) skim milk (6 g) respectively. Both were mixed using a magnetic stirring for 2 min to make a final concentration of 10% (w/w) skim milk samples. Aliquots of the mixed solutions (200 μL) were transferred into glass vials then the lids were closed. The glass vials were incubated at 35°C in an oven (Function Line incubator, Heraeus, Langenbold, Germany) and removed at different times (0 min, 15 min, 1 h and 24 h). The glass vials containing hydrolysed milk were then heating in a water bath at 100°C to inactivate the enzymes. To each glass vial 400 μL 8 M urea buffer pH 8 was added to disrupt any non-covalent protein bonds, and to solubilise the proteins. Then the samples were kept at 4°C until electrophoresis analysis.

3.8 Determination of protease activity

3.8.1 Standard protease assay

Protease activity was measured using the method of Kunitz (1947) with some modifications. Bovine casein was used as the substrate. Enzyme (100 μL) was mixed with 1.1 mL of 0.1 M Tris-HCl buffer (pH 7) containing 1% (w/v) casein, and vortexed for 30 seconds to ensure full dispersion. The reaction was performed at 35°C for 20 min in a water bath. It was then stopped by adding 1.8 mL 10% (w/v) trichloroacetic acid (TCA), and the mixture was allowed to stand for 30 min at room temperature. Mixtures were centrifuged at 10,300 × g for 20 min in a
Multifuge 3S-R centrifuge (Thermo Electron Corporation, Germany), then the absorbance of the supernatants was measured at 280 nm against a blank using a UV-Vis spectrophotometer (UV mini-1240, Shimadzu, Japan). The blank was prepared by adding TCA with enzyme solution first to inactive enzyme, then casein solution was added. One unit of protease activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per mL in 1 min under the assay conditions. A standard curve was made using 0-100 mg/L tyrosine (Hadj-Ali et al., 2007). Enzyme activity was defined as units/mg of protein.

3.8.2 Temperature optimum and stability

The effect of temperature on the enzyme activity was studied using casein as a substrate. The optimal temperature of the protease was determined using the standard enzyme assay (Section 3.8.1) over a temperature range from 0 to 100°C for 20 min. To investigate the protease temperature stability, enzyme was pre-incubated at temperatures of 0 to 100°C (at 10°C intervals) for 20 min, then the 1% (w/v) casein solution (same as in Section 3.8.1) was added and the standard enzyme assay performed under optimal pH and temperature conditions. The residual activity of enzyme after pre-incubation were express as a percentage of the control, which was the enzyme activity measured under optimal condition.

3.8.3 pH optimum and stability

The optimal pH value for the protease activity was studied using 50 mM buffers of different pH ranging from pH 6 to 14 at the optimal assay temperature. Preparation of buffers (Table 3.2) was based on the method of Attri et al. (2015) and the pH was measured using an Orion 320 PerpHecT pH meter connected to a model Orion 9106BNWP pH electrode (Massachusetts, USA).

<table>
<thead>
<tr>
<th>pH Range</th>
<th>Concentration (mM)</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>50</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>7-8</td>
<td>50</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td>9-10</td>
<td>50</td>
<td>Glycine-NaOH</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>Phosphate-NaOH</td>
</tr>
<tr>
<td>12-13</td>
<td>50</td>
<td>KCl-NaOH</td>
</tr>
<tr>
<td>14</td>
<td>1000</td>
<td>NaOH</td>
</tr>
</tbody>
</table>
Measurement of the pH stability of the protease was performed by pre-incubating 100 µL purified enzyme with 150 µL of the different pH buffers at 25°C for 20 min, then the remaining activity of the enzyme was detected under optimum assay condition. The results of residual enzyme activities were reported as a percentage of the control.

3.8.4 Effect of organic solvents on protease activity

Protease (100 µL) was mixed with different organic solvents (25% (v/v)) including isopropanol, methanol, ethanol, glycerol, dimethyl sulfoxide (DMSO), and chloroform. The mixtures were incubated at 25°C for 20 min. The residual activity of protease was then determined and compared to that of the control without organic solvent.

3.8.5 Inhibitors and effect of metal ions on protease activity

The enzyme inhibitors phenylmethylsulphonyl fluoride (PMSF, a serine protease inhibitor), p-chloromercuribenzoic acid (PCMB, a cysteine protease inhibitor) and ethylenediaminetetraacetic acid (EDTA, a metalloprotease inhibitor) were added to the standard assay at concentrations of 1 mM and 5 mM. The enzyme was pre-mixed with the inhibitors and incubated at room temperature for 20 min prior to the enzyme assay (Section 3.8.1).

The effect of different chloride salts on protease activity, which included Na⁺, Hg²⁺, Zn²⁺, Co²⁺, Ca²⁺, Mn²⁺, Mg²⁺ (1 mM and 5 mM), was studied. The enzyme was pre-incubated with the individual metal ions for 20 min at room temperature, followed by standard enzyme assay (Section 3.8.1). The results were expressed as the percentage of the protease activity compared to the absence of the inhibitors or the metal ions.

3.9 Preparation of casein hydrolysate

The method to prepare casein hydrolysate was based on Egito et al. (2007). Sodium caseinate was dissolved in 100 mM sodium phosphate buffer, pH 6.5 to make 10% and 11.25% (w/v) casein solutions. Rennet (0.1 mL calf rennet) was diluted with 0.9 mL Milli-Q water and vortexed for 1 min. Rennet (3 µL, diluted 10-fold) and tamarillo crude extract (350 µL, 1.17 ± 0.03 mg/mL) were added to 3 mL of 10% (w/v) and 11.25 % (w/v) casein solution, respectively, to make a final casein concentration of 10% (w/v). The casein solutions containing tamarillo protease or rennet were incubated at 35°C in a Function Line incubator (Heraeus, Langenselbold, Germany), and hydrolysate aliquots (200 µL) were removed at 15 min, 30 min,
1 h, 4 h and 24 h. The enzymatic reaction was stopped by heating the hydrolysates at 100°C for 5 min. Casein solution (10% w/v) was used as a standard. The hydrolysate solutions were stored at -80°C until analysed.

To prepare individual casein protein solutions, each of α-, β- and κ-casein were dissolved in 10 mM sodium phosphate buffer at pH 6.5. The final concentration of the casein solution (5 mL) was 10% (w/v). Purified tamarillo protease (1 mg) and rennet (10 μL, diluted 200-fold) were mixed with the casein solutions using a magnetic stirrer for 2 min. The mixtures were incubated at 35°C. Aliquots of hydrolysate (200 μL) were removed at different times: 15 min, 30 min, 1 h, 4 h, 10 h and 24 h. The hydrolysates were heated immediately at 100°C for 5 min to stop the enzymatic reaction. The hydrolysates were lyophilised using a freeze-dryer and kept in -80°C until analysed.

3.10 Chemical methods

3.10.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a widely used method to separate proteins based on their molecular weight using electrophoresis in a polyacrylamide gel. Proteins are denatured by being mixed with sodium dodecyl sulphate (SDS), which is an anionic detergent, breaking down most of the noncovalent bonds in the proteins. The SDS forms complexes with the proteins and gives them an overall negative charge. However, the migration of proteins in the gel is only dependent on their molecular weight (García-Descalzo et al., 2012). Under reducing condition, 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) is added to disrupt any the disulphide bonds.

The enzymes, milk samples, caseins and their hydrolysates were analysed by SDS-PAGE under reducing condition using a Mini-PROTEAN Tetra Cell and a PowerPac basic power supply (Bio-Rad Technologies, Inc., California, USA).

3.10.1.1 Preparation of polyacrylamide gel

The method for the preparation of the polyacrylamide gel was based on Laemmli (1970). Chemical reagents, 30% acrylamide, 1.5 M Tris-HCl buffer pH 8.8, 0.5 M Tris-HCl buffer pH 6.8, 10% (w/v) SDS, 10% (w/v) ammonium persulphate (APS) and tetramethylethylenediamine (TEMED), were prepared before making the polyacrylamide gel. The final concentrations of each reagent in the resolving and the stacking gels are shown in Table. 3.3.
Table 3.3 Composition of reagents applied in gel making.

<table>
<thead>
<tr>
<th>12% Resolving gel</th>
<th>4% Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% (v/v) Milli-Q water</td>
<td>56% (v/v) Milli-Q water</td>
</tr>
<tr>
<td>12% (v/v) acrylamide</td>
<td>4% (v/v) acrylamide</td>
</tr>
<tr>
<td>0.375 M Tris-HCl (pH 8.8)</td>
<td>0.125 M Tris-HCl (pH 6.8)</td>
</tr>
<tr>
<td>0.01% (w/v) SDS</td>
<td>0.01% (w/v) SDS</td>
</tr>
<tr>
<td>0.01% (w/v) APS</td>
<td>0.005% (w/v) APS</td>
</tr>
<tr>
<td>0.0004% (v/v) TEMED</td>
<td>0.001% (v/v) TEMED</td>
</tr>
</tbody>
</table>

The gels were made in glass cassettes, which consisted of a long glass plate and a short glass plate, fixed in a cell casting frame and casting stand. Resolving gel solution was injected into the cassette using a 1 mL autopipette until the level of the resolving gel solution was 1.5 cm below the edge of the short plate. A small amount of Milli-Q water was gently added to the top of resolving gel solution in the cassette to remove air bubbles. Then the resolving gel solution was kept at room temperature for at least 30 min to ensure complete gelation. After gelation of the resolving gel, the stacking gel (4%) solution was added on the top of resolving gel to reach the edge of the short plate. A plastic comb was slowly put into the cassette to cast wells between the two glass plates. The cassettes are stored at ambient temperature for at least 30 min to ensure the complete gelation of the stacking gel.

3.10.1.2 Performance of electrophoresis assay

The reagents for running the SDS-PAGE are shown in Table 3.4.
**Table 3.4** Composition of reagents used for running SDS-PAGE

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Running buffer</td>
<td>250 mM Tris, 250 mM glycine, 0.1 (w/v) SDS</td>
</tr>
<tr>
<td>Sample buffer</td>
<td>50 mM Tris-HCl (pH 6.38), 5% (v/v) 2-ME, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol</td>
</tr>
<tr>
<td>Staining solution</td>
<td>0.1% (w/v) Coomassie brilliant blue R-250, 40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) Milli-Q water</td>
</tr>
<tr>
<td>Destaining solution</td>
<td>40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) Milli-Q water</td>
</tr>
</tbody>
</table>

The enzymes, milk samples, caseins, and hydrolysates were mixed with sample buffer to make a final concentration of between approximately 1-4 mg/mL. The mixtures were boiled at 100°C for 3 min to accelerate protein denaturation and ensure the complete binding of SDS to the proteins and peptides. The samples were cooled down to room temperature before loading onto the gel.

The cassettes are removed from the cell casting frame and loaded into the electrode assembly, with the short plate side of the cassettes put towards the inside of the cell. The electrode assembly was then transferred into the mini tank. Running buffer (600 mL) was added to fill the space between the two cassettes first, and then the rest of buffer was added into the mini tank. The spacer combs were gently removed and 10 μL of prepared samples and molecular weight markers (Precision Plus Protein Standards, Bio-Rad Technologies, Inc., California, USA) were injected into each well. The migration of samples was performed at a constant voltage of 150V for approximately 3 h until the bromophenol blue marker migrated to approximately 0.5 cm above the bottom of the cassettes.

After electrophoresis, the gels were removed from the cassettes, placed in a tray with staining solution (Table 3.4) and shaken (IKA Laboratory Equipment, Staufen, Germany) overnight at room temperature. The gels were then washed with destaining solution at least three times using shaking to remove the excess Coomassie blue stain. The proteins or peptides showed up
in the gel as blue bands. Molecular weight makers were used to estimate the molecular weight of each band based on their migration distance.

### 3.10.2 Zymography

#### 3.10.2.1 Preparation of casein embedded polyacrylamide gel

The method to perform zymography was based on Raser et al. (1995) with minor modifications. The preparation of 4% stacking gel was the same as in Section 3.10.1.1, but 0.2% (w/v) casein was dissolved in the 12% resolving gel before TEMED was added. The preparation of the gels in the cassettes was as per Section 3.10.1.2.

#### 3.10.2.2 Performance of casein zymography

Lyophilised enzyme powder was mixed with sample buffer to achieve a final sample concentration of 2 mg/mL. As samples could not be heated without destroying their enzyme activity, the mixtures were vortexed for 20 min to ensure the complete binding of SDS instead of using a 100°C incubation. Samples (15 μL) were injected into sample wells in the 4% stacking gel. The migration of protein was carried out at a constant voltage of 120 V for approximately 4 h in an ice bath. The acrylamide gel was removed from the cassettes and washed firstly with Milli-Q water, and then twice with 2% (v/v) Triton X-100 to remove the SDS in order to preserve protein activity (Egito et al., 2007). The hydrolysis reaction of the embedded casein was performed at 37°C in 0.05 M Tris-HCl buffer (pH 7.5) containing 2 mM CaCl$_2$ for 24 h. Coomassie brilliant blue R-250 with 40% (v/v) methanol and 10% (v/v) acetic acid was used to stain the gel, followed by destaining with 40% (v/v) methanol and 10% (v/v) acetic acid. Colourless bands on the gel after destaining correlate to regions of caseinolytic activity, where the enzyme is located.

### 3.10.3 Determination of the tamarillo protease cleavage site using LC-MS and MS/MS

#### 3.10.3.1 Sample preparation

Sample preparation and liquid chromatography-mass spectrometry (LC-MS) were performed according to Egito et al. (2007). Standard κ-casein and hydrolysates of κ-casein (1 mg) (Section 3.9) were dissolved in 500 μL of 10 mM Tris-HCl pH 8.0 buffer containing 8 M urea, 40 mM trisodium citrate and 20 mM DTT. Mixtures were vortexed for 30 seconds, and then incubated in an oven at 37°C for 2 h.
For HPLC analysis solution A was prepared by mixing Milli-Q water with 0.1% trifluoroacetic acid (TFA) and the eluting solution B was prepared by mixing acetonitrile with 0.1% TFA. The sample mixtures (10 µL) were separated using a Phenomenex Jupiter C4 300 Å column (250 × 4.6 mm, 5 µm) on a reversed-phase liquid chromatography mass spectrometry (RP-LCMS) system (Agilent 1290, Agilent Technologies, Inc, Santa Clara, USA). Separation was achieved using a 12-64% gradient of acetonitrile containing 0.1% TFA, for 25 min at a flow rate of 0.5 mL/min. The LC eluant was directly injected into an Agilent 6460C Triple Quadrupole LC-MS (Agilent Technologies, Inc, Santa Clara, USA) with a Jet Stream electrospray ionization (ESI) ion source and a mass resolving range from m/z 700-2400 Da.

The molecular weights of the protein, peptide and hydrolysate were analysed by Agilent MassHunter Qualitative Analysis B. 07.00 version software (Agilent Technologies, Inc, Santa Clara, USA). The equations used to calculate the molecular weight of proteins, peptides and hydrolysates were according to Strupat (2005). In the mass-to-charge ratio figures, the value of two adjacent mass-to-charge ratios was identified as m/z\(_1\) and m/z\(_2\) (where m was the mass of the ion and z was the ion charge), and z\(_1\) = z\(_2\) + 1. These two adjacent mass-to-charge ratios were acquired by protonation (mass of proton H = 1.00794 u). The molecule of mass M was express as follow:

\[
m/z = (M + zH)/z \quad (1),
\]

Then M could be expressed as:

\[
M = z (m/z - H) \quad (2).
\]

Equations 1 and 2 were combined to obtain the charge state z\(_2\) of the ion signal at m/z\(_2\), then the combined equation was expressed as:

\[
z_2 = ((m_1/z_1) - H) / ((m_2/z_2) - (m_1/z_1)) \quad (3)
\]

Based on equation 3, the molecular weight (M) of the target protein, peptide and hydrolysates could be worked out as:

\[
M = z_2 (m_2/z_2 - 1) \quad (4)
\]
The resulting data was checked against the bovine entries from the UniProt Knowledgebase (UniProtKB) protein sequence database (32,236 entries as of January 2016).

### 3.10.3.2 Protein in-gel digestion

The protein bands in polyacrylamide gel (5% - 12%) were cut and digested by trypsin based on the method of Shevchenko et al. (1996) and Albright et al. (2009) with minor modifications.

### 3.10.3.3 Washing and dicing the gel

The polyacrylamide gel was washed with Milli-Q water at least three times to remove the destaining solution and the acetic acid. Then all the liquid was removed from the gel and the target bands was excised using a freshly cleaned scalpel blade and transferred into 1.5 mL Eppendorf tubes. The bands were diced into 0.5 – 1.0 mm cubes by using the scalpel against the tube walls.

### 3.10.3.4 Protein band destaining

Acetonitrile (100 μL) and ammonium bicarbonate (100 μL, 50 mM) was added to the tubes to cover the gel pieces and then vortexed for 30 seconds to achieve thorough mixing. The tubes were then placed in a shaker at 1400 rpm at 56°C for 10 min to remove the Coomassie blue stain from the gel pieces. The shaking and heating procedure was repeated for another 10 min if all the stain had not been removed. An auto-transfer pipette was used to carefully remove all the liquid from the tubes while avoiding pipetting out the gel pieces.

### 3.10.3.5 Dehydration and reduction

Acetonitrile (200 μL) was added to each tube to dehydrate the gel pieces, resulting in shrunken, opaque, and hard gel pieces. The acetonitrile was then removed from the tubes, and any residual acetonitrile was evaporated using an Eppendorf Thermomixer Comfort (Hamburg Germany) at 56°C with the tube lids open. During this heating step, the top of the tube was covered by the lid of the heater to keep out any dust. Fifty mL of 10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate was then added to each tube and heated at 56°C for 1 h, to reduce the disulphide bonds in the target proteins or peptides.
3.10.3.6 Alkylation

After the DTT solution was removed, the reduced proteins and peptides were then alkylated with 50 µL 50 mM iodoacetamide in 50 mM ammonium bicarbonate at room temperature for 45 min in the dark. Alkylation prevented the formation of disulphide bonds, and thus exposed more proteolysis sites. The alkylation solution was then removed.

3.10.3.7 Dehydration and tryptic digestion

Acetonitrile (200 µL) was then added into the tubes to dehydrate the gel pieces. After dehydration the acetonitrile was removed and the gel pieces then rehydrated with 25 µL of freshly prepared 12.5 ng/µL trypsin dissolved in 50 mM ammonium bicarbonate. Tryptic digestion was performed overnight at 37°C. The proteins or peptides in the gel pieces were hydrolysed to small fragments by the trypsin, which could diffuse easily into the gel. The digestion reaction was stopped by adding 1 µL of 50% formic acid to quench the trypsin, and the samples were then ready for LC-MS/MS analysis.

3.10.3.8 Determination of tamarillo protease cleavage site

Samples (10 µL) prepared in Section 3.10.3.7 were injected onto a C18 trap cartridge (LC Packings, Amsterdam, Netherlands) for desalting. The peptides in the sample were then separated on a 0.3×100mm 3.5u Zorbax 300SB C18 Stablebond column (Agilent Technologies, Santa Clara, CA, USA) using the following gradient at a flow rate of 6 µL/min: 0-3 min 10% solution B, 25 min 40% solution B, 26.5 min 98% solution B, 29 min 98% solution B, 30.5 min 10% solution B, 35 min 10% solution B. Solution A consisted of 0.1% formic acid in Milli-Q water and solution B consist of 0.1% formic acid in acetonitrile. The column eluate was ionised in the electrospray source of a QSTAR-XL Quadrupole Time-of-Flight mass spectrometer (TOF-MS, Applied Biosystems, Foster City, CA, USA). A TOF-MS scan from 330-1600 m/z was obtained, followed by three rounds of MS/MS on the three most intense multiply-charged precursors in each cycle. The resulting data was checked against the bovine entries from the UniProt protein sequence database (32,236 entries as at January 2016), using ProteinPilot v5.0 (Sciex, Foster City, CA, USA).
3.10.4 Protease characterisation using de novo peptide sequencing.

*De novo* peptide sequencing of the tamarillo protease was performed based on the method of Shevchenko et al. (1996). Tamarillo protease, prepared as in Section 6.3.2, was subject to electrophorisis using 4-12% (w/v) gradient precast NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) in an XCell SureLock Mini-Cell (Invitrogen) for 1 h with a constant voltage of 180V. The gels were stained with Coomassie brilliant blue R-250 and then destained twice with 50 mM NH₄HCO₃ in 50% methanol and washed three times with Milli-Q water.

The protein band corresponding to tamarillo protease was excised, and then dried and rehydrated twice with 100% acetonitrile and 100 mM NH₄HCO₃, respectively. Trypsin digestion of the protein band was performed overnight at 37°C in 10 μL of 12.5 ng/μL sequencing grade trypsin (Promega, Madison, WI, USA) in 50 mM NH₄HCO₃. Peptides were extracted twice using 5% formic acid in 50% acetonitrile. Pooled extracts were dried in a speed-vacuum (Thermo Electron, Waltham, MA, USA). The extracts were then redissolved in 50 μL 0.1% v/v formic acid, and desalted with a C18 ZipTip (Millipore, MA, USA).

The extracted peptides were analysed using a NanoLC-MS/MS on-line system which consisted of a nano-pump UltiMate™ 3000 UHPLC binary HPLC system (Dionex, Thermo Fisher) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher, Germany). Peptides were resuspended in 20 μL of sample buffer (3% acetonitrile, 0.1% formic acid) and a 2 μL sample was injected onto a pre-column 300 μm × 5 mm (Acclaim PepMap, 5 μm particle size). After loading, peptides were eluted onto an Acclaim PepMap100 C18 capillary column (75 μm × 15 cm, 100 Å, 3 μm particle size). Peptides were separated, at a flow rate of 300 nL/min, using a 40 min gradient from 5% to 40% mobile phase B and finally eluted into the MS. Mobile phase A was 0.1% formic acid in Milli-Q water and mobile phase B was 80% acetonitrile and 0.1% formic acid. The mass spectrometer was operated in positive and data-dependent mode, with a single MS scan (350-1400 m/z; at 60000 resolution at 200 m/z in a profile mode) followed by MS/MS scans on the 10 most intense ions at 15 000 resolution. Ions selected for the MS/MS scan were fragmented using higher energy collision dissociation (HCD) at a normalized collision energy of 28% and using an isolation window of 1.8 m/z.

The MS RAW files from the Q Exactive HF were analyzed via automated *de novo* sequencing using Peak Studio (v 5.0) software. The peptide sequences adjudged to be high-quality (score >0.65) were searched using a MS-BLAST strategy. Colour-based criteria (Waridel et al., 2007) was followed after merging the candidate sequences from *in silico de novo* sequencing.
from the Peak Studio software. The candidate sequences derived from the significant results were merged into a single query string, and submitted to MS-BLAST at http://genetics.bwh.harvard.edu/msblast/ (Balbuena et al., 2009) for a search against a nonredundant database (nrdb95). Only hits with a total BLAST score above 100 or with at least one high-scoring segment pair above 72 were considered as positive (Katz et al., 2007).

3.11 Physical methods

3.11.1 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) observation was based on Esteves et al. (2003b). Fluorescent dye solution (0.25% w/v) was prepared by mixing acridine orange with Milli-Q water for 20 min using a magnetic stirrer. The acridine orange solution was then mixed with the milk samples, and milk samples containing tamarillo extract, purified tamarillo protease or rennet, using a a magnetic stirrer for 2 min. More details on the different samples used in CLSM is given below and in Chapter 5 and Chapter 7. Aliquots (110 µL) of the stained milk samples were transferred into 76 mm × 26 mm × 1.35 mm glass slides with 0.6 - 0.8 mm depth and 18 mm diameter sample wells (Paul Marienfeld GmbH & Co., Lauda-Königshofen, Germany) by autopipette. Cover slips (18 mm × 18 mm, 0.17 mm thick) were used to cover the milk samples on the glass slides. The edge of the cover slips were sealed by nail polish to prevent evaporation. The glass slides were placed in a plastic box with wet paper towels at the bottom to prevent evaporation. The plastic box was incubated in an oven (Function Line incubator, Heraeus, Langenselbold, Germany) at 30°C (for milks prepared as per Section 3.2.1) for 1, 4 and 24 h, and at 35°C (milk prepared in Section 3.2.3) for 10 h. After each incubation time, the microstructures of the resulting gels were immediately observed using an Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan) with a X-Cite Series 120Q mercury halide lamp (Lumen Dynamics, Ontario, Canada) at a wavelength of 488 nm. Gel micrographs were obtained using a 60 × oil immersion objective lens. Nine 512 × 512 resolution images of Z-stack projections were obtained within a 2 µm depth. All micrographs of milk gel samples were analysed using the Olympus Fluoview 2.1 software (Olympus Corporation, Tokyo, Japan).
3.11.2 Rheology

Small and large dynamic oscillation rheology was performed on milk gels using an Anton Paar Physica MCR 301 stress-controlled rheometer (Anton Paar, Graz, Austria) (Figure 3.2A). The rheological properties of the gels was characterised by their viscoelastic behaviour, specifically, the storage (elastic) modulus $G'$ and the loss (viscous) modulus $G''$. A cup and bob geometry system (CC27), with a diameter of 26.5 mm and length of 48 mm for the bob and 27.5 mm of inner diameter of the cup (Figure 3.2 B) was used.

3.11.2.1 Sample preparation

Milk samples were prepared as per section Section 3.2.1. Further details on sample preparation are given in Chapter 5 and Chapter 7. The samples were mixed in Falcon tubes with rennet or tamarillo protein extract or purified protease as shown in Table 3.5. A sample volume of 18.5 mL was poured into the rheometer cup geometry and the bob was lowered. The surface of the sample was covered with a few drops of soya oil to prevent sample evaporation (Figure 3.2 C).

Table 3.5 Sample preparation for rheological measurements

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Milk concentration (w/w)</th>
<th>Composition (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>10% (w/w) skim milk + rennet</td>
<td>30 g of 10% (w/w) skim milk + 30 µL of 10 times diluted rennet</td>
</tr>
<tr>
<td></td>
<td>10% (w/w) skim milk + tamarillo crude extract (Laird’s Large)</td>
<td>30 g of 11.25% (w/w) skim milk + 3.5 mL crude extract of Laird’s Large tamarillo</td>
</tr>
<tr>
<td></td>
<td>10% (w/w) skim milk + tamarillo crude extract (Amber)</td>
<td>27 g of 11.11% (w/w) skim milk + 3 mL crude extract of Amber tamarillo</td>
</tr>
<tr>
<td></td>
<td>10% (w/w) skim milk + rennet</td>
<td>25 g of 10% (w/w) skim milk + 25 µL of 10 times diluted rennet</td>
</tr>
<tr>
<td></td>
<td>20% (w/w) skim milk + rennet</td>
<td>25 g of 20% (w/w) skim milk + 50 µL of 10 times diluted rennet</td>
</tr>
<tr>
<td>D₂O</td>
<td>10% (w/w) skim milk + purified tamarillo protease</td>
<td>12.5 g of 20% (w/w) skim milk + 50 mg protease dissolved in 12.5 g D₂O</td>
</tr>
<tr>
<td></td>
<td>20% (w/w) skim milk + purified tamarillo protease</td>
<td>20 g of 25% (w/w) skim milk + 100 mg protease dissolved in 5 g D₂O</td>
</tr>
</tbody>
</table>
3.11.2.2 Time sweep measurements

Time sweep measurements were performed as follows: the sample was loaded into the rheometer and held at a constant temperature of 25°C for 2 min to ensure temperature equilibration. The temperature was then increased to the target temperature (see details below). When the target temperature was reached, $G'$ and $G''$ values were recorded every minute, at a constant applied strain of 1% and a constant frequency of 0.1 Hz, using the Start Rheoplus software (Anton Paar, Graz, Austria). The time sweep measurements were usually recorded for 3 h, but in some cases they went up to 10 h.

Time sweep measurements allowed monitoring the kinetic of gelation through the increase of $G'$ and $G''$ with time. Further, the time at which $G' = G''$ could be defined as the gelation time, since this indicated a transition from liquid to gel.

The target temperatures for milk samples in H$_2$O were 20, 25, 27.5, 30, 35 and 40°C to investigate the effect of temperature on the milk gelation. These temperatures were reached from the starting temperature of 25°C by increasing the temperature at a rate of 5°C/min. In the case of milk samples prepared in D$_2$O, the initial temperature of the sample was set at 25°C, then increased to 35°C at a rate of 5°C/min. $G'$ and $G''$ were recorded at a constant frequency of 1 Hz and a constant applied strain of 1% as a function of time for 10 h. The applied strain of 1% was within the viscoelastic linear region.
3.11.2.3 Frequency sweep

The frequency sweep was performed at the end of the time sweep measurement. For milk samples prepared in H$_2$O, $G'$ and $G''$ were measured at a constant strain of 1%, while the frequency was applied from 0.01 to 10 Hz at a constant temperature of 30°C. For milk samples prepared in D$_2$O the frequency sweep was carried out from 0.1 to 10 Hz at a constant temperature of 25°C. The frequency sweep allows for the determination of the viscoelastic properties of the gelled milks. For example, if $G'$ and $G''$ did not vary with the frequency, with $G'$ higher than $G''$ by at least 10 fold, then the sample can be classified as a strong gel (Fernandes et al., 1991).

3.11.2.4 Strain sweep

At the end of the frequency sweep the temperature was set to 30°C for the milk samples prepared in H$_2$O. While the time sweep measurements (Section 3.11.2.2) were carried out at different temperatures, the strain sweep measurements were carried out at a constant temperature of 30°C to allow comparison between the samples. The strain sweep measurements were performed using strains varying from 0.1 to 10,000% at a constant frequency of 0.1 Hz.

Strain sweep measurements allow the determination of the linear viscoelastic region (LVR) which defines the strain range at which $G'$ and $G''$ are independent of the frequency. Further, the strain at which $G'$ and $G''$ cross-over can be defined as the strain at which the gel breaks down and starts to flow.

In the case of milk samples prepared in D$_2$O, the large deformation sweep measurements were performed with a constant shear rate (0.1 s$^{-1}$) for a total time interval of 2000 s at 25°C.

3.11.3 Analysis of casein micelles and milk gels internal structures

The internal structure of the casein micelles in milk gels resulting from the action of rennet or purified tamarillo protease was investigated using ultra small angle neutron scattering (USANS) and small angle X-ray scattering (SAXS). USANS was performed on the Kookaburra beamline at the OPAL reactor at the Australia National Science and Technology Organisation (ANSTO), Sydney, Australia (Rehm et al., 2013) and SAXS was performed on the BL19U2 BioSAXS beamline at the Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, China.
3.11.3.1 USANS

The Kookaburra beamline setup was based on the Bonse-Hart method (Bonse and Hart, 1965), which consisted of two channel-cut perfect Si single crystals named the monochromator and the analyser. The wavelengths of 4.74 Å (long neutron wavelengths) and 2.37 Å (short neutron wavelengths) were operated using Si111 and Si311 reflection from the two channels. Only milks prepared in D$_2$O were measured by USANS. The following parameters were used: a neutron wavelength $\lambda$=4.74 Å, a Cd aperture with a diameter of 30 mm, and a $Q$ range of 0.0003 to 0.1 nm$^{-1}$. The measurements were performed at room temperature.

The prepared milk samples were filled into demountable sample cells. The sample cells were then incubated in an oven (Laboratory Equipment PTY. Ltd. Australia) set at 35°C for 10 h. To obtain the milk gel curds and serums, milk gels were incubated similarly to the USANS cells above, but in 40 mL glass vials, which, after gelation, were cut using a thin spatula into approximately 2 to 5 mm$^3$ cubes. The cut gels were then transferred into 50 mL centrifuge tubes and centrifuged at 1,500 × g for 30 min at room temperature using an Eppendorf centrifuge 5810 R (North Ryde, Australia). Centrifugation separated the supernatant gel serum from the precipitated gel curd. The gel serum was then filtered through a 0.22 µm filter to be measured by USANS for baseline substraction.

To optimise the intensity of the neutron scattering, a cell pathlength of 1 mm was used for the measurement of the 10% (w/w) milks, their tamarillo or rennet-induced gels, and their serums. USANS cells with a 10 mm pathlength were used for the 1% (w/w) milks, 1% (w/w) milk gels, and 1% (w/w) milk serum. A cell pathlength of 0.5 mm was used for the 20% (w/w) milks, their gels and serums obtained after treatment with rennet or tamarillo protease.

3.11.3.2 SAXS

The samples used in the SAXS experiments were prepared similarly to those used in the USANS measurements (Section 3.11.3.1) except that the milk gels were made by transferring the milk-enzyme mixtures into 2 mm diameter capillaries (Charlessupper Company, Natick, USA). These were then sealed with Para-film and incubated at 35°C for 10 h in an BPZ-6123 oven (Shanghai Yiheng Scientific Instruments Co., Ltd. Shanghai, China). The serums prepared, similarly as for USANS measurements, were also measured in the same capillary tubes. However, the curds, since they could not be loaded into capillaries, were measured in 2
mm thick aluminium holders containing a 5 mm diameter drilled hole. The curds were restrained in the hole using cellotape.

SAXS was performed in a static mode at a wavelength of 1.033 Å and a sample-to-detector distance of 2234.4 mm, yielding a Q-range between 0.06 to 4 nm\(^{-1}\). The SAXS profiles of the samples were exhibited by subtracting the intensity of the background (serum) from the intensity of the samples (milk and milk gels) using the BioXTAS RAW software version 1.2.1 (Nielsen et al., 2009). All the samples were measured at room temperature.

### 3.12 Statistical analysis

All the experiments in this thesis were performed at least in duplicate. For the protease activity assay, samples were made in duplicates on two different days and the measurements were performed in duplicate. All the rheological measurements were carried out in duplicate on freshly made skim milk samples.
Chapter 4

4. Purification and characterisation of a protease (tamarillin) from tamarillo
Abstract

A tamarillo protease was obtained from tamarillo fruit by ammonium sulphate precipitation, followed by purification using diethylaminoethyl (DEAE)-Sepharose chromatography. A protease activity assay was performed on selected peak fractions using casein as a substrate. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the selected peak fractions showed that the chromatographic peak consisted of one protein with a molecular mass of approximately 70 kDa. The protease showed optimal activity at pH 11 and 60°C. The enzyme was sensitive to phenylmethylsulphonyl fluoride (PMSF) while ethylenediaminetetraacetic acid (EDTA) and p-chloromercuribenzoic acid (PCMB) had little effect on its activity, indicating that this enzyme was a serine protease. Hg²⁺ strongly inhibited enzyme activity, possibly due to the formation of mercaptide bonds with the thiol groups of the protease. This suggested that there may be some cysteine residues located close to the active site. *De novo* sequencing strongly indicated that the protease isolated from tamarillo was probably a subtilisin-like alkaline serine protease. The protease from tamarillo has been named ‘tamarillin’.
4.1. Introduction

Protein hydrolysis is a crucial factor in many biochemical processes, including cell growth and development (Callis, 1995). Proteolytic enzymes account for approximately 60% of the total global commercial enzyme business due to their wide range of substrate specificities, their wide range of pH activity and their relatively high tolerance to thermal treatment (Rajkumar et al., 2011). Moreover, proteases are widely applied in the food, pharmaceuticals, silk, detergent, leather, medicinal and biotechnological industries (Nallamestty et al., 2003; Banik and Prakash, 2004; Gupta et al., 2002). In the food industry, Sumantha et al. (2006) stated that proteases contributed to the improvement of functional and nutritional properties of proteins, and to the hydrolysis of gelatin, soy protein, casein and whey proteins. In terms of food processing, especially in the dairy industry, proteases play a significant role in milk coagulation, which is a key stage in cheesemaking.

Calf rennet is a traditional milk-clotting enzyme which has been used predominantly in the manufacture of different cheeses for centuries because of its high specificity to cleave the Phe105-Met106 peptide bond in κ-casein. The cleavage of κ-casein, which is located on the surface of the casein micelle, results in the initiation of milk coagulation due to the destabilization of the casein micelle (Pires et al., 1994; Vishwanatha et al., 2010). A worldwide shortage of rennet and increased global cheese production has resulted in the search to find new proteases with rennet-like properties as a substitute in cheese manufacture (Jacob et al., 2011).

Proteases from plant sources have shown a high potential as rennet substitutes and have already been used in cheese production processes (Huang et al., 2011; Katsaros et al., 2010). Most of the proteases purified from plant sources have been identified as cysteine proteases. These enzymes are typically active over a wide range of temperature and pH values, and exhibit great potential in the food, biotechnology and medical industries (Uchikoba et al., 1998; Shah et al., 2013). Cysteine proteases, such as actinidin purified from kiwifruit (Katsaros et al., 2010), ficin extracted from the latex of ficus (Devaraj et al., 2008), protein isolated from sunflower and albizia seeds (Egito et al., 2007) and papain from papaya (Saran et al., 2016), all showed milk-clotting activities and casein hydrolysis ability. However, air oxidation and metal ions are two factors that negatively affect the activity of cysteine proteases, and thus need to be considered during enzymatic reactions. Unfortunately, it is not convenient or economical to add reductants and chelating agents to cysteine proteases (Tomar et al., 2008).
Serine proteases, which were once thought to be rare in plants, have been found and extracted from different parts of various plants, including the latex, fruits, seed, root and leaves (Antão and Malcata, 2005; Shah et al., 2013). Recently, several serine proteases have been isolated and used for milk coagulation, including dubiumin, which is purified from the seeds of *Solanum dubium* (Ahmed et al., 2009a), streblin, extracted from *Streblus Asper* (Tripathi et al., 2011), and neriifolin, which is present in the latex of *Euphorbia neriifolia* (Yadav et al., 2011). Compared to cysteine proteases, serine proteases do not require the use of reductants and chelating agents. In addition, serine proteases have the ability to retain their activity and remain stable under relatively high temperatures, a wide range of pH values and even in the presence of both oxidizing agents and surfactants (Ahmed et al., 2009a). Therefore, serine proteases have high potential usage in the food industry and represent economical value as milk-clotting enzymes.

Tamarillo (or tree tomato, *Cyphomandra betacea* (F)) originated in South America. Tamarillo was introduced into New Zealand, and has been planted commercially since the late 19th century (Bohs, 1989b). Tamarillo is closely related to the genus *Solanum* which belongs to the *Cyphomandra* (Solanaceae) species (Bohs, 1989a; Bohs, 1994). In the present work, an alkaline serine protease isolated from tamarillo was purified and characterised. To the best of our knowledge, a tamarillo protease and its properties have never been reported before.

4.2. Material and methods

4.2.1 Material

Tamarillo fruit (Laird’s Large cultivar) were obtained from Maungatapere, New Zealand. Rennet was purchased from RENCO New Zealand Laboratory, Eltham, New Zealand. Casein from bovine milk was purchased from Sigma-Aldrich (Auckland, New Zealand). DEAE-Sepharose was bought from GE Healthcare, Sweden. All chemicals were of chemical grade and were purchased from Sigma-Aldrich.

4.2.2 Protease purification

Tamarillo fruit (300 g) were homogenized for 20 min using a Polytron PCU2 laboratory homogeniser (Brinkmann Instruments, Luzern, Switzerland), followed by filtration through two layers of cheesecloth to remove insoluble material. The filtrate was mixed with 80 mL sodium citrate (0.05 M, pH 5.5). The mixture was centrifuged at 15,000 × g at 4°C for 20 min. According to the method of Burgess et al. (2009), 61.69 g of ammonium sulphate was added
to 155 mL supernatant to make a 65% saturation ammonium sulphate solution, mixed gently for 20 min using a magnetic stirrer, followed by standing in an ice bath overnight. The precipitated proteins were collected by centrifugation at 15,000 × g at 4°C for 20 min and re-dissolved in 30 mL 0.05 M citrate buffer (pH 5.5). Ammonium sulphate was removed by dialysing the protein solution in a DEAE cellulose tube (molecular weight cut-off, 12,000 Da) against the same citrate buffer at 4°C for 24 h, with the dialysis buffer being changed three times.

Ion-exchange chromatography on a DEAE-Sepharose resin packed in a 22 × 5 cm column equilibrated with sodium citrate buffer (0.05 M, pH 5.5) overnight was used for protein purification. Tamarillo protein solution (40 mL) was applied to the column. After washing the column with 240 mL of citrate buffer the protein was eluted using a 200 mL linear gradient of 0.0-1.0 M NaCl in citrate buffer at a flow rate of 1.0 mL/min. Eluant fractions (3 mL) were collected in glass tubes and their absorbance measured at 280 nm using an UVmini-1240 spectrophotometer (Shimadzu Corporation, Australia). Consecutive tubes giving similar absorbance readings were pooled together as a single fraction and dialysed against Milli-Q water for 24 h. The protein in each fraction was concentrated by freeze-drying and stored at -80°C for subsequent analysis.

4.2.3 Electrophoresis and protein content

The molecular mass of the purified protease was determined using 4-12% (w/v) polyacrylamide SDS-PAGE (Laemmli, 1970), under denaturing and reducing conditions. The gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250. The molecular mass was calculated by comparison to standard protein markers.

The protein content of the different fractions collected from the ion-exchange chromatography was estimated using the absorbance at 280 nm. Protein was quantitatively measured by the Bradford method (Bradford, 1976) using a calibration curve of bovine serum albumin (BSA).

4.2.4 Proteolytic activity assay

Proteolytic enzyme activity was measured using the method of Kunitz (1947) with some modifications, and with casein as the substrate. Protein preparation (100 μL) was mixed with 1.1 mL of 0.1 M Tris-HCl buffer (pH 7) containing 1% (w/v) casein, vortexed and then incubated at 35°C in a water bath for 20 min. The reaction was stopped by adding 1.8 mL 10%
(w/v) trichloroacetic acid (TCA), and the solution was then allowed to stand for 30 min at room temperature. Precipitated protein was removed by centrifugation at 10,300 × g for 20 min, and the absorbance of the supernatant was measured at 280 nm against the blank. The blank was prepared by mixing TCA and enzyme solution, and then adding casein solution. One unit of protease activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per milliliter in 1 min under the assay conditions. A standard curve was made by using 0-100 mg/L tyrosine (Hadj-Ali et al., 2007), and a standard specific enzyme activity was defined in units/mg of protein.

4.2.5 Optimal temperature, optimal pH and stability

The effect of temperature on the enzyme activity was studied using casein as the substrate. The range of pH and temperature studied was based on Attri et al. (2015). The standard enzyme assay (Section 4.2.4 above) was performed over a temperature range from 0 to 100°C for 20 min. The temperature stability of the enzyme was investigated by pre-incubating the enzyme at specific temperatures ranging from 0 to 100°C for 20 min, followed by measurements using the enzyme assay under optimum pH and temperature conditions. The remaining protease activity was expressed as a percentage of the control.

The optimal pH value for enzyme activity was also studied using 50 mM buffers of different pH values ranging from 6 to 14 following the method of Attri et al. (2015). The buffers used in the standard proteolytic activity assays were: phosphate buffer, pH 6; Tris-HCl buffer, pH 7-8; glycine-NaOH buffer, pH 9-10; phosphate-NaOH buffer, pH 11; KCl-NaOH buffer, pH 12-13 and 1 M NaOH, pH 14. The pH stability of the protease was determined by pre-incubating 100 μL of the purified enzyme with 150 μL of the different pH buffers at 25°C for 20 min, and the remaining enzyme activity was then measured using the optimum assay conditions. The results of the residual enzyme activities were expressed as a percentage of the control activity. Protease activities were not studied at pH 4 and 5 as the isoelectric point of casein is at approximately 4.6. Casein did not dissolve in buffer at pH 4 and 5 even after overnight string.

4.2.6 Effect of organic solvents, inhibitors and metal ions on protease activity

Purified protease (100 μL) was mixed with 25% (v/v) of different organic solvents including isopropanol, methanol, ethanol, glycerol, dimethyl sulfoxide (DMSO), and chloroform, and the
mixture was then incubated at 25°C for 20 min. The enzyme was assayed (Section 4.2.4) and the activity was compared to the control without organic solvent.

Inhibitors (1 mM and 5 mM) such as phenylmethylsulphonyl fluoride (PMSF), for serine proteases, \( p \)-chloromercuribenzoic acid (PCMB), for cysteine proteases, and ethylenediaminetetraacetic acid (EDTA), for metalloproteases, were added to the enzyme activity assay. The enzyme was pre-mixed with the inhibitors and incubated at room temperature for 20 min. The residual activity was measured by the standard enzyme assay. The results were expressed as the percentage of the protease activity measured without inhibitors.

The effect of different chloride salts on enzyme activity, which included Na\(^+\), Hg\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\) (at concentrations of 1 mM and 5 mM), was studied. The enzyme was pre-incubated with the individual metal ions for 20 min at room temperature, followed by the standard enzyme assay. The results were expressed as the percentage of the protease activity measured without metal ions.

**4.2.7 Kinetic parameters determination**

The relationship between increasing substrate concentration and hydrolysis reaction velocity of tamarillo proteolytic enzyme was studied in 50 mM KCl-NaOH buffer, pH 11 at 60°C. Casein was used as the substrate over the range of 0-800 \( \mu \text{M} \). The measurements were performed using the standard protease assay. Blanks were made by mixing the casein substrates with the enzyme inactivated by TCA. The Michaelis–Menten plot (Lineweaver et al., 1934) and the Hanes plot (Hanes, 1932) were used to calculate the kinetic parameter, \( K_m \).

**4.2.8 Protease characterisation using de novo peptide sequencing**

*De novo* peptide sequencing of the tamarillo protease was performed based on the method of Shevchenko et al. (1996). Tamarillo protease, prepared as in Section 4.3.2, was subjected to electrophorisis using 4-12% (w/v) gradient precast NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) in an XCell SureLock Mini-Cell (Invitrogen) for 1 h with a constant voltage of 180V. The gels were stained with Coomassie brilliant blue R-250 and then destained twice with 50 mM \( \text{NH}_4\text{HCO}_3 \) in 50% methanol and washed three times with Milli-Q water. The protein band corresponding to tamarillo protease was excised, and then dried and
rehydrated twice with 100% acetonitrile and 100 mM NH₄HCO₃, respectively. Trypsin digestion of the protein band was performed overnight at 37°C in 10 μL of 12.5 ng/μL sequencing grade trypsin (Promega, Madison, WI, USA) in 50 mM NH₄HCO₃. Peptides were extracted twice using 5% formic acid in 50% acetonitrile. Pooled extracts were dried in a speed-vacuum (Thermo Electron, Waltham, MA, USA). The extracts were then redissolved in 50 μL 0.1% v/v formic acid, and were desalted with a C18 ZipTip (Millipore, MA, USA).

The extracted peptides were analysed using a NanoLC-MS/MS on-line system which consisted of a nano-pump UltiMate™ 3000 UHPLC binary HPLC system (Dionex, Thermo Fisher) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher, Germany). Peptides were resuspended in 20 μL of sample buffer (3% acetonitrile, 0.1% formic acid) and a 2 μL sample was injected onto a pre-column (Acclaim PepMap, 300 μm × 5 mm, 5 μm particle size). After loading, peptides were eluted onto an Acclaim PepMap100 C18 capillary column (75 μm × 15 cm, 100 Å, 3 μm particle size). Peptides were separated and eluted into the MS, at a flow rate of 300 nL/min, using a 40 min gradient from 5% to 40% mobile phase B. Mobile phase A was 0.1% formic acid in Milli-Q water and mobile phase B was 80% acetonitrile and 0.1% formic acid. The mass spectrometer was operated in positive and data-dependent mode, with a single MS scan (350-1400 m/z at 60000 resolution; at 200 m/z in a profile mode) followed by MS/MS scans on the 10 most intense ions at 15000 resolution. Ions selected for the MS/MS scan were fragmented using higher energy collision dissociation (HCD) at a normalized collision energy of 28% and using an isolation window of 1.8 m/z.

The MS RAW files from the Q Exactive HF were analysed via automated de novo sequencing using Peak Studio (v 5.0) software. The peptide sequences of high-quality (score >0.65) were searched using a MS-BLAST strategy. Colour-based criteria (Waridel et al., 2007) were followed after merging the candidate sequences from in silico de novo sequencing from the Peak Studio software. The candidate sequences derived from the significant results were merged into a single query string, and submitted to MS-BLAST at http://genetics.bwh.harvard.edu/msblast/ (Balbuena et al., 2009) for a search against a nonredundant database (nrdb95). Only hits with a total BLAST score above 100 or with at least one high-scoring segment pair above 72 were considered as positive (Katz et al., 2007).
4.3 Results and discussion

4.3.1 Purification and molecular mass of tamarillo protease

As shown in Figure 4.1, two absorbance peaks eluted from the DEAE-Sepharose column with absorbance maxima for the 150 and 212 fractions. Maxima for the protease activity did not match the protein profile, with maximum activity in fraction 126, a shoulder at fraction 150 and then a second, smaller maximum at fraction 225. Fractions for the two different peaks were pooled (fractions 113-137, peak 1; and fractions 204-221, peak 2) and the first peak showed a higher protease activity (0.043 ± 0.003 units/mL) compared to the second peak (0.006 ± 0.002 units/mL). Compared with the crude extract, the first peak showed an approximate 5.6-fold increase in protease activity as shown in Table 4.1, while the second peak contained more protein but only a low level of activity was measured.

![Figure 4.1 Elution curve of tamarillo protease on anion-exchange chromatography resin. DEAE-Sepharose was pre-equilibrated with 0.05 M sodium citrate buffer at pH 5.5. The unbound proteins were washed out with sodium citrate buffer and column was eluted with a linear gradient of 0.0-1.0 M NaCl at the same pH. Fractions of 3 mL volume were collected. Protein content (■) and proteolytic activity (■) were measured.](image-url)
Table 4.1 Purification of tamarillo protease.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol (mL)</th>
<th>Protein Concentration (mg/mL)</th>
<th>Total (mg)</th>
<th>Protein Activity Concentration (units/mL)</th>
<th>Total (units)</th>
<th>Specific Activity (units/mg protein)</th>
<th>Purification Factor</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sample⁹</td>
<td>155</td>
<td>0.94 ± 0.01</td>
<td>145.0</td>
<td>0.112 ± 0.005</td>
<td>17.3</td>
<td>0.119 ± 0.002</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Crude extract⁸</td>
<td>120</td>
<td>1.17 ± 0.03</td>
<td>140.0</td>
<td>0.143 ± 0.009</td>
<td>17.1</td>
<td>0.121 ± 0.005</td>
<td>1.0</td>
<td>99</td>
</tr>
<tr>
<td>Fraction I</td>
<td>213d</td>
<td>0.06 ± 0.00</td>
<td>13.3</td>
<td>0.043 ± 0.003</td>
<td>9.1</td>
<td>0.682 ± 0.072</td>
<td>5.6</td>
<td>53</td>
</tr>
<tr>
<td>Fraction II</td>
<td>147d</td>
<td>0.10 ± 0.02</td>
<td>14.7</td>
<td>0.006 ± 0.002</td>
<td>0.8</td>
<td>0.050 ± 0.013</td>
<td>0.4</td>
<td>5</td>
</tr>
</tbody>
</table>

⁹ Tamarillo pulp after homogenization, filtration and centrifugation

b After ammonium sulphate precipitation and dialysis

c DEAE-Sepharose fractions 113-137

d Only 40 mL was loaded onto the DEAE-Sepharose column for each run; this is the accumulation of three runs

e DEAE-Sepharose fractions 204-221

The protease in Fraction I migrated as a single protein band on a 4-12% polyacrylamide gel under both denaturing and reducing conditions. The molecular mass of the purified enzyme was approximately 70 kDa under denaturing conditions (Figure 4.2, Lane 1 and 2, non-reducing), while under reducing condition the main band migrated at approximately 23 kDa.

The molecular mass of the tamarillo protease was similar to that of the subtilisin-like serine endoproteases from barley (Terp et al., 1999) and to a typical pyrolysin-like subtilase from tomato (*Solanum lycopersicum*) plants (Ottmann et al., 2009). Antão and Malcata (2005) indicated that the molecular mass of serine proteases in plants varied from 19 to 110 kDa with the majority having molecular masses between 60 and 80 kDa.
4.3.2 Enzyme pH and temperature optima and stability

When the assay for the purified tamarillo protease was conducted using different pH buffers, the enzyme displayed protease activity over a wide range of pH values, from pH 6 to 12, with a maximum hydrolysis activity at pH 11 (Figure 4.3). Although at pH 10 only 60% of the maximum activity was observed, the protease displayed broad activity maxima with approximately 90% of this activity being retained at pH 12.

The stability of the tamarillo protease after incubation at different pH values, followed by activity analysis at the optimal pH of 11, showed that the enzyme was pH stable between pH values 7-11, and its stability then started to decrease markedly at pH 12 (Figure 4.3). These two sets of results, regarding enzyme activity and stability, indicated that the purified tamarillo protease belongs to the plant alkaline protease family.

Other plant proteases with high pH optima include those from the fruit of *Maclura pomifera* (Rudenskaya et al., 1995), the dandelion, *Taraxacum officinale* webb (Rudenskaya et al., 1998) and *Cucurbita ficifolia* (Curotto et al., 1989), which have pH stability ranges of 6 - 9, 7 - 9 and 8 - 11, respectively. These are all subtilisin-like serine proteases. A survey of the literature
suggested that serine proteases extracted from plant sources that exhibit optimum enzyme activity and stability at high pH values are very rare. The serine proteases, dubiumin, isolated from the seed of fresen (Ahmed et al., 2009a) and a protease from Cucumis trigonus Roxburghi (Asif-Ullah et al., 2006) also showed optimal activity at pH 11 (Table 4.2). Moreover, a pyrolysin-like subtilase from tomato plants also exhibited a high level of stability at alkaline pH values (Ottmann et al., 2009). In terms of food processing, pH is an important parameter that affects the usage of most enzymes, especially under alkaline conditions. This is because most enzymes are unstable in alkaline pH, and thus are unable to catalyse hydrolysis reactions under high pH conditions, which might limit their usefulness in food applications, even when used as cheese-making coagulants (Lamas et al., 2001).

Figure 4.3 Effect of pH on enzyme activity (■) and stability (○) ranging from pH 6-14.
Table 4.2 Characteristics of reported plant serine proteases

<table>
<thead>
<tr>
<th>Plant source (protease name)</th>
<th>Mr (kDa)</th>
<th>pH optima</th>
<th>Temperature optima (°C)</th>
<th>pH stability</th>
<th>Temperature stability (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamarillo protease&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75</td>
<td>11.0</td>
<td>60</td>
<td>7.0-12.0</td>
<td>60</td>
</tr>
<tr>
<td><em>Cucumis trigonus</em></td>
<td>67</td>
<td>11.0</td>
<td>70</td>
<td>4.0-10.0</td>
<td>65</td>
</tr>
<tr>
<td><em>Cucumis melo</em> (cucumisin)</td>
<td>54</td>
<td>7.1</td>
<td>70</td>
<td>4.0-11.0</td>
<td>50</td>
</tr>
<tr>
<td><em>Taraxacum officinale</em> (taraxilisin)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67</td>
<td>8.0</td>
<td>40</td>
<td>6.0-9.0</td>
<td>40</td>
</tr>
<tr>
<td><em>Solanum dubium</em> (dubiumin)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>66</td>
<td>11.0</td>
<td>70</td>
<td>3.0-12.0</td>
<td>67-70</td>
</tr>
<tr>
<td><em>Euphorbia milii</em> (milin)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51.4</td>
<td>8.0</td>
<td>60</td>
<td>5.0-12.0</td>
<td>25-65</td>
</tr>
<tr>
<td><em>Maclura pomifera</em> (macluralisin)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>65</td>
<td>8.5</td>
<td>58</td>
<td>7.0-9.0</td>
<td>25-75</td>
</tr>
<tr>
<td><em>Ipomoea camea</em> (camein)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>80.2</td>
<td>6.5</td>
<td>65</td>
<td>3.0-10.0</td>
<td>35-75</td>
</tr>
</tbody>
</table>

<sup>a</sup>This study  
<sup>b</sup>Asif-Ullah et al. (2006)  
<sup>c</sup>Yadav et al. (2006).  
<sup>d</sup>Rudenskaya et al. (1998).  
<sup>e</sup>Ahmed et al. (2009a)  
<sup>f</sup>Kaneda et al. (1975).  
<sup>g</sup>Rudenskaya et al. (1995).  
<sup>h</sup>Patel et al. (2007).

According to the results shown in Figure 4.4, 60°C was the optimal temperature of the tamarillo protease. The enzyme activity increased exponentially when the temperature was increased from 0 to 60°C, then decreased nearly linearly to zero activity when the temperature was further increased to 100°C, although it retained nearly 40% of its activity at 80°C.

The activity of the enzyme also remained stable when it was incubated at different temperatures for 20 min, up to a temperature of 60°C. Above this temperature the activity decreased nearly linearly as the temperature was increased to 100°C. This suggested that the optimal tamarillo protease activity was limited by the stability of the protein at temperatures above 60°C.
The plant serine protease from *Euphorbia milii* (Yadva et al., 2006) showed a similar optimal temperature. The optimum temperature of some other plant serine proteases were also high, for example, both dubiumin from seed of *Solanum dubium* Fresen (Ahmed et al., 2009a) and the protease from *Cucumis trigonus* Roxburghi (Asif-Ullah et al., 2006) displayed optimum activity at a temperature of 70°C (Table 4.2).

![Figure 4.4](image)

**Figure 4.4** Effect of temperature on enzyme activity (■) and stability (●) ranging from 0 to 100°C. Assay conditions are as described in the Methods section.

### 4.3.3 Effect of organic solvents, inhibitors and metal ions on the enzyme activity

The effect of different organic solvents (25% (v/v)) on the activity of the purified protease from tamarillo are reported in Figure 4.5. The enzyme retained ~96% of its original activity in the presence of ethanol, and up to 89% and 85% of its activity in methanol and glycol, respectively. In the presence of isopropanol, DMSO, and chloroform the enzyme activity retained was ~76%, ~77%, and ~62%, respectively. The activity of enzymes in the presence of organic solvents can be affected by many factors. Under organic solvents, depending on the concentration, enzymes tend to be inactivated and unstable (Ghorbel et al., 2003). As reported by Barberis et
al. (2002), in the presence of organic solvents, the catalytic processes of proteases are affected by the disruption of the hydrogen bonds, the hydrophobic interactions and the charges, which in turn affect the dynamics and conformation of the proteases. While the tamarillo protease remained active in the presence of some of the organic solvents investigated here, unfortunately these results cannot be compared to other plant serine proteases due to the lack of other published data.

![Figure 4.5](image) Effect of different organic solvents on tamarillo protease activity.

Inhibition studies can provide insight into the nature of an enzyme, its cofactor requirement, and the nature of the active center (Sedmak and Grossberg, 1977; Sigma and Mooser, 1975). As can be seen in Figure 4.6, EDTA and PCMB (1 mM and 5 mM) had little effect on the activity of the tamarillo protease, indicating that this protease was neither a cysteine protease nor a metalloprotease. However, the typical serine protease inhibitor PMSF inhibited the enzyme activity by 70% at 1 mM PMSF and 80% at 5 mM PMSF. Serine residues in the active site of a serine protease covalently bind PMSF. PMSF can therefore be used as a label to determine the active site in serine proteases (James, 1978; Fahrney and Gold, 1963). Thus, it
is likely that the purified tamarillo protease is a serine protease. Other plant serine proteases, including a subtilisin-like protease from *Cucumis trigonus* Roxburghi (Asif-Ullah et al., 2006), dubiumin from seed of *Solanum dubium* Fresen (Ahmed et al., 2009a) and milin from the medicinal plant *Euphorbia milli* (Yadav et al., 2006), were reported to be strongly inhibited by PMSF.

![Figure 4.6](image)

**Figure 4.6** Effect of different inhibitors (■ 1 mM and □ 5 mM) on the proteolytic activity of the tamarillo protease. Inhibitors were pre-incubated with tamarillo protease for 20 min at room temperature.

Metal ions, namely Na⁺, Zn²⁺, Co²⁺, Ca²⁺, and Mn²⁺ (at 1mM or 5mM), showed negligible effect on tamarillo protease activity (Figure 4.7). These results illustrated that there was no requirement for the tamarillo protease to be activated by these metal ions. However, the protease activity was strongly inhibited by the Hg²⁺ metal ion, with the enzyme retaining only ~32% and ~30% of its original activity after 20 min incubation with 1 mM and 5 mM Hg²⁺, respectively. According to Ahmed et al. (2009a), dubiumin, a serine protease from the seeds of *Solanum dubium*, was inhibited by Hg²⁺, indicating that dubiumin contained cysteine residues near its active site. Similar findings were reported for a subtilisin-like protease from *Bacillus cereus* (Moriyama et al., 1998).
Figure 4.7 Effect of different metal ions ( ■ 1 mM and □ 5 mM) on proteolytic activity of the tamarillo protease. The tamarillo protease was pre-incubated with various metal ions for 20 min.

4.3.4 Kinetic parameters determination

The effect of increasing the casein concentration on the hydrolysis reaction velocity can be described using the Michaelis-Menten plot (Figure 4.8A) and the Hanes plot (Figure 4.8B). The activity of the tamarillo protease reached saturation at a high substrate concentration (800 μM casein). The value of $K_m$ was 32.3 μM for casein as the substrate. The $K_m$ value gave an indication of the binding affinity of the substrate to the enzyme and was very comparable with the $K_m$ values of some other plant serine proteases. $K_m$ values of ~330, ~33.3 and ~66 μM were reported for the serine proteases benghalensis (Sharma et al., 2009), milin (Yadav et al., 2006), and religiosin (Kumari et al., 2010) extracted from the latex of the medicinal plant *Ficus benghalensis*, the latex of *Euphorbia milii*, and the latex of *Ficus religiosa* respectively, using casein as the substrate. Note that, the $K_m$ values of these plant serine proteases were obtained under different temperature and pH conditions.
4.3.5 Protease characterization using *de novo* protein sequencing

The functional specificity of the tamarillo protease was further characterized by identifying amino acid sequence similarities between the purified tamarillo protease and known proteases from other species. Tryptic digested peptides fragments (quality score $>0.65$) from the tamarillo protease were subject to *de novo* sequencing using Peak Studio software followed by MS-Blast analysis against the nrdb95 database. The *de novo* sequences of seven peptide fragments are...
listed in Table 4.3. The sequences were found to match an identified subtilisin-like protease from two *Solanum tuberosum* database sequences (gi|565386017 and gi|75180715) with BLAST scores greater than 87 (Figure 4.9A and B). These results strongly indicated that the protease purified from tamarillo was a subtilisin-like serine protease.

Table 4.3 De novo sequences from tamarillo protease

<table>
<thead>
<tr>
<th>Chain number</th>
<th>Observed m/z</th>
<th>De novo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>480.897</td>
<td>EGAFESEGAYRPK</td>
</tr>
<tr>
<td>2</td>
<td>509.800</td>
<td>AGVVVEGLK</td>
</tr>
<tr>
<td>3</td>
<td>543.795</td>
<td>LTYQVTFSK</td>
</tr>
<tr>
<td>4</td>
<td>561.758</td>
<td>GVCESLTKN</td>
</tr>
<tr>
<td>5</td>
<td>566.809</td>
<td>NAPIVAFFSR</td>
</tr>
<tr>
<td>6</td>
<td>669.346</td>
<td>STHPDWSAVIK</td>
</tr>
<tr>
<td>7</td>
<td>671.828</td>
<td>TTVNVGDATSSYK</td>
</tr>
</tbody>
</table>

A

>gi|565386017|ref|XP_006358840.1| PREDICTED: subtilisin-like protease SBT1.2 [Solanum tuberosum]
MGFFKILVFIFCSFPWPPTIQSGLETYIVHVESPLESTIQSSLTDLSYLLSLPLKTTTATTSSRNGEAATMIYSSYHNVKGFARLTAEQVKEMEKKHFVSAQKRILLSLDTHTSLGQLQNMGGWKSDNYGKVIGVIDTGLIPHPSTVDPGKPPAKWVGCEFLNIPYKCNKLTRGARYKRGNSPIDGNHGTATSTAGAFVKGANYGNANTGAVGAPLAIHAVYKVCQSGCCSSDIALAMDSAAIDGDVDVLISLGSGFNSFYDPIALGAYATARGVILGSACNSGRGLLAHSVGAAPWILTVGASTIDRMRKVAKLTKGRENFEPEGASAPVPLSNTFTFLFADARKHDQSETYPCPKPCPLNVFKVILCLAGGGVSSVAKQGQVVKDDAVGOMI

B

>gi|75180715|sp|Q9LWA3| Q9LWA3_SOLLC Subtilisin-like protease
MMAQYSSVLTIIIGLICVLFSPTTHAAEWAQNSQIYIYVCEFPSEGERTAYQDLEWSYLPLPTTSVSSREAPRIYISYNRLTQPAKLDSEIDKEMXKGEFGVSARFPQFCFVSHTHSVNLQLQNM5GFWSKSDNYGKVIGVQLDGILPDDPSPFGSVDGTPAPKNGGCEFLNIPYKCNKLTRGARYKRGNSPIDGNHGTATSTAGAFVKGANYGNANTGAVGAPLAIHAVYKVCQSGCCSSDIALAMDSAAIDGDVDVLISLGSGFNSFYDPIALGAYATARGVILGSACNSGRGLLAHSVGAAPWILTVGASTIDRMRKVAKLTKGRENFEPEGASAPVPLSNTFTFLFADARKHDQSETYPCPKPCPLNVFKVILCLAGGGVSSVAKQGQVVKDDAVGOMI

Figure 4.9 MS-BLAST result, peptide matching sequences for subtilisin-like protease from *Solanum tuberosum* sequences (A) (gi|565386017) and (B) (gi|75180715). Sequences highlighted in yellow indicate matches.
4.4 Conclusion

Overall, to the best of our knowledge, this is the first study on the purification and characterisation of a protease from tamarillo fruit. This protease exhibited activity over a broad range of pH values, had good temperature stability, and displayed high activity optima for pH (pH 11) and temperature (60°C). In addition, there was no requirement for the tamarillo protease to be activated by metal ions, Hg²⁺ ions inhibited the protease activity and the typical serine protease inhibitor PMSF strongly inhibited the enzyme activity. Amino acid sequences from the tamarillo protease matched other identified plant subtilisin-like proteases. This subtilisin-like alkaline serine protease might be used in food applications, such as cheese-making, or other applications which require alkaline pH environments. Finally, we suggest that this tamarillo protease be named ‘tamarillin’.
Chapter 5

5. Effect of temperature and pH on the properties of skim milk gels made from a tamarillo protease extract and rennet
Abstract

Reconstituted skim milk was gelled with a crude protease extract from tamarillo (*Cyphomandra betacea*) fruit and compared to gels prepared with calf rennet. The effects of temperature and pH on the gelation of skim milk was investigated by small deformation oscillatory rheology. The tamarillo extract-induced gels had a faster rate of increase in the elastic modulus $G'$ at the early stage of gelation than rennet-induced milk gels. This was most probably due to the broader proteolytic activity of the tamarillo protease extract as shown by SDS-PAGE analysis. Confocal microscopy also showed that the milk gels resulting from the addition of the tamarillo extract had larger voids than rennet-induced milk gels. The proteolytic activity of the tamarillo extract was found to be optimal at pH 11. For both rennet and tamarillo extracts, the aggregation time ($t_{ag}$) was similar between pH 6.7 and 6.5, but $t_{ag}$ of rennet-induced milk gels was lower than those of milks gels obtained by the addition of tamarillo extracts at pHs lower than 6.5. An increase in temperature was found to have a significant effect on $t_{ag}$, particularly at 20°C where rennet in 3 h did not coagulate milk, but the tamarillo extracts coagulated milk within 2 h. The results of this study suggest that extracts from tamarillo fruit could be used for milk gelation, particularly under lower temperature or high pH conditions.
5.1 Introduction

Calf rennet is a traditional milk-clotting enzyme which has been used predominantly in the manufacture of different cheeses for centuries. Its high specificity to cleave the Phe$_{105}$-Met$_{106}$ peptide bond in κ-casein and its relatively low general proteolytic activity results in destabilizing the casein micelle surface leading to milk coagulation or clotting (Pires et al., 1994; Vishwanatha et al., 2010). A world-wide shortage of this enzyme has been predicted due to an increase in the production of cheeses and the reduced availability of suckling calves’ stomachs (Lopes et al., 1998). In addition, bovine spongiform encephalopathy (BSE) has resulted in reducing both the supply and the demand for bovine rennet (Roseiro et al., 2003). There are also consumers’ restrictions on the use of animal rennet for different reason. Vegetarians prefer to have no animal products or animal ingredients in their food. For religious reasons, the use of animal products can be also prohibited (Whitaker, 1959; Gordin and Rosenthal, 1978; Roseiro et al., 2003). Furthermore, countries such as Germany and the Netherlands forbid the use of recombinant calf rennet since it is considered to be a genetically engineered food (Roseiro et al., 2003). These restrictions on the use of rennet have led to a growing interest in other milk-clotting agents, especially vegetable coagulants.

To meet the increased demand for natural, rennet-like clotting agents, a new source of proteases has attracted a lot of interest. Proteases from plant sources have shown great potential as rennet substitutes and have been used in cheesemaking (Huang et al., 2011; Katsaros et al., 2010). Some plant substitutes, such as actinidin extracted from the kiwifruit, have relatively high specific activity for milk-clotting (Mazorra-Manzano et al., 2013). In addition, dried cardoon flowers of Cynara cardunculus L. and Cynara humillis L. have been used for centuries in Spain and Portugal to prepare certain varieties of cheese with a creamy soft-texture and specific flavours (Roseiro et al., 2003). However, some common proteases from plants such as papain, ficin and bromelain exhibit low milk-clotting activity, and their broad casein proteolytic activity affects the texture, flavour and yield of cheese (Garg and Johri, 1994; Jacob et al., 2011).

In cheesemaking the temperature and pH are important factors which affect the cheese quality. Unfortunately, there are few studies on the effect of temperature or milk pH on the rheological properties of milk gels (Esteves et al., 2003a; Esteves et al., 2003b). In this paper, we report on the ability of tamarillo protease extracts to clot milk. Tamarillo fruit is native to South America.
and was introduced to New Zealand in the late 1800s. The milk-clotting activity of two different tamarillo cultivars, namely Laird’s Large with red skin and Amber with yellow skin, were investigated (Figure 5.1) and compared to calf rennet. Proteins were crudely extracted from these two tamarillo cultivars, then investigated for their proteolytic activity on caseins using SDS-PAGE, and their effect on milk-clotting, using confocal microscopy and rheological measurements. The effects of temperature (20–40°C) and pH (5.0–6.7) were also considered.

Figure 5.1 Pictures of Laird’s Large tamarillo (A), and Amber tamarillo (B) fruits. (C) Picture of a milk gel induced by tamarillo extract.

5.2. Material and methods

5.2.1 Material and milk samples preparation

Low heat skim milk powder (SMP) was obtained from Synlait Milk Ltd., Rakaia, New Zealand. The calf rennet (Strength, 280 International Milk-clotting Units (IMCU) mL⁻¹) used in this study was supplied by RENCO New Zealand Laboratory (RENCO New Zealand, Eltham, New Zealand). Red cultivar Laird’s Large and yellow cultivar Amber tamarillo fruit, hand-picked from the trees, were purchased from Maungatapere, New Zealand. All other chemicals and reagents were purchased from Sigma-Aldrich Ltd., Auckland, New Zealand.

5.2.2 Tamarillo crude extraction

Tamarillo protease extraction was based on the method of McDowall (1970) with some modifications. Approximately 300 g of tamarillo fruit (Laird’s Large and Amber) was
homogenized for 30 min using a Polytron PCU2 laboratory homogeniser. The homogenate was filtered through two layers of cheesecloth, and the insoluble material was discarded. To approximately 85 mL of the filtrate, 80 mL of 0.05 M sodium citrate buffer pH 5.5 were added. The mixture was centrifuged using a Sorvall LYNX Superspeed Centrifuge (Thermo Fisher Scientific, USA) at 15000 × g at 4°C for 20 min. The supernatant (155 mL) was precipitated by adding 61.69 g of ammonium sulphate to achieve 65% ammonium sulphate saturation, followed by centrifugation under the same condition as above. The precipitate (approximately 85 g) was dissolved in 30 mL 0.05 M citrate buffer (pH 5.5) and dialyzed through a cellulose membrane tube (molecular weight cut-off 12,000 Da) against Milli-Q water at 4°C overnight to remove salt. Tamarillo extract was stored at -80°C for subsequent studies.

5.2.3 Sample preparation.

Reconstituted skim milks were obtained by mixing skim milk powders with Milli-Q water. The skim milk powder and Milli-Q water were mixed in proportion to achieve a total solid concentration of 10%, 11.11% or 11.25%. The powder-water mixture was stirred using a magnetic stirrer for at least 2 h at room temperature to ensure complete dispersion. The reconstituted milk mixtures were kept at 4°C overnight to ensure full hydration. Prior to use, the reconstituted milk was left at room temperature for at least 4 h to ensure temperature equilibration.

To investigate the effect of pH, reconstituted milk was pre-mixed with appropriate amounts of glucono-δ-lactone (GDL), using a magnetic stirrer for 2 min to reach a target pH varying between pH 5 and 6.7. To initiate milk gelation only one concentration of rennet or tamarillo extract was used. For rennet, 30 μL of rennet, diluted 10 times in Milli-Q water, was added to 30 mL of 10% reconstituted milk. For the tamarillo extracts, 3.5 mL of Laird’s Large extract and 3 mL of Amber extract was added to 30 mL of 11.25% reconstituted milk and 27 mL of 11.11% reconstituted milk respectively, to give a final concentration of 10% milk. The concentrations of rennet and the tamarillo extracts were chosen using a preliminary rheological experiment where the rennet concentration was varied to achieve an aggregation onset time of 30 min for 10% milk at pH 6.7 at 30°C. The tamarillo extract concentrations were varied to achieve the same aggregation time onset under the same conditions as achieved with rennet.
5.2.4 Chemical analysis

Crude tamarillo extracts were analysed for protein, lipid, carbohydrate and ash contents. The protein content was determined by the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as a standard at concentrations of 0.2-1.2 mg/mL. Lipid content was measured by the Soxhlet method (Shahidi, 2001). Total carbohydrate content was determined by the phenol-sulphuric acid method (DuBois et al., 1956) and glucose was used as a standard. Ash content was measured gravimetrically after incinerating the tamarillo extracts in a KSL-1100X high temperature muffle furnace (Richmond, USA). The temperature was increased to 250°C gradually and kept at 250°C for one hr for carbonisation, then the temperature was increased to 500°C and maintained for 1 h.

5.2.5 Caseinolytic protease activity assay

Enzyme activity was measured using casein as the substrate and 50 mM buffers of different pH values ranging from 5 to 14 based on the method of Attri et al. (2015). The buffers used were phosphate buffer (pH 5-6), Tris-HCl buffer (pH 7-8), glycine-NaOH buffer (pH 9-10), phosphate-NaOH buffer (pH 11), KCl-NaOH buffer (pH 12-13), and 1 M NaOH (pH 14). Crude extract solution (100 μL) was mixed with 1.1 mL of 0.1 M buffer containing 1% (w/v) casein by vortexing for 30 seconds. The reaction was carried out at 35°C in a water bath for 20 min. Then 1.8 mL 10% (w/v) trichloroacetic acid (TCA) was added to stop the reaction. The mixture was allowed to stand for 30 min at room temperature, followed by centrifugation at 10,300 × g for 20 min. The absorbance of the supernatant was then measured at 280 nm against the blank using a UVmini-1240 spectrophotometer (Shimadzu Corporation, Australia). The blank was prepared by mixing TCA and enzyme solution, and then adding the casein solution.

5.2.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (Laemmli, 1970) was used to characterise the tamarillo extracts and the milk hydrolysates under non-reducing condition. The running gel was a 4-12% gradient polyacrylamide gel in 0.375 M Tris-HCl (pH 8.8) and 0.01% SDS, and the stacking gel contained 4% acrylamide in 0.125 M Tris-HCl (pH 6.8) and 0.005% SDS. The electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS (pH 8.16).
For tamarillo extracts, 40 μg was mixed with loading buffer, containing 50mM Tris-HCl (pH 6.8), 2% SDS, 10% (v/v) glycerol and 0.02% (w/v) bromophenol blue, to make a final concentration of 2 mg/mL. The mixture was boiled at 100°C for 3 min and the denatured proteins (15 μL) were added into each well. The electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS (pH 8.16).

For the SDS-PAGE of milk hydrolysates, samples were prepared according to the method of Jin and Park (1996). Rennet and tamarillo extracts were added to a 10% (w/w) milk sample (6g), followed by stirring for 2 min. Aliquots were then transferred into glass vials and incubated at 35°C in an oven. Glass vials were removed from the oven at 0 min, 15 min, 1 h and 24 h and the hydrolysis reaction was stopped by heating the vials at 100°C for 5 min. The milk gel in each glass vial was dissolved in 400 μL 8 M urea buffer pH 8 by vortexing for 2 min. Dissolved milk gel solutions were diluted 15 times with the Laemmli sample buffer.

Electrophoresis was carried out at a constant voltage of 110 V until the bromophenol blue band reached the bottom of the gel. The gel was stained with 0.1% Coomassie brilliant blue R-250, 40% methanol and 10% acetic acid, and was destained with 40% methanol and 10% acetic acid. Molecular weight markers (Precision Plus Protein Standards) were used for an indication of the molecular weight of the bands.

5.2.7 Rheology

An Anton Paar Physica MCR 301 stress-controlled rheometer (Anton Paar, Graz, Austria) was used to monitor the viscoelastic properties of the milk gels. A cup and bob geometry consisting of a 25 mm external diameter bob with a 27.1 mm internal diameter cup was used. Milk samples (18.5 mL) were loaded inside the cup preset at a temperature of 25°C. The bob was lowered and the sample was covered with a thin layer of soya oil to minimise evaporation. To investigate the effect of temperature, after loading the sample, the target temperature (from 20 to 40°C) was set using the rheometer Peltier heating/cooling system. The effects of pH were studied at a constant temperature of 30°C.

The rheological measurements consisted of a time sweep measurement, followed by a frequency sweep measurement, then a strain sweep measurement. The time sweep measurement was carried out at a constant frequency of 0.1 Hz and a constantly applied strain of 1%, during which the elastic modulus $G'$ and the loss or viscous modulus $G''$ were recorded.
every minute at the target temperature. At the end of the time sweep measurement, the temperature was set to 30°C for 10 min to ensure sample temperature equilibration, and the frequency sweep was carried out by varying the frequency from 0.01 to 10 Hz and at a constant strain of 1%, where $G'$ and $G''$ are recorded as a function of the frequency. This was followed by a strain sweep where the strain was varied from 0.1 to $10^4\%$ at a constant frequency of 1 Hz, were $G'$ and $G''$ are recorded as a function of the strain. Note that the frequency sweep and the strain sweep are measured at a constant temperature of 30°C to allow comparison between the samples gelled at different temperatures. While for the time sweep the temperature was varied from 20 to 40°C to investigate the effect of temperature on the kinetic of milk gelation.

5.2.8 Confocal Laser Scanning Microscopy (CLSM)

CLSM was used for observing the microstructure of the milk gel network following the method of Lucey et al. (1998) with some modifications. Acridine orange, the fluorescent protein dye, was dissolved in Milli-Q water to make a 0.25% (w/v) dye solution. A few drops of dye solution were added to the skim milks and stirred for approximately 2 min. The milk samples were prepared in the same way as for the rheological measurements. Rennet and tamarillo extracts were added to the stained milks, stirred for another 2 min, then an aliquot of these milks was transferred into the well of the microscope, slides were covered with a glass coverslip and sealed using nail polish. The slides were transferred into a plastic box containing wet paper towels to prevent evaporation, the lid was closed and the box incubated at 30°C. The microstructure of gels were measured by CLSM with a 60 × oil immersion objective after 1 h, 4 h and 24 h incubation, using an Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan) with a X-Cite Series 120Q mercury halide lamp (Lumen Dynamics, Ontario, Canada) at wavelength 488 nm. For each sample, nine 512 × 512 resolution images of Z-stack projection were obtained within 2 µm depth.

5.3 Results and Discussion

5.3.1 Characterisation of tamarillo extracts

Table 5.1 presents the dry weight based composition of tamarillo extracts. According to Table 5.1, the protein content of Laird’s Large and Amber extracts was 81.6 ± 0.2% (w/w) and 83.1 ± 0.2% (w/w), respectively. Carbohydrates contents were 15.9 ± 0.0 % (w/w) and 14.4 ± 0.1%
(w/w) for Laird’s Large and Amber extracts, respectively. As expected, low lipid amounts (<0.5% w/w) were measured for both extracts.

Analysis of the tamarillo extracts by SDS-PAGE (Figure 5.2A) show that the protein profiles in both the Laird’s Large (lane 2) and Amber (lane 3) tamarillo crude extracts were similar with protein bands of molecular weights varying between 10 to 75 kDa, and the main bands at approximately 14 kDa. Another two faint bands at approximately 30 and 70 kDa were also observed. Note that Figure 5.2B, dealing with milk hydrolysis by tamarillo crude extracts and rennet, will be discussed in the next section.

**Table 5.1** Composition of extract from Laird’s Large and Amber (% w/w dry basis). STDEV = standard deviation.

<table>
<thead>
<tr>
<th>Tamarillo extracts</th>
<th>Carbohydrates (%)</th>
<th>Protein (%)</th>
<th>Lipid (%)</th>
<th>Ash (%)</th>
<th>Residue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laird’s Large</td>
<td>15.9 ± 0.00</td>
<td>81.6 ± 0.20</td>
<td>0.41 ± 0.06</td>
<td>0.72 ± 0.01</td>
<td>1.38 ± 0.26</td>
</tr>
<tr>
<td>Amber</td>
<td>14.4 ± 0.10</td>
<td>83.1 ± 0.20</td>
<td>0.45 ± 0.04</td>
<td>0.76 ± 0.04</td>
<td>1.23 ± 0.13</td>
</tr>
</tbody>
</table>

**Figure 5.2** (A) SDS-PAGE of tamarillo crude extracts: lane 1 is molecular markers, lane 2 is crude extract from Laird’s Large and lane 3 is the crude extract from Amber. (B) SDS-PAGE of milk hydrolysis by rennet and crude extract from Laird’s Large tamarillo: Lanes 1 to 4 are rennet hydrolysed milk, lane 5 is molecular markers and lanes 6 to 9 are tamarillo crude hydrolysed milk. Lanes 1 and 6 are milk, lanes 2 and 7; 3 and 8; and 4 and 9; are milks hydrolysed for 15 min; 1 h; and 24 h; respectively. Milk protein bands are indicated by α-casein (α-CN), β-casein (β-CN), κ-casein (κ-CN), β-lactoglobulin (β-Lg) and α-lactalbumin (α-Lac).
The proteolytic activities of the tamarillo extracts as a function of pH are shown in Figure 5.3, along with that of rennet. The activity of rennet was optimal at low pH (≤6.5) with a maximum at pH 5.5. Activity then started to decrease sharply when the pH was higher than pH 7, reaching a minimum activity at a pH ≥8. These results showed that the optimal pH of rennet on casein hydrolysis was acidic. This was in agreement with Humme (1972) who reported that the optimum pH for rennet hydrolysis of κ-casein was between 5.1 - 5.3. According to Chitpinityol and Crabbe (1998), rennet is an aspartic protease known to have an acidic optimal pH (Silva and Malcata, 2005).

In comparison, the proteolytic activities of the tamarillo extracts increased markedly when the pH was increased from pH 5, reaching a maximum at pH 11, and then decreasing to a minimum at pH 13. These results suggested that the optimal hydrolytic activity of the extracts from both tamarillo cultivars was at alkaline pH. Similar results were also found in extracts from plants with alkaline optimal pH, such as a serine protease extract from Cucumis trigonus Roxburghi which exhibited optimal caseinolytic activity at 11 (Asif-Ullah et al., 2006) and a serine milk-clotting protease extract from Solanum dubium Fresen that also had optimal activity at pH 11 (Ahmed et al., 2009a).

![Figure 5.3 Caseinolytic activity as a function of pH for rennet (■), crude extracts from (●) Laird’s Large tamarillo and (▲) Amber tamarillo.](image-url)
5.3.2 Milk gel microstructure and SDS-PAGE analysis

The micrographs of the milk gels obtained by the addition of rennet or tamarillo crude extracts are shown on Figure 5.4. The milk proteins were stained white in the micrographs. For both rennet and tamarillo extracts, after 1 h incubation the milk protein formed a dense network with very small voids. The voids increased in size after 4 h incubation, and there appeared to be little differences between the rennet and tamarillo extract-containing gels. After 24 h incubation the milk gels appeared to be composed of smaller protein aggregates.

Figure 5.4 Confocal scanning laser micrographs of 10% skim milk gels made at 30°C at pH 6.7 for 1 h (a, d, g), 4 h (b, e, h) and 24 h (c, f, i). Milks were gelled by rennet (a, b, c), Laird’s Large tamarillo extract (d, e, f) and Amber tamarillo extract (g, h, i). Protein matrix is white and dark areas (voids) are the aqueous phase. Scale bar = 20 μm.
To further understand what happens to the different milk proteins during milk gelation, SDS-PAGE was performed on the milks gelled with either added rennet or tamarillo extracts from the Laird’s Large variety (Figure 5.2B). No obvious effect was observed on the whey proteins even after 24 h treatment (approximately 18 kDa for β-lactoglobulin and 14 kDa for α-lactalbumin). However, the caseins were affected markedly. There was a reduction in the area of the κ-casein band (near 25 kDa) and the appearance of a peptide band of approximately 13 kDa for milk treated with rennet (Figure 5.2B, lane 2, 3 and 4), and a peptide band of approximately 18 kDa for the milk treated with the tamarillo extract (Figure 5.2B, lane 7 and 8). For the rennet-induced gels, the increase in incubation time resulted in further hydrolysis of κ-casein and the increase in the area of the peptide band around 13 kDa. This band was assumed to be para κ-casein and has also been identified by Egito et al. (2007) who reported a molecular weight of 12,268 Da. Even after 24 h incubation, the other caseins, α- and β-casein, were not affected. This confirms the specificity of rennet to hydrolyse κ-casein (Hang et al., 2016).

In the milks treated with the tamarillo extracts however, all the caseins were susceptible to hydrolysis as can be seen by the reduction in the intensity of α-, β- and κ-casein bands. The peptide band at approximately 18 kDa, (lane 9) attained maximum band density after 1 h incubation time, after which it appeared to be subject to further hydrolysis. There was also the appearance in the 24 hr incubation sample of a faint band of approximately 15 kDa migrating just above the α-lactabumin band. The origin of this band was not known. The SDS-PAGE results clearly indicated that the tamarillo crude extract has a broad specific activity on bovine caseins. This broad specificity has been also reported for other plant-based proteases such as lettucine extracted from Lactuca sativa leaves (Lo Piero et al., 2002), and enzyme extracts from Helianthus annus seed and Albizia lebbeck (Egito et al., 2007).

5.3.3 Rheological measurements

Small deformation oscillatory measurements allow for the accurate monitoring of the gelation kinetics of milks under the action of rennet (Hemar et al., 2004; Esteves et al., 2002; Mellema et al., 2002) and during acidification (Lucey and Singh, 2003; Renault et al., 2000; Liu et al., 2014). As shown in Figure 5.5A, there was a lag period (30 min) after the addition of the rennet or tamarillo extracts to the milk, where the elastic modulus $G'$ remained constant. After this lag time, $G'$ increased markedly between 30 min and 1 h, and then plateaued at longer times. To
ensure that the lag-time (onset of aggregation) at 30°C is the same for milks with added rennet or tamarillo extracts, preliminary experiments were performed where the amount of the tamarillo extracts was varied to achieve an aggregation time similar to that resulting from the addition of rennet. The amounts of tamarillo extract needed to mimic the rennet (30 μL, diluted 10-fold) were 3.5 and 3 mL for the Laird’s Large and Amber extracts respectively.

The lag-time was set at 30 min, long enough to allow changes to be monitored when the milk pH or the temperature of gelation are varied, but not too long to ensure that it remains relevant for cheesemaking. According to Figure 5.5A, at the initial stages of gelation, both tamarillo extract-induced gels had a faster rate of increase of the elasticity ($G'$) than rennet. Esteves et al. (2002) reported a similar result on plant coagulant-induced milk gels that exhibited a higher rate of increase of $G'$ at the initial stages than that of rennet-induced gels. Their explanation was that the broader proteolytic activity of plant coagulants on caseins resulted in a faster rate of increase in $G'$. The microstructures of the tamarillo protease treated milk gels also displayed larger voids than those of rennet-induced gels after 1 h incubation (Figure 5.4). SDS-PAGE (Figure 5.2B lane 8) indicated that there was extensive hydrolysis of the caseins, where tamarillo extract not only hydrolysed κ-casein, but also hydrolysed α- and β-casein, while rennet hydrolysed mainly κ-casein (Figure 5.2, lane 3). It is commonly accepted that κ-casein is located on the surface of the casein micelles milk (Holt and Horne, 1996), however, Dalgleish et al. (1989) and Diaz et al. (1996) stated that some α- and β-caseins were also located close to the surface of the casein micelles. Since the tamarillo extracts hydrolysed all the caseins, it was possible that these extracts were more effective than rennet at destabilising the casein micelles leading to faster milk aggregation than rennet.

After 3 h gelation the frequency sweep measurement (Figure 5.5B) showed that all the milks have gelled. This was indicated by $G'$ being higher than $G''$. However, these gels can be classified as weak-gels since the difference between $G'$ and $G''$ was less than ten-fold, and also that both $G'$ and $G''$ are not constant with the frequency (Lapasin and Pricl, 1995). The gel strength was also higher for the milk gels made with added Laird’s Large tamarillo extract, compared to those made with either rennet or Amber tamarillo extract. The strain sweep measurement (Figure 5.5C) carried at the completion of the frequency sweep, showed that for applied strains ≤10% both $G'$ and $G''$ were independent of the strain. This strain range indicated that the time sweep and the frequency sweep measurements, both performed at a constant strain
of 1%, where within the Linear Viscoelastic Region; in other words, the applied strain was very small and did not affect the gels microstructure. When the applied strain was higher than 70%, both $G'$ and $G''$ started to decrease markedly due to the breakup of the gels under the large deformations. With increasing applied strain both $G'$ and $G''$ crossed over at approximately 100% for Amber, 389% for Laird’s Large and 631% for rennet-induced gels, indicating that the gels started to flow under the action of large deformation.
Figure 5.5 (A) Elastic modulus as function of time, (B) frequency sweep, and (C) strain sweep for 10% (w/w) milk gels induced by rennet, Laird’s Large and Amber tamarillo extracts. Symbols are: rennet-induced gel (■), Laird’s Large extract-induced gel (○) and Amber extract-induced gel (▲). In (B) and (C) symbols are: rennet-induced gel (■, ○), Laird’s Large extract induced gel (●, ○) and Amber extract induced gel (▲, △), with solid symbols corresponding to $G'$ and open symbols are for $G''$. 
Time sweep measurements were used to investigate the effect of the initial milk pH and temperature on the onset of aggregation time ($t_{ag}$) at which $G'$ starts to increase. $t_{ag}$ of skim milks increased with the increase in pH (Figure 5.6A). A reduction of the pH of milk resulted in the decrease of the charge density of $\kappa$-casein, which provides electrostatic repulsion between casein micelles (De Kruif, 1999; Van Hooydonk, 1986). Compared to the tamarillo extracts, the addition of rennet to milk resulted in shorter $t_{ag}$ times at pH<6.5. This could be explained by the proteolytic activity of rennet that increased sharply as the pH was decreased from 7 to 5, with an optimal pH at approximately 5.2 (Figure 5.3). Conversely, the caseinolytic activity of the two tamarillo extracts decreased in the same pH region (pH below 7).

When the temperature of the gelation was increased from 20 to 30°C, the $t_{ag}$ time decreased markedly, then decreased slightly when the temperature was further increased from 30 to 40°C (Figure 5.6B). For example, the $t_{ag}$ value of milk with added Laird’s Large tamarillo extract decreased from 90 to 30 and then 22.5 min as the temperature was increased from 20 to 30 and then to 40°C. This decrease in $t_{ag}$ with temperature was expected since enzymatic hydrolysis reactions generally accelerate with temperature (Esteves et al., 2003a; Kokate, 2011). An important observation was that while both tamarillo extracts resulted in milk aggregation at 20°C within 2 h, the milk with added rennet did not aggregate until after 3 h. This again might be due to the ability of the tamarillo extracts to hydrolyse all the caseins, compared to rennet which specific hydrolyses $\kappa$-casein.
Figure 5.6 Aggregation time as a function of temperature for milk gels obtained by the addition of rennet (■), Laird’s Large extract (●), and Amber extract induced gel (▲). Error bars correspond to standard deviations. Tamarillo extract-induced gel and rennet-induced gel at different pH (A) and temperature (B) values.
5.4 Conclusions

This study showed that crude extracts from tamarillo fruit exhibited broader caseinolytic activity compared to rennet, and that the proteolytic activity of the tamarillo extracts increased markedly when the pH was increased to reach a maximum at pH 11. The broader hydrolytic activity of the tamarillo extracts was also observed through the results of the rheological measurements and the microscopic observations of the milk gels. While the addition of rennet to milk resulted, as expected, mainly in the hydrolysis of κ-casein, the tamarillo extracts hydrolysed all the caseins, as early as 15 min after the addition of the extracts. The extensive hydrolysis of the caseins by the tamarillo extracts resulted in the microstructure of milk gels presenting larger voids (at 1 h gelation), compared to the milk gels obtained by rennet addition. Further, at the initial stage of gelation, the increase in the elasticity ($G'$) of the milk gels made with the tamarillo extracts was faster than those made by rennet. For both rennet and tamarillo extracts, a decrease of the milk pH or an increase in temperature resulted in the decrease of the onset of aggregation time ($t_{ag}$). It is noteworthy to report that at 20°C, there was no milk aggregation 3 h after the addition of rennet, while the milk with added tamarillo extracts coagulated within 2 h.

While this study demonstrated that tamarillo crude extract could hydrolyse caseins and coagulate milk, further studies are needed to identify the active tamarillo protease, and to investigate its use in food applications. For instance, while milk coagulation is applied in the early stages of cheesemaking, later stages such as cheese ripening are also very important. Thus, the long term effect of the tamarillo extract on the properties of the caseins and the texture and sensory attributes of the cheese need investigating, to compare the tamarillo extract not only to rennet, but also to other plant extracts (e.g. extract from cardoon flowers) which are currently used commercially for cheesemaking. Other potential uses of the tamarillo extracts might include applications were alkaline and low temperature protein hydrolysis conditions are required.
Chapter 6

6. Protease activity of enzyme extracts from tamarillo fruit and their specific hydrolysis of bovine caseins
Abstract

The characterisation of a serine protease isolated from tamarillo fruit and its milk casein hydrolysis activity were investigated. Compared with calf rennet, a crude extract from tamarillo exhibited wider caseinolytic activity on sodium caseinate. The purified protease was named ‘tamarillin’ and revealed proteolytic activity toward purified α-, β- and κ-casein. Similar to calf rennet, tamarillin preferably hydrolysed κ-casein, but, unlike calf rennet, it also displayed high proteolytic activity towards both α- and β-casein. The major peptide generated from κ-casein by tamarillin was analysed by gel electrophoresis and liquid chromatography mass spectrometry to confirm its molecular mass as 14,290 Da. The cleavage site was confirmed by in-gel tryptic digestion and time-of-flight mass spectrometry analysis to be at Asn123-Thr124. This was in contrast to the Phe105-Met106 cleavage site of rennet hydrolysis.
6.1 Introduction

Milk coagulation is one of the basic steps in cheese making (Guinee and Wilkinson, 1992; Macedo et al., 1993), and is usually achieved using chymosin (EC 3.4.23.4), the main protease component in calf rennet. Chymosin hydrolyses the Phe$_{105}$-Met$_{106}$ bond of $\kappa$-casein with high specificity, resulting in instability of the casein micelles and subsequent milk coagulation (Jollès et al., 1963; Egito et al., 2007). Most commercial milk-clotting enzymes are aspartic proteases that display chymosin-like cleavage specificity. However, two proteases, from Cryphonectria parasitica and the microbial Endothia parasitica have been reported to have good milk-clotting activity with the hydrolysis site on $\kappa$-casein at Ser$_{104}$-Phe$_{105}$ (Jacob et al., 2011; Drohse and Foltmann, 1989).

Milk curd can also be produced using proteases from other animal, microbial, plant sources, and by using the chymosin genes from calf stomachs that have been genetically engineered into microbial hosts (Jacob et al., 2011). A world-wide shortage of calf rennet has been predicted due to the increased production of cheese and a simultaneous decrease in the general availability of suckling calves' stomachs (Jacob et al., 2011). The usage of animal rennet has been also restricted by religion in countries such as India and Israel (Whitaker, 1959; Gordin and Rosenthal, 1978), and because of vegetarianism and opposition against genetically engineered foods (e.g. Germany and Netherlands forbid the application of recombinant calf rennet) (Roseiro et al., 2003). This has led to the search for alternative milk-clotting sources (Roseiro et al., 2003), particularly for proteases which are natural and cost effective, such as proteases isolated from plants (Shah et al., 2014; Nasr et al., 2016).

The milk-clotting activities of a number of plant coagulants have been recently investigated. These include extracts from Callotropis procera (sodom apple) (Aworh and Muller, 1987), from artichoke (Cynara scolymus) flowers (Chazarra et al., 2007), from twigs of Streblus asper (Senthilkumar et al., 2006), from latex of Euphorbia microsciadia (Rezanejad et al., 2015), actinidin from kiwifruit (Lo Piero et al., 2011), from bentong ginger (Zingiber officinale var. Bentong) (Nafi et al., 2014), extract from the root of Jacaratia corumbensis O. Kuntze (Duarte et al., 2009), a protease from the seed of Solanum dubium (Ahmed et al., 2009b), religiosin B from the latex of Ficus religiosa (Kumari et al., 2012) and lettucine from the leaves of Lactuca sativa (Lo Piero et al., 2002). However, most of these plant coagulants have been found to be unsuitable for cheese making due to their broad, non-specific hydrolysis activity on caseins,
resulting in low yields and a bitter taste in the final cheese product. The aspartic protease cardosin A, extracted from the flowers of *Cynara carduculus*, has been used as a coagulant in ovine cheese making in Portugal and Spain (Macedo et al., 1993; Fernández-Salgueiro and Sanjuán, 1999; Carmona et al., 1999). Cardosin A has similar characteristics to rennet with a highly specific cleavage on the Phe\textsubscript{105}-Met\textsubscript{106} site of κ-casein (Macedo et al., 1993). Unlike rennet however, cardosin A has also been found to hydrolyse α- and β-casein (Silva and Malcata, 1998; Silva and Malcata, 1999). Therefore, cardosin A has not been used for the production of bovine milk cheese due to its broad hydrolytic activity, resulting in a bitter taste of the cheese made using bovine milk (Macedo et al., 1996).

In this paper we report for the first time the effect of a serine protease extracted from tamarillo fruit (tamarillin) on bovine caseins. The effect of crude tamarillo extract on caseinates and that of the purified tamarillo extract on individual caseins are investigated by SDS-PAGE to determine the extent of hydrolysis in comparison with rennet. The molecular masses of the peptides resulting from the hydrolysis of κ-casein by the tamarillin were characterised using mass spectrometry, and its specific cleavage site on κ-casein was elucidated using time-of-flight mass spectrometry (TOF-MS).

### 6.2 Materials and methods

#### 6.2.1 Materials and sodium caseinate preparation

Casein standards, α-, β- and κ-casein, were purchased from Sigma-Aldrich Ltd., (Auckland, New Zealand). Calf rennet (280 International Milk-clotting Units (IMCU) mL\textsuperscript{-1}) was supplied by RENCO New Zealand Laboratory (RENCO New Zealand, Eltham, New Zealand). Low heat skim milk powder (SMP) was obtained from Synlait Milk Ltd., Rakaia, New Zealand. All chemicals were of analytical grade.

Sodium caseinate was prepared by the method of Lucey et al. (2000) with some modifications. Skim milk powder (10% w/w) was mixed with Milli-Q water for 2 h using a magnetic stirrer, then the reconstituted milk was kept in the fridge one day before use to ensure full hydration. The reconstituted skim milk was acidified to pH 4.6 by adding 2 M HCl drop-wise at room temperature. The resulting curd was separated from the whey, then washed five times with Milli-Q water and dewatered using two layers of cheesecloth. The washed curd was re-
dissolved with Milli-Q water in a 1:1 ratio, and the pH of the mixture was adjusted to 6.8 using 2 M NaOH under slow stirring. The sodium caseinate solution was freeze-dried for future use.

6.2.2 Tamarillo crude and purified protease extracts

The method for preparing a crude tamarillo protease extract was based on McDowall (1970) with some modifications. Laird’s Large (Cyphomandra betacea) tamarillo fruit (~300 g) were homogenized for 20 min using a Polytron PCU2 laboratory homogeniser (Brinkmann Instruments, Luzern, Switzerland), followed by filtration through two layers of cheesecloth to remove insoluble material. Tamarillo filtrate (~85 mL) was mixed with 80 mL 0.05 M pH 5.5 sodium citrate buffer by gentle stirring, then centrifuged at 15,000 × g for 20 min at 4°C. Protein from the supernatant (155 mL) was precipitated overnight at 4°C using a 65% saturated ammonium sulphate solution (61.69 g). The precipitated protein was centrifuged at 15,000 × g for 20 min at 4°C, and then dissolved in 30 mL 0.05 M sodium citrate buffer (pH 5.5). One volume of the resulting solution was dialyzed 3 times against five volumes of the same buffer for 24 h at 4°C. The dialysed solution was freeze-dried and called the crude extract. The protein concentration of the crude extract solution was 1.17 ± 0.03 mg/mL.

Crude tamarillo extract solution (40 mL) was loaded onto a DEAE-Sepharose Fast Flow ion exchange column (22 × 5 cm) pre-equilibrated with 0.05 M sodium citrate buffer (pH 5.5). The individual proteins were eluted using a 200 mL linear gradient of 0-1.0 M NaCl at a 1.0 mL/min flow rate. Fractions (10 mL) were collected and their protein content was measured by absorbance at 280 nm using a spectrophotometer (UVmini-1240, Shimadzu Corporation, Australia). Fractions corresponding to peaks of high caseinolytic activity (as shown in Chapter 4, Section 4.3.1) were pooled and dialysed three times against Milli-Q water for 24 h at 4°C to remove salt. The principal pooled protease fraction was then freeze-dried and stored at -80°C. The purified tamarillo protease was given the name ‘tamarillin’.

6.2.3 Hydrolysis of caseins by rennet and tamarillo extracts

Casein hydrolysates were prepared based on Egito et al. (2007). For sodium caseinate hydrolysed by crude tamarillo extract or rennet, the sodium caseinate was dissolved in 100 mM sodium phosphate buffer, pH 6.5 using a magnetic stirring for 2 h to obtain a concentration of 10 mg/mL. Tamarillo crude extract (350 µL) or rennet (10 µL, diluted 10-fold with Milli-Q water) were added to 3 mL sodium caseinate solution to make a final sodium caseinate
concentration of 10 mg/mL. The hydrolysis reactions were carried out at 35°C (Function Line Incubator, Heraeus, Langenselbold, Germany), and aliquots of hydrolysates were removed at 15 min, 30 min, 1 h, 4 h and 24 h. The hydrolysis was stopped by heating the hydrolysate aliquots at 100°C for 5 min in a water bath.

For the hydrolysis of individual caseins by tamarillin, α-, β- and κ-casein were dissolved in 10 mM sodium phosphate buffer (pH 6.5) to obtain a protein concentration of 10 mg/mL. Tamarillin (1 mg) or rennet (10 µL, diluted 200-fold with Milli-Q water) were added to 5 mL of the α-, β- and κ-casein solutions. The hydrolysis step was performed as described above. The hydrolysates were further freeze-dried and kept in –80°C until analysis.

6.2.4 Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12% (w/v) polyacrylamide resolving gel in 0.375 M Tris-HCl buffer, pH 8.8 containing 0.1% (w/v) SDS and with a 5% (w/v) polyacrylamide stacking gel in 0.125 M Tris-HCL, pH 6.8, according to Laemmli (1970). The electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% (w/v) SDS, pH 8.16, and electrophoresis was carried out at a constant voltage of 150 V for 2 h. After electrophoresis, the gel was stained in 0.1% (w/v) Coomassie brilliant blue R-250 overnight, following by destaining in 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) Milli-Q water for three h.

For sample preparation, one volume of each hydrolysate solution was added to three volumes of 50 mM Tris-HCL buffer, pH 6.8, containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.1% (w/v) bromophenol blue. Proteins were denatured by heating at 100°C for 3 min before loading (10 µL) onto the SDS-PAGE. Precision Plus Protein Dual Color Standards (Bio-Rad, Auckland, New Zealand) were used to generate a protein molecular mass calibration curve.

6.2.5 κ-Casein peptide molecular mass characterisation

The molecular masses of κ-casein and peptides hydrolysed from κ-casein were determined using reversed-phase liquid chromatography-mass spectrometry (LC-MS) according to Egito et al. (2007) with some modifications. 1 mg of κ-casein and hydrolysed κ-casein (by rennet and tamarillo protease) was dissolved in 500 µL of 10 mM Tris-HCl pH 8.0 buffer containing
8 M urea, 40 mM trisodium citrate and 20 mM dithiothreitol (DTT), and incubated at 37°C for 2 h. The samples (10 µL) were then loaded onto a Phenomenex Jupiter C4 300 Å column (250 × 4.6 mm, 5 µm) using an Agilent 1290 HPLC system (Agilent, United States) and eluted with a 12-64% gradient of acetonitrile with 0.1% trifluoroacetic acid for 25 min at a flow rate of 0.5 mL/min. The masses of the eluted peaks were determined using an Agilent 6460C Triple Quadrupole MS (Santa Clara, CA, USA) with Jet Stream electrospray ionization (ESI) as an ion source, a scanning range from m/z 700-2400 Da and data analysis and spectra deconvolution using the Agilent Mass Hunter Qualitative Analysis B.07.00 software.

6.2.6 In-gel digestion

In-gel tryptic digestion of peptide bands was adapted from the methods described by Shevchenko et al. (1996) and Albright et al. (2009). The bands corresponding to either κ-casein or major peptides resulting from the action of rennet or tamarillin on κ-casein, were cut from the gel and diced into pieces. Each sample was transferred to a 1.5 mL Eppendorf tube, 200 µL of 50% acetonitrile: 50% 50 mM ammonium bicarbonate was added to cover the gel pieces, which were then mixed by vortexing for 30 seconds. The tubes were then shaken (1400 rpm at 56°C for 10 mins), and the supernatant was discarded. 200 µL of acetonitrile was added to each tube to dehydrate the gel into hard, white pieces, and the supernatant was removed. The gel samples were then reduced by adding 50 µL of 10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate and heating at 56°C for 1 h. The DTT solution was replaced with 50 µL of 50 mM iodoacetamide in 50 mM ammonium bicarbonate, and the sample alkylated in the dark at room temperature for 45 min. The alkylation solution was then removed, acetonitrile (200 µL) was added into each tube to shrink the gel, and then 200 µL acetonitrile containing 25 µL of freshly prepared 12.5 ng/µL trypsin in 50 mM ammonium bicarbonate was added. The digestion was incubated overnight at 37°C, then stopped by adding 1 µL 50% formic acid to quench the trypsin, and the samples were analysed by LC-MS/MS.

6.2.7 Cleavage site of tamarillin on κ-casein

Samples (10 µL) were injected onto a C18 trap cartridge (LC Packings, Amsterdam, Netherlands) for desalting prior to chromatographic separation on a 0.3x100 mm 3.5u Zorbax 300SB C18 Stablebond column (Agilent Technologies, Santa Clara, CA, USA) using the following gradient at a flow rate of 6 µL/min: 0-3 min 10% solution B, 25 min 40% solution
B, 26.5 min 98% solution B, 29 min 98% solution B, 30.5 min 10% solution B, 35 min 10% solution B, where solution A was 0.1% formic acid in water and solution B was 0.1% formic acid in acetonitrile. The column eluate was ionised in the electrospray source of a QSTAR-XL Quadrupole Time-of-Flight mass spectrometer (TOF-MS, Applied Biosystems, Foster City, CA, USA). A TOF-MS scan from 330-1600 m/z was performed, followed by three rounds of MS/MS on the three most intense multiply-charged precursors in each cycle. The resulting data was searched against the bovine entries from the Uniprot protein sequence database (32,236 entries as at January 2016), using ProteinPilot v5.0 (Sciex, Foster City, CA, USA).

6.3 Results and discussion

6.3.1 Hydrolysis of sodium caseinate by crude tamarillo extract

The hydrolysis of sodium caseinate by crude tamarillo extract and rennet was monitored by SDS-PAGE (Figure 6.1). Sodium caseinate migrated as a broad band (at approximately 27 kDa) below a discrete, sharper band (Figure 6.1, lane 2). The higher molecular mass band was identified as α-casein, while the broad band consisted of β-casein running just above κ-casein. There was also a faint unidentified band running just below the κ-casein band. Hydrolysis of sodium caseinate with rennet (Figure 6.1A) resulted, by 15 minutes, in the loss of the κ-casein band and the appearance of a band at approximately 13 kDa. This band corresponded to the well-recognised cleavage of κ-casein at Phe105-Met106 to generate para-κ-casein (12,268 Da) and glycomacropeptide (Egito et al., 2007). Glycomacropeptide was difficult to detect by SDS-PAGE as it has a highly acidic polyhydroxylic nature and thus it has difficulty interacting with the gel matrix (Coolbear et al., 1996).
Figure 6.1 SDS-PAGE of bovine sodium caseinate hydrolysed at different times by rennet (A) and tamarillo crude extract (B). Lane 1: protein marker; Lane 2: sodium caseinate; Lane 3: 15 min hydrolysis; lane 4 and 5: 30 min hydrolysis; lane 6 and 7: 1 h hydrolysis; lane 8 and 9: 4 h hydrolysis and lane 10: 24 h hydrolysis.

When the sodium caseinate was hydrolysed using crude tamarillo extract the κ-casein was once again all hydrolysed within 15 minutes. A protein band at approximately 13 kDa was not observed however, which suggested that the κ-casein cleavage pattern was not the same as for the rennet hydrolysis. Although after 15 minutes there did not appear to be any change in the α- and β-casein bands, the α-casein band was not visible after 4 h and the β-casein band was greatly reduced, while at the same time a number of smaller bands with molecular masses between 15-20 kDa appeared (Figure 6.1B, lane 8 and 9). However, after 24 h proteolysis by the crude tamarillo extract, all the bovine caseins had been degraded and no peptide bands were observed in the SDS-PAGE gel (Figure 6.1B, lane 10).

Thus, α- and β-casein appeared to be initially more resistant to crude tamarillo extract hydrolysis when compared to κ-casein, and the hydrolysis of sodium caseinate by the crude tamarillo extract initially produced a number of large peptides, including three peptides at approximately 21, 18 and 15 kDa (Figure 6.1B lane 3 to 9). Similar results were reported for protease extracts from *Albizia lebbeck* (Egito et al., 2007), *Lactuca sativa* (Lo Piero et al., 2002), *Onopordum acanthium* (Brutti et al., 2012), *Albizia julibrissin*, *Euonymus sieboldianus* and *Celastrus orbiculatus* (Otani et al., 1991), which also resulted in the hydrolysis of caseins into large peptides.
6.3.2 Hydrolysis of individual caseins by tamarillin

To further understand the action of the tamarillo protease on caseins, the effects of the purified tamarillo protein extract, tamarillin, on the individual caseins, namely α-, β- and κ-casein, was investigated using SDS-PAGE (Figure 6.2D, E and F). The results were also compared to the action of rennet on these caseins under the same conditions (Figure 6.2A, B and C).

With the exception of κ-casein, the caseins were more resistant to hydrolysis by rennet than by tamarillin. Using rennet, no α-casein was hydrolysed until after 1 h incubation (Figure 6.2A, lane 5), and a very faint band, close to the main α-casein band, appeared after 4 h (Figure 6.2A, lane 6). McSweeney et al. (1993) indicated that in bovine α\textsubscript{s1}-casein the Phe\textsubscript{23}-Phe\textsubscript{24} peptide bond was the most sensitive to the activity of rennet, which generated α\textsubscript{s1}(f\textsubscript{1-23}) (molecular mass ~2,764.23 Da) and (f\textsubscript{24-119}) (molecular mass ~20,228.66 Da). Bovine α\textsubscript{s2}-casein has been reported to be the most resistant casein protein to the action of rennet (Grappin et al., 1994).

Similarly, β-casein was resistant to hydrolysis by rennet for the first 1 h, then a peptide band close to the β-casein band was clearly seen after 4 h (Figure 6.2B, lane 6). This band corresponds closely to the work of Sousa and Malcata (1998), who reported that rennet cleaved bovine β-casein (23,980 Da) at Leu\textsubscript{192}-Tyr\textsubscript{193} and Ala\textsubscript{189}-Phe\textsubscript{190} resulting in macropeptides of 21,474 Da and 21,146 Da respectively (Broyard and Gaucheron, 2015). The hydrolysis of β-casein also clearly resulted in the generation of a peptide band of approximately 13 kDa. This band was similar in size to the rennet breakdown products of β-casein reported by Trujillo et al. (1998), namely β-III, which was 15,708 Da (f\textsubscript{1-139}) and 14365 Da (f\textsubscript{1-127}).

In the case of κ-casein, rennet exhibited high specific hydrolysis to produce a para-κ-casein band within the first 15 min (Figure 6.2C lane 3), with no further hydrolysis observed after this time.

In comparison to rennet, tamarillin started hydrolysing α-casein noticeably by 15 min (Figure 6.2D, lane 4) when a peptide band of approximately 15 kDa first appeared. With longer hydrolysis times two other bands at approximately 12 and 13 kDa were also produced (Figure 6.2D, lanes 5-9). After 24 h hydrolysis (Figure 6.2D, lane 9), nearly all the α-casein was hydrolysed with the prominent bands remaining being those observed at approximately 12, 13 and 14 kDa.
β-casein was more resistant to hydrolysis by tamarillin with the first peptide bands of approximately 13 kDa and 17 kDa observed after 1 h (Figure 6.2E, lane 6). The degree of hydrolysis of β-casein increased from 1 h to 10 h, and β-casein was complete degraded after 24 h. Two major hydrolysis products at approximately 13 kDa and 17 kDa were present in the SDS-PAGE gel from 1 h (Figure 6.2E, lane 6) until 10 h (Figure 6.2E, lane 8). After this, the bands were further hydrolysed and became barely discernible after 24 h hydrolysis. Compared to α-casein, while the initial hydrolysis was slower, tamarillin displayed more extensive degradation of β-casein.

The action of tamarillin on κ-casein is shown in Figure 6.2F. A major band of approximately 15 kDa was rapidly generated by 15 min incubation. Only trace amounts of κ-casein were present after 4 h hydrolysis (Figure 6.2F, lane 7) and κ-casein was complete degraded after 10 h incubation (Figure 6.2F, lane 8). While the density of the major 15 kDa band increased as κ-casein was degraded from 15 min to 4 h, it then appeared to be gradually further hydrolysed to a faint band by 24 h of hydrolysis (Figure 6.2F, lanes 6, 8, 9).
Figure 6.2 SDS-PAGE of bovine individual caseins, namely α-casein (A and D), β-casein (B and E) and κ-casein (C and F) hydrolysed by rennet (A, B and C,) or tamarillin (D, E and F). Lane 1 is protein marker and lane 2 are caseins. In A, B and C, lanes 3-8 are hydrolysis times from 15, 30 min, 1, 4, 10 and 24 h, respectively. While, in D, E and F, lanes 3-9 are hydrolysis times from 0, 15, 30 min, 1, 4, 10 and 24 h, respectively.

The effect of tamarillin on the different caseins was very similar to that reported by Tavaria et al. (1997), using two extracts (A and B) from *Centaurea calcitrapa* (a plant from the Compositae family). They observed a higher degree of hydrolysis for α-casein than for β-casein and κ-casein. Specifically, within the same degradation time (24 h), extract B from *Centaurea*
calcitrapa hydrolysed α-casein bovine more rapidly than β-casein. Extract A also degraded α-casein to a higher extent than β-casein and κ-casein after 72 h hydrolysis. In contrast however, the proteolytic action of extracts from Cynara cardunculus, which have been used in cheese making in Portugal and Spain, resulted in extensive hydrolysis of ovine β-casein while extensive degradation of α-casein only occurred under certain condition (Silva and Malcata, 1998).

Digestion of κ-casein by tamarillin was very similar to that observed for other plant-based enzymes. Lufrano et al. (2012) reported that the actions of recombinant procirsin from Cirsium vulgare on κ-casein generated a 15 kDa peptide. Chazarra et al. (2007) compared the action of calf rennet to a crude extract of artichoke flowers, and the purified cynarases A, B and C from Cynara scolymus, and also reported that the resulting bands from the hydrolysis of κ-casein were approximately 15 kDa. Lettucine (from Lactuca sativa leaves) also cleaved κ-casein to yield a 15,100 Da band (Lo Piero et al., 2002) and a milk-clotting enzyme from Solanum dubium seeds hydrolysed κ-casein producing a 16 kDa peptide band after 5 min incubation (Ahmed et al., 2010).

6.3.3 Characterisation of the main peptides generated from the hydrolysis of κ-casein by rennet and tamarillin.

Using the reversed-phase LC-MS, the molecular mass of κ-casein was calculated to be 19,009.8 Da (Figure 6.3A), which was close to the 19,006 Da reported by Farrell et al. (2004). For the rennet-hydrolysed κ-casein, glycomacropeptide and para-κ-casein had elution times and molecular masses of 16.4 min and 17.8 min, and 6789.6 Da and 12,270.3 Da, respectively (Figure 6.3B and C). The molecular masses were similar to the theoretical values stated by Egito et al. (2007).

The principal peptide hydrolysed from κ-casein by tamarillin eluted at 17.9 min and was observed in the 1, 4 and 10 hr samples. After 24 h, although a corresponding band was still observed by SDS-PAGE (Figure 6.2F, lane 9), the level was too low to be detected by LC-MS. The molecular mass of the peptide was 14,289.7 Da (Figure 6.3D), similar to the size of the band observed by SDS-PAGE in Figure 6.2C and there was only a trace of this 14,289.7 Da band present after 24 h hydrolysis.
Figure 6.3 Mass analysis of κ-casein and its hydrolysis products using ESI-MS (spectra and deconvoluted masses). A is κ-casein; B is para-κ-casein generated by rennet hydrolysed κ-casein; C is glycomacropeptide generated by rennet hydrolysed κ-casein; D is peptide produced by tamarillin hydrolysed κ-casein. The deconvoluted masses of the peptides were calculated using Agilent MassHunter Profinder B.06.00 software.
To further identify the site of action of tamarillin on κ-casein, in-gel trypsin digestion followed by peptide mass fingerprinting with TOF-MS was performed. The major hydrolysis band generated from κ-casein (Figure 6.2F) was excised from the gel and analysed. The peptides were identified by TOF-MS analysis (Table 6.1) and a coverage map of the sequence of κ-casein generated (Figure 6.4). The in-gel tryptic digested band corresponded to approximately 72.8% of the κ-casein sequence from the N-terminal end, which also correlated to the 14,289.7 Da peptide identified by LC-MS. There was 100% peptide coverage of the digested band. Based on the result of the in-gel digestion, the cleavage site of tamarillin on κ-casein was confirmed to be at Asn₁₂₃-Thr₁₂₄. The hydrolysis of similar peptide bonds (Asn- Thr) in κ-casein has previously been reported by Reid et al. (1995). As opposed to the Asn₁₂₃-Thr₁₂₄ cleavage site reported here, they observed that the Asn₁₆₀-Thr₁₆₁ bond at the C-terminal of κ-casein was quite susceptible to the action of PIII-type lactococcal proteases while PI-type proteases displayed high specific hydrolysis at the Asn₈-Thr₉ bond (Reid et al., 1995). Thus, the preferred cleavage site of tamarillin on κ-casein (Asn₁₂₃-Thr₁₂₄) is the first to be reported for a rennet-like protease from plant sources.

**Table 6.1** Ionisation fragment analysis for the in-gel trypsin digestion of tamarillin peptide from κ-CN using quadrupole time-of-flight mass spectrometry.

<table>
<thead>
<tr>
<th>Measured MW (Da)</th>
<th>Theoretical MW (Da)</th>
<th>Peptide fragments</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1399.73</td>
<td>1399.73</td>
<td>112 - 123</td>
<td>KNQDKTEIPTIN</td>
</tr>
<tr>
<td>1607.84</td>
<td>1607.84</td>
<td>98 - 111</td>
<td>HPHPHLSFMAIPPK</td>
</tr>
<tr>
<td>3283.66</td>
<td>3283.64</td>
<td>69 - 97</td>
<td>SPAQILQWQVLSNTVPACK</td>
</tr>
<tr>
<td>4009.08</td>
<td>4009.06</td>
<td>35 - 68</td>
<td>SCQAAQPTTMAR</td>
</tr>
<tr>
<td>1250.67</td>
<td>1250.70</td>
<td>25 - 34</td>
<td>YIPIQYVLSR</td>
</tr>
<tr>
<td>1011.55</td>
<td>1011.54</td>
<td>17 - 24</td>
<td>FFSDKIAK</td>
</tr>
<tr>
<td>2068.94</td>
<td>2068.92</td>
<td>1 - 16</td>
<td>QEQNQEOPIRCEKDER</td>
</tr>
</tbody>
</table>
Specificity studies have reported that the cleavage sites of most principal milk-clotting proteases are generally toward the Phe$_{105}$-Met$_{106}$ peptide bond in bovine $\kappa$-casein (Huppertz et al., 2006). An exception was *E. parasitica*, where only the Ser$_{104}$-Phe$_{105}$ bond was hydrolysed. The different cleavage site of the *E. parasitica* protease however, did not affect its milk-clotting ability (Drohse and Foltmann, 1989). Although plant enzymes as rennet substitutes have been studied for a long time, few investigations on the specificity of plant protease action on $\kappa$-casein have been reported. Other plant coagulants (Table 6.2), such as cardosins A and B extracted from *Cynara cardunculus* (Sousa and Malcata, 1998) and a protease extracted from *Helianthus annuus* (Egito et al., 2007), were shown to possess the same cleavage site as rennet at Phe$_{105}$–Met$_{106}$. Lettucine, which was extracted from *Lactuca sativa* leaves, was assumed to have cleavage sites on bovine $\kappa$-casein at Arg$_{97}$–His$_{98}$, Lys$_{111}$–Lys$_{112}$, or Lys$_{112}$–Asn$_{113}$ (Lo Piero et al., 2002). In addition, cleavage sites Lys$_{116}$–Thr$_{117}$ and Ser$_{104}$–Phe$_{105}$ were the preferred targets of enzymes extracted from *Albizia lebbeck* seed (Egito et al., 2007) and *Solanum dubium* seed (Ahmed et al., 2010) respectively.
Table 6.2 Reported specificity of plant milk-clotting enzymes on bovine κ-casein

<table>
<thead>
<tr>
<th>Name/origin of protease</th>
<th>Sources</th>
<th>Preferential cleavage site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamarillin – tamarillo fruit</td>
<td>Cyphomandra betacea</td>
<td>Asn₁₂₃-Thr₁₂₄</td>
<td>Present study</td>
</tr>
<tr>
<td>Lettuce - lettuce</td>
<td>Lactuca sativa</td>
<td>Arg₉⁷-His₉₈, Lys₁₁₁–Lys₁₁₂, or Lys₁₁₂–Asn₁₁₃</td>
<td>Lo Piero et al., 2002</td>
</tr>
<tr>
<td>Cardosins A and B</td>
<td>Cynara cardunculus</td>
<td>Phe₁₀₅–Met₁₀₆</td>
<td>Sousa and Malcata, 1998</td>
</tr>
<tr>
<td>Albizia seed</td>
<td>Albizia lebbeck</td>
<td>Lys₁₁₆-Thr₁₁₇</td>
<td>Egito et al., 2007</td>
</tr>
<tr>
<td>Sunflower seed</td>
<td>Helianthus annuus</td>
<td>Phe₁₀₅–Met₁₀₆</td>
<td>Egito et al., 2007</td>
</tr>
<tr>
<td>Solanum dubium seed</td>
<td>Solanum dubium</td>
<td>Ser₁₀₄-Phe₁₀₅</td>
<td>Ahmed et al., 2010</td>
</tr>
</tbody>
</table>

6.4 Conclusion

Both crude extract and purified protease (tamarillin) from tamarillo fruit exhibited broad hydrolysis on bovine casein proteins. Compared with α- and β-casein, κ-casein was more susceptible to the action of tamarillin with the major peptide being formed after only 15 min hydrolysis. Tamarillin also displayed high proteolytic activity towards both α- and β-casein. SDS-PAGE and LC-MS results indicated that κ-casein was hydrolysed by tamarillin to generate a major 14,290 Da peptide. The cleavage site of this peptide fragment was confirmed by TOF-MS to be at Asn₁₂₃-Thr₁₂₄. This is the first report of the hydrolysis of κ-casein by a plant protease with the Asn₁₂₃-Thr₁₂₄ peptide bond as the preferred cleavage target.

The result from this study suggested that plant coagulants may have other important cleavage sites of hydrolysis on κ-casein, which could contribute to milk-clotting in a manner similar to that caused by the breakdown of the Phe₁₀₅–Met₁₀₆ bond. Also, the specificity studies of milk-clotting enzymes indicated that there were possible new mechanisms for rennet-like behaviour on κ-casein, which need to be studied further in the future.
Chapter 7

7. Rheological and structural properties of coagulated milks reconstituted in D$_2$O: Comparison between rennet and tamarillo protease (tamarillin)
Abstract

The physicochemical properties and structural characteristics of milk gels incorporating deuterium oxide (D₂O) and induced by rennet and tamarillo protease (tamarillin) were investigated. SDS-PAGE analysis showed that both proteases hydrolysed κ-casein while tamarillin also exhibited a broader specificity on α- and β-casein. Small and large deformation rheological measurements showed that rennet-induced milk gels at low milk solids content (10% w/w) displayed higher elasticity than the gels induced by tamarillin. In high concentration milk gels (20% w/w), the aggregation time was sharply decreased and the elasticity was increased for both rennet and tamarillin-induced gels. In addition, large deformation experiments indicated that rennet-induced gels were more brittle than those made with tamarillin for both 10 and 20% (w/w) milk gels. The microstructures of the milk gels displayed more porosity in the gels made with tamarillin, compared to those made with rennet, likely due to the broader caseinolytic activity of tamarillin. Ultra-small angle neutron scattering (USANS) showed that there are no differences on the large-scale structure of tamarillin and rennet-induced gels. However, small-angle X-ray scattering (SAXS) revealed that rennet-induced gels have a denser protein network than that of tamarillin-induced gels.
7.1 Introduction

Cheese is one of the traditional processed dairy products obtained by first coagulating the milk (Omotosho et al., 2011). The milk-clotting enzyme rennet is usually used in the cheesemaking industry because of its high specificity to cleave the Phe\textsubscript{105}-Met\textsubscript{106} peptide bond in κ-casein. This cleavage affects the stability of the interactions between the casein micelles, resulting in the aggregation of casein micelles and subsequent milk-clotting (Pires et al., 1994; Vishwanatha et al., 2010). However, an increase in the demand for cheese products and a world-wide shortage of rennet has resulted in the increase of the price of rennet (Jacob et al., 2011). Other factors such as the restriction of the use of animal rennet for religious and diet (vegetarianism) reasons, motivated the search for rennet substitutes (Roseiro et al., 2003). Although bacterial and genetically engineered rennet have been proven to be good milk coagulants, recently the interest in plant proteases has grown (Shah et al., 2014). Plant-based coagulants, such as extracts from the flower of 	extit{Cynara cardunculus}, have been traditionally used in ovine and caprine cheeses (Pires et al., 1994; Trujillo et al., 1994; De Sa and Barbosa, 1972).

Some plant proteases share the same hydrolysis site in κ-casein as rennet (Phe\textsubscript{105}-Met\textsubscript{106}), but most plant proteases also have broader proteolytic activity on α-, β- and κ-casein (Macedo et al., 1993). Ahmed et al. (2010) investigated the action of a milk-clotting enzyme from the seeds of 	extit{Solanum dubium} on bovine caseins, and reported that all caseins are more sensitive to the action of this enzyme compared to rennet. They also reported that β- and κ-casein are more susceptible to hydrolysis than α-casein. Egito et al. (2007) studied the proteolytic activity of extracts from the seeds of 	extit{Albizia lebbeck} and 	extit{Helianthus annuus}, and also reported that α- and β-casein are more susceptible to the action of these plant extracts than rennet. The extract from 	extit{Helianthus annuus} has the same cleavage site on κ-casein than rennet, while the Lys\textsubscript{116}-Thr\textsubscript{117} bond was the preferred target of an extract from 	extit{Albizia lebbeck}. In addition, an actinidin extract from kiwifruit preferably hydrolysed β-casein to κ-casein (Lo Piero et al., 2011).

Mazorra-Manzano et al. (2013) compared the milk-clotting behaviour of extracts from kiwifruit, melon and ginger with rennet. Their results showed that the curds induced by an extract from kiwifruit had similar properties to that produced by rennet. However, the melon extract-induced curds were more fragile and had a lower yield than that produced by rennet. The properties of curds were related to the proteolytic activities of these enzymes, with the
melon extract displaying a higher proteolytic activity compared to the kiwifruit extract and rennet. Esteves et al. (2001) also stated that plant coagulant extracts from the flowers of *Cynara cardunculus* and *Cynara humilis* exhibited higher proteolytic activities than that of rennet, which resulted in lower milk gel firmness due to higher casein hydrolysis by the plant coagulants.

In this study tamarillin, a serine protease isolated from tamarillo fruit, with rennet-like properties, was investigated. The effect of tamarillin on the physicochemical properties and structural characteristics of milk gels was compared to those obtained by the addition of rennet. In addition, the effect of milk concentration on the gel properties was also considered. The milks were prepared by reconstituting skim milk powders in D$_2$O to allow fast ultra-small angle neutron scattering measurements (USANS) measurements. SDS-PAGE analysis was performed to assess the extent of casein hydrolysis by rennet and tamarillin, and confocal laser scanning microscopy (CLSM) was employed for observation of the gel network. Rheological measurements, both under small and large deformation, were performed to determine the mechanical properties of the resulting milk gels. Small-angle X-ray scattering (SAXS) measurements were performed to probe the fine-structure of the gel protein network, while USANS was used to probe the protein network on a larger scale on the milk gels. Note that although the milk gel formation induced by acidification has been studied using SAXS and USANS before (van Heijkamp et al., 2010; Moitzti et al., 2010), to the best of our knowledge these methods have never been previously used to study milk gels obtained by enzymatic action.

### 7.2 Materials and methods

#### 7.2.1 Materials

Low heat skim milk powder (SMP) was obtained from Synlait Milk Ltd., Rakaia, New Zealand. Deuterium oxide (D$_2$O) was produced in Cambridge Isotope Laboratories, Inc., Andover, USA. Rennet (280 International Milk-clotting Units) was ordered from RENCO, Eltham, New Zealand. Capillary tubes (2 mm I.D) were purchased from Charlessupper Company, Natick, USA. All other chemicals and reagents were purchased from Sigma-Aldrich Ltd., Auckland, New Zealand. Laird’s Large tamarillo fruit were hand-picked from trees in an orchard in Maungatapere, New Zealand.
7.2.2 Tamarillin extraction and purification

Purification of tamarillin was based on McDowall (1970) and Aminlari et al. (2009). Tamarillo fruit (300 g) were homogenized for 20 min using a Polytron PCU2 laboratory homogeniser (Brinkmann Instruments, Luzern, Switzerland), followed by filtration through two layers of cheesecloth to remove the insoluble material. The tamarillo filtrate (approximately 85 mL) was mixed with 80 mL of 0.05 M sodium citrate (pH 5.5) buffer by gentle stirring, then centrifuged at 15,000 × g for 20 min at 4°C in a Sorvall LYNX superspeed Centrifuge (Thermo Fisher Scientific, USA). The resulting supernatant (approximately 155 mL) was precipitated with (NH₄)₂SO₄ (65% saturation) overnight at 4°C, using gentle stirring. The precipitated proteins were collected by centrifugation at 15,000 × g for 20 min at 4°C and then dissolved in 30 mL 0.05 M sodium citrate buffer (pH 5.5), for 24 h at 4°C.

The crude tamarillo extract (40 mL, 1.17 ± 0.03 mg/mL) obtained above was loaded on a DEAE-Sepharose fast flow (GE Healthcare Life Sciences, New Zealand) ion exchange column (22 cm×5 cm) pre-equilibrated overnight with 0.05 M sodium citrate buffer (pH 5.5). The proteins were separated with a 200 mL linear gradient of 0-1.0 M NaCl at a flow rate of 1.0 mL/min. Fractions (10 mL) were collected and the protein content was determined using a spectrophotometer (Shimadzu Corporation, Australia) at 280 nm. Fractions were assayed for protease activity (as in Section 3.8.1) and peak areas with the highest activity (tamarillin) were pooled and dialysed against Milli-Q water to remove salt for 24 h at 4°C. The tamarillin was then freeze-dried (Labconco Corporation, Missouri, USA) and stored at -80°C until further use.

7.2.3 Preparation of reconstituted milks and milk gels

Reconstituted milks were prepared by mixing skim milk powder with D₂O at milk solids concentrations of 1%, 2%, 10%, 20% and 25% (w/w) using a magnetic stirring (Heidolph Instruments, Germany) for at least 2 h at room temperature to ensure full dissolution. The mixtures were kept at 4°C overnight to ensure complete hydration and then equilibrated to room temperature for at least 4 h before use.

Milk samples (1% w/w) were prepared by mixing either 5 g of 1% (w/w) milk with 10 µL rennet (diluted 100-fold) or 2.5 g of 2% (w/w) milk with 2.5 g tamarillin (2 mg dissolved in 5 g D₂O). The 10% (w/w) milk gel samples were prepared by mixing either 25 g of 10% (w/w) milk
milk with 25 µL rennet (diluted 10-fold) or 12.5 g of 20% (w/w) milk with 50 mg tamarillin pre-dissolved in 12.5 g D₂O. For the 20% (w/w) milk samples, either 25 g of 20% (w/w) milk was mixed with 50 µL rennet (diluted 10-fold) or 20 g of 25% (w/w) milk was mixed with 100 mg tamarillin pre-dissolved in 5 g D₂O. The milk and enzyme concentrations were chosen to keep a constant enzyme to protein ratio for each desired final concentration of milk.

To obtain the milk serums, milk gels (10% and 20% w/w) were incubated at 35°C for 10 h in 40 mL volume glass vials. Then, gels were cut using a thin spatula into roughly 10 mm³ cubes. The cut gels were transferred into 50 mL centrifuge tubes and centrifuged at 1,500 × g for 30 min at room temperature using Eppendorf centrifuge 5810R (North Ryde, Australia). Centrifugation separated the supernatant serum from the precipitated gel curd. The serum was collected and then further filtered through a 0.22 µm filter (TISCH Scientific, Ohio, USA) to be measured by USANS and SAXS for baseline subtraction.

7.2.4 Analysis of casein hydrolysis by SDS-PAGE

Aliquots (100 µL) of the milks with added rennet or tamarillin, as described in Section 7.2.3 above, were transferred into glass vials and incubated at 35°C for 10 h in an incubator (Function Line incubator, Heraeus, Langenselbold, Germany). After 10 h hydrolysis, the reaction was stopped by heating the glass vials at 100°C for 3 min in a water bath (Ahmed et al., 2010). Urea buffer (8 M, pH 8) was added to each sample with vortexing for 5 min to break the non-covalent bonds between the proteins and peptides. To achieve the same final protein concentration, 1%, 10% and 20% (w/w) milk gel samples were added to 100 µL, 400 µL and 900 µL urea buffer respectively. Aliquots (100 µL) of the mixtures were mixed with 200 µL SDS-PAGE sample buffer, consisting of 50 mM tris-HCl (pH 6.8), 2% (w/w) SDS, 0.1% (w/w) bromophenol blue, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol.

Following Laemmli (1970), SDS-PAGE was performed using a 4% acrylamide stacking gel and a 12% acrylamide resolving gel containing 0.1% SDS. Samples (10 µL) were loaded into the gel wells. Electrophoresis was conducted at 150 V for 2 h in 0.025 M Tris-HCl, 0.192 M glycine buffer containing 0.1% (w/v) SDS using a Mini-PROTEAN Tetra Cell (Bio-Rad Technologies, Inc., California, USA). After running the SDS-PAGE, the protein bands in the gels were stained using 0.1% Coomassie brilliant blue R-250 overnight followed by destaining using 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) Milli-Q water.
7.2.5 Rheological measurements

Rheological characterisation was conducted in an Anton Paar Physica MCR 301 stress-controlled rheometer (Anton Paar, Graz, Austria), equipped with a stainless-steel cup and bob geometry system (a cup with an inner diameter of 27.5 mm and a 26.5 mm diameter and 48 mm length bob). Milk samples (18.5 mL, prepared as described in Section 7.2.3) treated with rennet or tamarillin, was loaded into the cup and bob geometry. The samples were stirred for 2 minutes on a magnetic stirrer to mix the protease solution and the milks and soya oil (2-3 drops) was added to cover the surface of milk sample to prevent water evaporation during the measurements. The following three rheological characterisation protocols were performed: (1) A time sweep was performed by first equilibrating the sample temperature at 25°C for 2 min, followed by increasing the temperature to 35°C at a rate of 2°C/min. The elastic modulus $G'$ (storage) and the viscous (loss) modulus $G''$ values were recorded at a constant frequency of 1 Hz and a constant applied strain of 1% every minute for 10 h. (2) At the end of the time sweep, the temperature was lowered to 25°C for 10 min, and a frequency sweep measurement was performed where $G'$ and $G''$ values were measured as a function of frequency from 0.1 to 10 Hz at a constant strain of 1% at 25°C. (3) The frequency sweep measurement, was followed by a large-deformation sweep performed with a constant shear rate 0.1 s$^{-1}$ for a total interval time of 2000 s at 25°C. During this large-deformation sweep test the stress was measured, while the strain (=shear rate×time) was varied from 0 to 200%.

7.2.6 Confocal laser scanning microscopy

The microstructures of milk gels were investigated by confocal laser scanning microscopy (CLSM) following the method of Esteves et al. (2003b) with minor modifications. The milk samples were prepared as for the rheological measurements. A few drops of 0.25% (w/v) acridine orange were mixed with the milk samples using a magnetic stirrer for 2 min at room temperature. Aliquots of mixtures (100 µL) were transferred into a slide with a cavity. A coverslip was used to cover the milk sample and was sealed by nail polish to prevent evaporation. The slides were then incubated in an oven for 10 h at 35°C. The microstructure of gels was observed using an Olympus FV1000 CLSM microscope (Olympus Corporation, Tokyo, Japan) with an X-Cite series 120Q mercury halide lamp (Lumen Dynamics, Ontario, Canada) at a wavelength of 488 nm. Images of gel microstructure were obtained using a 60×
oil immersion objective lens. Each sample was prepared in duplicate and at least three representative areas from the same sample were observed.

7.2.7 Ultra small angle neutron scattering (USANS)

UANS measurements, based on the Bonse-Hart method (Bonse and Hart, 1965), were performed on the Kookaburra beamline at ANSTO (Sydney, Australia). It consisted of two channel-cut perfect Si single crystals, a monochromator and an analyser. Milk in D$_2$O samples were measured by USANS at a neutron wavelength $\lambda=4.74$ Å and a Cd aperture with a diameter of 30 mm, at a $Q$ range of 0.0003 to 0.1 nm$^{-1}$, at room temperature. The milk samples were filled into demountable cells. Then the sample cells were incubated at 35°C in an oven (Laboratory equipment PTY. Ltd. Australia) for 10 h. Note that to optimise the intensity of the neutron scattering, cell path lengths of 1 mm and 0.5 mm were used for the 10% (w/w) and 20% (w/w) milks and milk gels, respectively.

7.2.8 Small angle X-ray scattering (SAXS)

The samples used in SAXS experiments were prepared similarly to those used in USANS measurements. The main difference was that the milk gels were prepared by transferring the milks-enzyme mixtures into 2 mm inner diameter capillary quartz tubes (Charlessupper Company, Natick, USA), sealed by Para-film, followed by incubation at 35°C for 10 h in a BPZ-6123 oven (Shanghai Yiheng Scientific Instruments Co., Ltd. Shanghai, China). The serums of the milk gels were also measured in the same capillary tubes.

Synchrotron small-angle X-ray scattering (SAXS) measurements were performed on the BL19U2 BioSAXS beamline at the National Centre for Protein Science Shanghai (NCPSS) at the Shanghai Synchrotron Radiation Facility (SSRF, Shanghai, China). A monochromatic beam of 1.033 Å wavelength ($\lambda$) was used and sample-to-detector distance of 2234.4 mm, yielding a $q$-range ($q=4\pi\sin\theta/\lambda$, $\theta$ is the scattering angle) between 0.06 to 4 nm$^{-1}$. The SAXS profiles of the samples were analysed by subtracting the background of the corresponding serum using the BioXTAS RAW software version 1.2.1 (Nielsen et al. 2009). All the samples were measured at room temperature.
7.3 Results and discussion

7.3.1 Protein profile and proteolytic activity

The extent of skim milk hydrolysis by rennet or tamarillin after 10 h incubation was investigated by SDS-PAGE. For rennet treated milk there was a small amount of κ-casein remaining for 1% (w/w) milk (Figure 7.1, lane 2) compared with untreated milk (Figure 7.1, lane 8), and a peptide band appeared at approximately 14 kDa. With 10 and 20% (w/w) milks (Figure 7.1, lanes 3 and 4 respectively), κ-casein was completely hydrolysed and a peptide band at approximately 14 kDa was observed. This new peptide band was assumed to be attributed to para-κ-casein, as has been reported for the action of rennet on milk by Timotijević et al. (2006). Both α- and β-casein in all three milks (Figure 7.1, lanes 2, 3 and 4) were not affected by rennet treatment (Figure 7.1, lane 8). This result was expected, as previous studies also reported that, compared to κ-casein, α- and β-casein were less susceptible to the action of rennet (Fox et al., 1994).

For milk samples treated with tamarillin however, all the caseins were hydrolysed (Figure 7.1, lane 5, 6 and 7) after 10 h incubation. κ-caseins was fully hydrolysed, and only small amounts of α- and β-casein remained. The amounts of the non-hydrolysed α- and β-casein appeared to be dependent on the concentration of milk, with the higher the milk concentration, the higher the amount of these caseins remaining. Compared to the action of rennet, more peptide bands between 10 and 25 kDa were generated, particularly peptides with molecular weights of approximately 13, 16 and 18 kDa. This clearly indicated the broad hydrolytic activity of tamarillin. Similar findings were reported for the action of other of plant proteases on caseins, including lettucine extracted from leaves of Lactuca sativa (Lo Piero et al., 2002), and protein extracts from seeds of Albizia lebbeck and Helianthus annuus (Egito et al., 2007).
Figure 7.1 SDS-PAGE of skim milk hydrolysed by rennet and tamarillin, after 10 h incubation. Lane 1 are protein markers, lanes 2, 3 and 4 are rennet-treated 1, 10 and 20% (w/w) skim milks, respectively. Lanes 5, 6 and 7 are 1, 10 and 20% (w/w) skim milks treated with the tamarillin respectively. Lane 8 is 10% (w/w) skim milk. Milk protein bands are indicated by α-casein (α-CN), β-casein (β-CN), κ-casein (κ-CN), β-lactoglobulin (β-Lg) and α-lactalbumin (α-Lac).

7.3.2 Rheological measurements

The complex modulus $G^* = ((G')^2 + (G'')^2)^{1/2}$ as a function of time for the 10 and 20% (w/w) milks treated with rennet or tamarillin are reported in Figure 7.2A. The 1% milk samples treated with rennet and tamarillin were not subject to rheological analysis as they did not form gels. Qualitatively, the gelation behaviours measured by rheology were similar to those previously reported for milk treated with rennet (Waungana et al., 1998). As can be seen on Figure 7.2A, the kinetics of gelation for the 20% (w/w) milks was faster than for the 10% (w/w) milks. Further, at the same milk concentration, the kinetics of gelation of milks treated with rennet was also faster than that of the milks treated with tamarillin. Similar findings were reported by Esteves et al. (2002), who showed that ~1 h after the addition of enzyme coagulant to milk at 32°C, the increase rate of $G'$ of rennet-induced gel were faster than plant coagulants extracts from Cynara cardunculus and Cynara humilis induced gels.

After 10 h gelation the value of $G^*$ was 222.0 ± 0.0 Pa and 86.4 ± 0.7 Pa, for 10% (w/w) milks treated with rennet and tamarillin respectively and 1895.0 ± 35.4 Pa and 1645.0 ± 7.0 Pa, for...
20% (w/w) milks treated with rennet and tamarillín, respectively. The faster gelation kinetics for the high concentration milks compared to the low concentration milks could be understood in terms of the number of caseins micelles involved, and how close they are to each other (Culioli and Sherman, 1978; McMahon et al., 1993; Waungana et al., 1996). After 10 h hydrolysis, $G^*$ values were also higher for the high concentration milks, probably due to the greater number of bonds between the milk protein aggregates in the gel network (Waungana et al., 1996; Waungana et al., 1998). Ong et al. (2013) stated that rennet-induced milk gels formed with concentrated milks were much firmer, with the explanation that, as the concentration of milk protein increased, and the distance between casein micelles decreased. Green and Grandison (1993) indicated that the increase in gel firmness was most probably due to the increase in number and strength of the casein micelle network. The lower $G^*$ values observed for tamarillo protease treated milks compared to rennet treated milks, was most likely due to the broad hydrolysis of tamarillín. In other words, as shown by SDS-PAGE analysis, more caseins are hydrolysed by tamarillín than rennet, resulting in less intact caseins present in the protein network. Esteves et al. (2002) reported that the $G'$ values in rennet-induced gels were higher than those obtained from plant coagulant (from Cynara cardunculus and Cynara humilis) induced gels. They suggested that this was because of the higher extent of rearrangements in the milk gels obtained by the action of plant coagulants, compared to rennet-induced gels (Esteves et al., 2002).

To investigate the viscoelastic behaviour of the milks gels, a frequency sweep was performed at the end of the time sweep (Figure 7.2B). For all gels, $G'$ values were higher than $G''$ values, and both $G'$ and $G''$ values increased only slightly with the increase in frequency. This confirmed the gel-like behaviour of the milk gels obtained by the addition of rennet or tamarillín, however, because $G'$ values were larger than $G''$ values by less than ten-fold, this indicated that these were weak gels (Fernandes et al. 1991; Lapasin and Pricle, 1995).

Strain deformation measurements were performed after the completion of the frequency sweep to investigate the large deformation rheological behaviour of the milk gels (Figure 7.2C). All the samples exhibited a similar behaviour within a low strain region, where the stress was proportional to the strain. This linear region occurred at strain values <10%, and confirmed that the frequency sweep measurements, performed at 1% strain, were carried out in the Linear Viscoelastic Region. This linear region was followed by a non-linear region at the end of which the stress reached a maximum. This non-linear region, where the stress increased with the strain,
indicated that these gels behaved as strain-hardening systems (Groot et al., 1996). After a maximum stress was reached, the stress started to decrease with the increase in the strain, indicating that the gels were starting to break.

To compare the different milk gels, the values of maximum stress ($\sigma_{\text{max}}$) and maximum strain ($\gamma_{\text{max}}$) were determined (Figure 7.3C). For the 10% (w/w) milk gels, induced by rennet and tamarillin, the values of $\sigma_{\text{max}}$ and $\gamma_{\text{max}}$ were 52.7 ± 8.3 Pa and 44.7 ± 2.1%, and 74.7 ± 3.0 Pa and 96.0 ± 2.1% respectively. Similarly, the 20% (w/w) milk gels, coagulated by rennet and tamarillin, had $\sigma_{\text{max}}$ and $\gamma_{\text{max}}$ values of 267.5 ± 10.6 Pa and 45.7 ± 0.7%, and 560.0 ± 8.5 Pa and 93.0 ± 0.7% respectively. For both milk concentrations, the $\sigma_{\text{max}}$ and $\gamma_{\text{max}}$ values of milks gelled by tamarillin were higher than those of the milks gelled by rennet. These results do not contradict those obtained by the time sweep and the frequency sweep, obtained by small deformation rheological measurements, where the gels made with rennet were found to be more elastic than those made with tamarillin. The results obtained by the large strain experiments indicated that milk gels made with tamarillin were less brittle than those made with rennet. This could be a consequence of the extensive hydrolysis of the casein by tamarillin. In this instance the protein network in the tamarillin-induced milk gels were made of “weaker” casein aggregates, which would deform more under larger strains before breaking.
Figure 7.2 (A) $G^*$ values as a function of time, (B) frequency sweep, and (C) large deformation rheological measurements of milks gelled with the addition of rennet or tamarillin. Symbols correspond to: 10% (w/w) rennet-induced milk gel (■); 10% (w/w) tamarillin-induced milk gel (●); 20% (w/w) rennet-induced milk gel (▲); and 10% (w/w) tamarillin-induced milk gel (◆). In (B) $G'$ values are solid symbols and $G''$ values are represented by open symbols.
7.3.3 Confocal laser scanning microscopy (CLSM) observations

The confocal micrographs for the different milk gels are depicted in Figure 7.3. The protein network, due to the staining of the proteins, appeared white, while the voids, composed of the milk serum, are dark. After 10 h incubation, the micrographs of the 10% (w/w) milk gels made with both rennet and tamarillin showed a protein network with small voids distributed throughout the gel (Figure 7.3A and B) although the voids were larger in the tamarillin-induced gels. The larger porosity observed for tamarillin-induced gels might be attributed to the extensive hydrolysis of the caseins. This was more evident in the 20% (w/w) milk gels, where the micrograph of milk gelled with rennet show smaller voids and a more homogeneous protein network (Figure 7.3C), while the micrograph of the 20% (w/w) milk gelled by tamarillin had a protein network with larger voids (Figure 7.3D).

Microscopy observations remain qualitative and cannot be extrapolated into an explanation of the macroscopic behaviour of the milk gels, as obtained by rheology. However, these CLSM experiments clearly demonstrated that all the milks with added rennet or tamarillin resulted in a protein network. Furthermore, they indicated that the voids in the milk gels obtained by tamarillin treatment were bigger than those observed in the milks gelled by rennet. This might explain the larger deformation behaviour of these gels (Figure 7.2C), where a gel with a denser structure (rennet milk gels) may be expected to break at lower strains, compared to the more flexible gels with larger voids such as those obtained by using tamarillin.
7.3.4 USANS and SAXS investigations

Small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) have been extensively employed to characterise the structure of casein micelles over a wide range of length scales. SAXS and SANS have been used to determine the nanostructures of casein micelles in their native and unperturbed states avoiding the complications of sample preparation steps such as drying and oxidative dying that are usually used in various
microscopy techniques (Nakagawa and Kagemoto, 2013). Here, by combining ultra-small angle neutron scattering (USANS) and SAXS, the hierarchical milk gel structures, induced by rennet and tamarillin, in length scales ranging from a few nanometres to several microns can be probed. Firstly, USANS was used to examine the large-scale structures of the different milk gels. The USANS data (Figure 7. 4A and B) were obtained for a wavevector of $5 \times 10^{-4} \text{Å}^{-1} < q < 10^{-2} \text{Å}^{-1}$, corresponding to a probed length scale of 60 nm to 1.2 µm. The scattering curves of the milk gels showed a scattering intensity, $I(q)$, that followed a power law decay equation given as:

$$I(q) \sim q^{-m}$$

Where $m$ is the power law exponent, from which the nature and geometry of the scattering object can be determined. For example, $0 < m < 3$ may refer to mass fractal structures (three dimensional self-similarity over a large scale lengths), which indicates the compactness of the object (Martin and Hurd, 1987).

Across the USANS $q$-range, all milk gels with different milk solid contents and coagulants (rennet or tamarillin) demonstrated two power-law regimes as shown in Figure 7.4A and B. More specifically, in the $q$-range of approximately $5 \times 10^{-5}$ to $3 \times 10^{-3}$ Å$^{-1}$, a power law exponent of $m \sim 2$ was observed for both rennet and tamarillin coagulated milk gels. In this $q$-range, 10 and 20% (w/w) milk gels formed by both rennet and tamarillin may be described as mass fractal as indicated by a power law exponent of $m$, 2.10 ± 0.03 and 2.20 ± 0.06, and 2.15 ± 0.05 and 2.20 ± 0.04, respectively. The similarities in exponent, $m$, in the low $q$ range that characterised the gel large-scale structure suggested similar morphologies of the milk gel networks coagulated by rennet and tamarillin for both 10 and 20% (w/w) milk. This mass fractal behaviour was also observed in acid induced skim milk gels (van Heijkamp, et al., 2010) and other colloidal aggregates systems (Schaefer et al., 1984).
Figure 7.4 (A, B) Ultra-small angle neutron scattering (USANS) patterns and (C, D) small-angle X-ray scattering (SAXS) patterns of milks and milk gels coagulated by rennet or tamarillin at milk solid content of 10% and 20%.

In the high-\(q\) region, there was a distinct crossover to a second power law regime with an exponent of \(\sim 4\). This slope was originally calculated by Porod for two-phase systems with sharp boundaries (Guimer and Fournet, 1955). For both rennet and tamarillin coagulated milk gels, the crossover point was approximately \(q=0.003\ \text{Å}^{-1}\), corresponding to the scattering size of 200 nm, a typical size of the casein micelles (Holt, 2016). The fact that the crossover point and the Porod slope was the same, suggested that the casein micelles remain intact in the milk gels (Schaefer, et al., 1984).

In terms of milk, both 10% and 20% (w/w) milk demonstrated similar USANS scattering patterns. The \(I(q)\) over the low \(q\) regime (approximately \(5\times10^{-4}\ \text{Å}^{-1}\) to \(3\times10^{-4}\ \text{Å}^{-1}\)) could be fitted by the Guinier function (Hayter and Penfold, 1983), and decayed by a \(q^{-4}\) power law in high \(q\) regime. The radius of gyration \(R_g\) of the casein micelles determined by the Guinier analysis...
for 10 and 20% (w/w) milks was 83 ± 3 and 82 nm, respectively. This compared well with another study where a value of $R_g \approx 100$ nm was reported for casein micelles (Pignon et al., 2004). The scattering profile decayed by a $q^{-4}$ power law in the high $q$ regime in a manner typical of Porod-like casein micelles spheres with sharp interfaces between the casein micelles and the surrounding solvent (Day et al., 2017).

To extend the structural characterisation down to the nm scale and even smaller, SAXS was employed to further analyse the milk gels induced by rennet or tamarillin. The SAXS scattering profiles of milk (Figure 7.4C and D) showed similar scattering characteristics as those reported in previous SAXS studies (Day et al., 2017; De Kruif, 2014; Liu et al., 2017). Generally, the SAXS scattering pattern of both 10 and 20% (w/w) milk samples coagulated with rennet or tamarillo protease were similar. In the low $q$ region, between 0.008 to 0.02 Å$^{-1}$ the scattering intensity was observed to decay closely following a $q^{-4}$ power law, suggesting a relatively smooth and sharp interface of the casein micelles (Mata et al., 2011). This agreed with previous observations for casein micelles using SAXS experiments on dilute milk solutions (Day et al., 2017; Liu et al., 2017). A distinctive shoulder at $q \sim 0.08$ Å$^{-1}$ observed in the milk samples has been commonly attributed to the scattering of colloidal calcium phosphate (CCP) nanoclusters. However, De Kruif (2014) argued that the scattering magnitude from CCP nanoclusters was too small to be visible in the scattering profiles and instead the author believed that this shoulder should be attributed to protein inhomogeneities at the 1-3 nm scale. A recent resonant soft X-ray scattering study confirmed that the shoulder at $q \sim 0.08$ Å$^{-1}$ was due to the presence of protein inhomogeneities within the casein micelles (Ingham et al., 2015). In the high $q$ region (0.08-0.18 Å$^{-1}$), all the scattering profiles followed a power law $q^{-2.5}$ decay, demonstrating a non-globular internal structure with a fractal dimension of 2.5 (Marchin et al., 2007). The scattering profile transition from approximately $q^{-4}$ to $q^{-2.5}$, suggested characteristics of Porod-like spheres with sharp interfaces between the casein micelles and surrounding solvent (D$_2$O) in the low $q$ region, to a much less dense internal structure with a fractal dimension around 2.5 in the high $q$ region (Day et al., 2017).

Upon coagulation, a peak appeared at approximately 0.035 and 0.030 Å$^{-1}$ for 20% (w/w) milk coagulated by rennet and tamarillin, respectively. This feature was also observed in SANS experiments (Holt et al., 2003) and SAXS of dried milk powders (Mata et al., 2011), however it was usually absent in SAXS of casein micelles in aqueous solution (Day et al., 2017). In SANS experiments, the peak at approximately 0.035 Å$^{-1}$ was most prominent when the solvent
was contrast matched to the protein. While in SAXS experiments, the peak at approximately 0.035 Å\(^{-1}\) was observed in dried milk powders other than milk solutions which suggested that the peak was due to the higher packing density of casein micelles found in dried milk powder (Mata et al., 2011). A recent resonant soft X-ray scattering study suggested that this feature corresponded to the average separation distance between CCP nanoclusters (Ingham et al., 2015), which was consistent with previous SANS studies (Holt et al., 2003).

With the formation of milk gels by the addition of rennet or tamarillin to milk, the appearance of the peak at approximately 0.035 Å\(^{-1}\) in the milk gels could be attributed to the increment of local packing density of casein micelles and hence CCP nanoclusters in the gel state compared to the solution (Mata et al., 2011). Indeed, similar features have also been observed in SAXS scattering profiles of casein micelles suspension following ultrafiltration, where the casein micelles packing density was greatly enhanced after ultrafiltration (Pignon et al., 2004). Also, the 20% (w/w) milk coagulated with tamarillin demonstrated a lower-\(q\) peak position (0.030 Å\(^{-1}\)) compared to that of rennet sample (0.035 Å\(^{-1}\)). Hence, the CCP nanoclusters separation distance was larger in tamarillin coagulated milk (~210 Å\(^{-1}\)) than that of the rennet sample (~180 Å\(^{-1}\)), according to Bragg’s law (\(d=2\pi/q\)) (Windsor, 1988). These results further confirmed the CLSM observations that rennet-induced denser, smaller voids than tamarillin, which also contributed to the higher complex modulus of milk gel networks made with rennet. In 10% (w/w) milk samples with rennet and tamarillin addition, this peak was less obvious compared to the corresponding 20% (w/w) milk gels. This was especially evident for the 10% (w/w) milk-tamarillin gels, where only a shoulder at approximately ~0.030 Å\(^{-1}\) could be observed (Figure 7.4C). This could be due to the packing density of casein micelles, and hence CCP nanoclusters, in 10% (w/w) milk, with rennet and tamarillin addition being much lower than that of the corresponding 20% (w/w) milk samples.

### 7.4 Conclusion

SDS-PAGE demonstrated that tamarillin exhibited a broader casein hydrolysis than rennet, when added to 1%, 10% and 20% (w/w) milk samples prepared in D\(_2\)O. In small deformation rheological measurement, the gelation kinetics of the high concentration milks (20% w/w) was faster than that of the low concentration milks (10% w/w) and the complex modulus \(G^*\) value of the high concentration milk gels were higher than that of the low concentration milk gels. These results could be explained by the higher number and density of casein micelles at higher
milk concentrations, which would result in reduced distances between the casein micelles, and the formation of more protein-protein bonds in the gel networks. Typical strain-hardening behaviour of all the milk gels formed by tamarillin and rennet was observed under large shear deformation conditions. Compared to low concentration milk gels, a denser protein network was observed in the micrographs of the high concentration milk gels induced by both rennet and tamarillin. However, the tamarillin-induced gels showed more porosity and larger voids than the rennet-induced gels, most probably due to the higher extent of casein hydrolysis. Rennet-induced gels were found to have a denser structure than those treated with tamarillin; this was reflected in the higher elasticity but more brittle behaviour of these milk gels. Large-scale structure, as probed by USANS, of both rennet- and tamarillin-induced, 10 and 20% (w/w) milk gels had similar morphologies of milk gel networks. However, for the 20% (w/w) gels, at smaller scales, SAXS revealed a peak in the tamarillin-induced gel spectra at 0.030 Å⁻¹ and in the rennet-induced gel spectra at 0.035 Å⁻¹. These peaks were associated with the distance of the CCP nanoclusters in the milk gels, providing evidence that the protein network of rennet-induced milk gels (20% w/w) were denser than their tamarillin counterparts. Finally, the differences found between rennet- and tamarillin-induced milk gels may affect the texture properties of cheese, thus offering the possibility for the development of new cheese varieties with different texture characteristics.
Chapter 8

8. Conclusions and future work
8.1 Conclusions

The overall objective of this research was to investigate the coagulation of reconstituted skim milk by the addition of a tamarillo protease, in a crude and purified form. The effect of this protease was compared to rennet, a conventional protease used in cheesemaking. The extent of hydrolysis of the individual caseins by the purified tamarillo protease and rennet was also studied; particularly, their hydrolysis effects on $\kappa$-casein. The research work focused on milk aggregation and casein hydrolysis, since milk aggregation (coagulation) is one of the first steps in cheesemaking and the hydrolysis of caseins plays an important role on cheese quality. This tamarillo protease, which has not been identified previously, will be refereed to as tamarillin.

In Chapter 4 the preparation from Laird’s Large tamarillo fruit of both a crude enzyme extract (an ammonium sulphate (AS) protein precipitate) and a purified protease (using AS followed by ion-exchange chromatography) was described. The purified protease with the highest caseinolytic activity was collected and indentified using SDS-PAGE, which indicated that the molecular weight was approximately 70 kDa (under non-reducing conditions) and 23 kDa (under reducing conditions). The stability and activity of the purified tamarillo protease under different environmental conditions were studied. The results showed that purified tamarillo protease had a wide range of thermal stability, up to 60°C, and the optimal activity of this protease was also at the same temperature of 60°C. In addition, the purified tamarillo protease remained stable between pH 7 and 11, with an optimal activity in the alkaline range, at pH 11. This alkaline optimum pH is very rare for plant proteases, and to the best of our knowledge only serine proteases from Cucumis trigonus Roxburghi (Asif-Ullah et al., 2006) and Solanum dubium dubiumin (Ahmed et al., 2009a) were reported to have an optimal activity at the same pH value (pH 11). The caseinolytic activity of tamarillo protease could only be strongly inhibited in the presence of phenylmethylsulfonyl fluoride (PMSF), a typical serine inhibitor. This indicated that the tamarillo protease was a serine protease. Except for PMSF, the tamarillo protease exhibited stability under most of the investigated environmental factors, such as organic solvents and metal ions. However, the activity of tamarillo protease was strongly inhibited when Hg$^{2+}$ was present. According to Ahmed et al. (2009a) and Moriyama et al. (1998), serine proteases activity is inhibited by Hg$^{2+}$ due to the presence of cysteine residues close to their active site. De novo protein sequencing, a method to study proteins when the genome has not been sequenced (Sun et al., 2010), identified amino acid sequences within the purified tamarillo protease that were highly matched to a subtilisin-like protease. Overall this chapter helped to demonstrate that the tamarillo protease was a subtilisin-like serine
protease with high stability under wide environmental conditions, and with high optimal pH and temperature activity.

Chapter 5 investigated the hydrolysis of 10% (w/w) reconstituted bovine milk by the crude protein extracts (AS precipitates) from the Laird’s Large and Amber tamarillo fruits, in comparison to rennet. The proteins in these crude extracts and their proteolytic abilities, determined using 10% (w/w) milk as substrate, were studied by SDS-PAGE. Results showed that for the crude protease extracts from both tamarillo varieties there were three main protein bands present in the SDS-PAGE gel at ~75 kDa, ~30 kDa and ~14 kDa. In addition, while rennet mainly hydrolysed κ-casein, the proteases from tamarillo hydrolysed all the caseins, with hydrolysis being observed as early as 15 min after the addition of tamarillo extract. The pH effect on the activities of the tamarillo extracts and rennet were investigated. Similar results to Chapter 4 were obtained showing that the caseinolytic activity of these plant extracts increased when the pH was increased to 11. The properties and microstructures of milk gels induced by the tamarillo protease extracts and rennet were investigated using rheology and observation by Confocal Scanning Laser Microscopy (CLSM). Microstructures of milk gels were obtained at 1, 4 and 24 h after the addition of enzymes and incubation at 30°C. After 1 h gelation, the milk gels induced by rennet have less porosity than those gelled by the addition of tamarillo extracts, due to the broader hydrolytic activity of the tamarillo extracts on caseins. However, after 4 h, the porosity of all enzyme-induced gels became similar. After 24 h incubation nearly all the caseins had been further hydrolysed by both rennet and tamarillo extracts although to different degrees. The properties of the gels induced by the two tamarillo extracts and effect on $t_{ag}$ (milk aggregation time) under different pH values or temperatures were also investigated and compared to those obtained by the addition of rennet. The results showed that at the initial stage of milk aggregation, the elastic modulus $G'$ of milk gels induced by tamarillo extracts increased rapidly when compared to rennet milk gels. This was assumed to be due to the broader hydrolysis of the bovine caseins by the tamarillo extracts. In addition, either pH or temperature did significantly influence $t_{ag}$, in the case of both rennet and the tamarillo extracts. For all coagulants, increasing temperature or decreased pH resulted in reducing $t_{ag}$. Especially, at 20°C there was no milk aggregation, even after 3 h after the addition of rennet, while the tamarillo extracts-induced gels were formed within 2 h.

Chapter 6 was dedicated to the hydrolysis of individual caseins, namely α-, β-casein, and κ-casein, by the purified protease from the Laird’s Large tamarillo fruit. Purified protease from
tamarillo fruit exhibited broader hydrolysis on bovine caseins, when compared to rennet. Compared to α- and β-casein, κ-casein was more susceptible to the action of the purified protease as the hydrolysis of this casein was observed already 15 min after the addition of the tamarillo protease. Moreover, the tamarillin also exhibited high proteolytic activity on both α- and β-casein. According to SDS-PAGE and LC-MS results, a major peptide was generated from κ-casein under the action of the tamarillin. The molecular weight of this major peptide was 14,289.7 Da, which corresponds to the amino acid fraction 1-123 from the C-terminal of the κ-casein primary sequence. The cleavage site of this peptide fragment was confirmed by TOF-MS to be at Asn_{123}-Thr_{124}. This is the first time that a target bond on κ-casein located at Asn_{123}-Thr_{124} by a plant protease has been identified. This cleavage site may accelerate the milk-clotting and may contribute to the further elucidation of the milk-clotting mechanism.

Chapter 7 investigated milks (1%, 10% and 20% w/w) reconstituted in D_{2}O hydrolysed by the action of tamarillin or rennet, and the properties of the resulting milk gels (10% and 20% w/w). According to SDS-PAGE results, the tamarillin exhibited similar hydrolysis on κ-casein to rennet, while it had a broader hydrolysis specificity on α- and β-casein. The whey proteins in the milks (α-lactalbumin and β-lactoglobulin) were less susceptible to the action of either rennet or tamarillin. Similar results were also obtained by CLSM, which showed that the microstructure of gels induced by the tamarillin were more porous than the gels induced by rennet. Small deformation rheological measurements, showed that the high concentration milk (20% w/w) gels displayed faster gelation kinetics and higher complex modulus G* than that of low concentration milk (10% w/w) gels. This was presumed to be because the high concentration milk contained larger numbers and a higher density of casein micelles, which resulted in more protein-protein bonds formed in the gel network and the reduced casein micelle-casein micelle distance. Large shear deformation rheology unravelled the strain-hardening behaviours of all the milk gels formed by the tamarillo protease and rennet. Compared to tamarillo protease-induced milk gels, rennet-induced milk gels had a denser structure and less porosity, which was reflected in the rennet-induced milk gels having a higher elasticity but more brittleness than those treated by tamarillo protease. Ultra-Small Angle Neutron Scattering (USANS), which characterizes large scaled gel structures (in the μm range), showed that both 10% and 20% (w/w) milk gels-induced by the tamarillo protease and rennet exhibited similar gel network morphologies. However, Small-Angle X-ray Scattering (SAXS),
revealed that the rennet-induced milk gels have a denser protein network than that those made by the addition of the tamarillo protease.

8.2 Future works

Building on the findings of this thesis, and given that the tamarillo protease tamarallin has not been investigated before, several future works could be suggested:

Microstructure and textural properties of milk gels obtained by the addition of tamarillin

In Chapter 5 and Chapter 7 the microstructure of the milk gels induced by rennet or tamarillo protease were observed using CLSM. While CLSM showed the difference between the milk gels; i.e. their porosity, this microscopy technique does not give any information on the milk gels finer structure, such as the casein micelle. Scanning electron microscopy (SEM) could be used to further investigate the structure of these milk gels. Several studies (Kalab and Harwalkar, 1973; Gastaldi and Lagaude, 1996; Renault et al., 2000) used SEM to investigate milk gels and the state of the casein micelles in milk gels, so this technique could be used to study the microstructure of the milk protein networks and casein aggregates in milk gels obtained by the addition of the tamarillo protease. Alternatively, other microscopic method such as Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) should be also considered.

Application of tamarillin in cheesemaking

According to the results obtained in Chapter 4, similarly to other plant proteases, tamarillin exhibited a broad specificity for the hydrolysis of caseins. If applied to cheesemaking, hydrolysis and especially the residual activity of tamarillin in the curd, should be controlled to avoid generating bitter peptides and reducing the cheese yield. The parameters which may affect the hydrolysis and residual activity of this protease in cheese curd are pH and temperature. Because the optimal pH of tamarillin is in the alkaline range (Chapter 4), acidic pH might be considered to inhibit the activity of tamarillin. Temperature is also an important factor (Chapter 4 and 5), and it might be used to control tamarillin hydrolysis and milk gel formation. Note that the addition of food-grad additives might offer a way of controlling tamarillin activity. For example, whey proteins have been reported to inhibit the residual activity of rennet in milk (Kosikowski and Mistry, 1997).
Sensory test of cheeses made with tamarillin

Compared to rennet, most of the proteases from plant sources, such as papain, ficin and bromelain, display either broad or nonspecific hydrolysis of caseins resulting in bitter flavours, low yield of cheese, and can affect the texture of the cheeses (Garg and Johri, 1994; Jacob et al., 2011). It could be worth producing model cheeses with tamarillo protease and investigating their sensory properties. Naser and Hamid (2016) has conducted sensory evaluation on cow or goat milk white soft cheese induced by an protease isolated from the seeds of sunflower (*Helianthus annuus*) or rennet. The sensory evaluation including colour, flavour, texture and saltiness based on the method of Larmond (1977). The results showed that the milk source can significantly affect the colour and flavour of white soft cheese, which may be due to the variation of fat content in milk (Carpino et al., 2004). In addition, there was a highly significant difference between the plant coagulant and rennet with regard to colour, flavour, texture and saltiness. This might be because plant coagulants have a broader hydrolysis of caseins resulting in bitter flavours and texture defects (Roseiro et al., 2003). Moreover, Nuñez et al. (1991) found that La Serena cheese (soft cheese made with ewe milk), made by a plant coagulant from *Cynara cardunculus*, had more pronounced texture and higher flavour quality than that made by rennet. Therefore, soft or semi-hard cheese could be produced by the addition of tamarillo protease. Sensory evaluation, which would include colour, texture, flavour, taste and saltiness, could then be compared with rennet-induced cheese. Based on results from the sensory evaluation, process conditions such as temperature, pH, salt and aging time could be further adjusted to control the proteolytic activity and residual activity of tamarillo protease. Other cheeses which are usually not produced by rennet could also be investigated.

X-ray crystallography study of tamarillin

The characterisation of tamarillin was conducted in Chapter 4, which showed that this protease was a serine protease with subtilisin-like specificity. Based on these results, it would be of interest to investigate the basic molecular mechanisms of this protease. Similar results on proteases isolated from plants have been reported, such as crinumin, a serine protease extract from the plant *Crinum asiaticum* (Singh et al., 2011), and cardosin A, an aspartic protease extract from *Cynara cardunculus*. L. (Bento et al., 1998). By performing X-ray crystallography, the structure of the tamarillo protease could be obtained. According to Patel et al. (2007), protease crystallography can contribute to the understanding of the three-dimensional structural organization and the mechanism of enzymatic action. In addition, the structure-function
relationship and insight into the exceptional stability of the protease could be obtained. Ottmann et al. (2009) crystallized and elucidated the structure of a serine protease from tomato, which was subtilase 3. Based on the understanding of the structure, they have investigated the function of the PA-domain and identified the effect of binding Ca$^{2+}$ ion on its activity and stability. A further investigation of the crystallography of tamarilllin could give a better understanding of the inhibition of the protease activity by Hg$^{2+}$, which was described in Chapter 4.

**Detection of protease in different parts of tamarillo fruit**

In this thesis, the tamarillo protease was isolated from the whole tamarillo fruit. The protease content in skin, pulp and seed should be investigated. Milk-clotting protease, such as extracts from sunflower (*Helianthus annuus*) and albizai (*albizia lebbeck*) seeds (Egito et al., 2007), dubiumin from the seeds of *Solanum dubium* Fresen (Ahmed 2009a) and cardosins A isolated from *Cynara cardunculus* seeds (Faro et al., 1995), were reported to exist in the seeds of these plants. It was thus suggested that a high protein content may exist in the seeds. Determining the amount of the protease from the different parts of the tamarillo fruit will help maximise the extraction process. Further, knowledge of the presence of the tamarillo protease in different parts of the fruit, may offer an opportunity in the use of wastes (skin and seeds) resulting from the tamarillo bio-processing.

**Effect of growth stage and cultivars on tamarillin**

The effects of seasonal variations and growth stages on the chemical composition of tamarillo fruit should be investigated. This study would contribute to the decision of when is the best time to harvest tamarillo fruits which gives the highest yield of protease and protease activity. Similar research has been conducted, for example, Retamal et al. (1987) analysed the chemical composition in young and mature cladodes of *Opuntia ficus-indica*. The highest crude protein values were found in the young cladodes. Tosun et al. (2008) investigated the chemical changes during the ripening of blackberry fruit. Their results showed that significant compositional changes were found in green (unripe), red and black (ripe) fruits, especially protein content which decreased with the ripening of blackberry fruits. The protease content of tamarillo fruits obtained from young (unripe), on the tree (ripe), and from the ground (over ripe) fruits should be analysed.
The protease used in this thesis (Chapter 4 to 7) were obtained from Laird’s Large tamarillo fruits. Protease obtained from Amber tamarillo fruits were also studied in Chapter 5. Other cultivars such as Mulligan should be also studied, as some cultivars might contain higher protease amount.

*Tamarillin as a meat tenderiser protease*

Ficin extract from fig tree (*Ficus carica*) has been suggested for use in meat tenderisation because its proteolytic hydrolysis results in increased muscle protein solubility (Ramezani et al., 2003). Lewis and Luh (1998) stated that compared to papain (from papaya), actinidin (from kiwifruit) has lower hydrolysis activity on myofibrillar proteins with no over-tenderization effect on the meat surface. They also suggested that the extent of actinidin-induced hydrolysis on myofibrillar proteins can be controlled before cooking. Aminlari et al. (2009) investigated the increase in the tenderisation of cattle beef after 2 h incubation at 37°C with actinidin, a sulfhydryl protease from kiwifruit. Because actinidin hydrolysed beef proteins into smaller units, it increased the protein solubility. The increase of the solubility of myofibrillar proteins could result in improving the water holding capacity and tenderness of beef meat (Aminlari et al., 2009). In addition, the hydrolysed protein increased the viscosity of the beef meat emulsions, which resulted in the improvement in the beef meat product texture. Tamarillo protease also exhibited non-specific activity in Chapter 4 and 6, and thus could be considered as a meat tenderiser. A study on the effect of tamarillo protease on meat could be performed on protein solubility and texture.
References


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