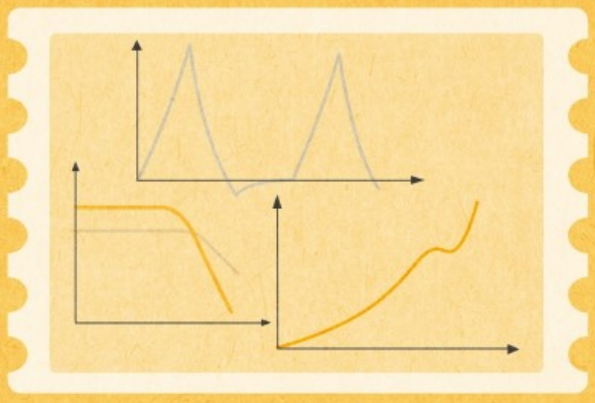
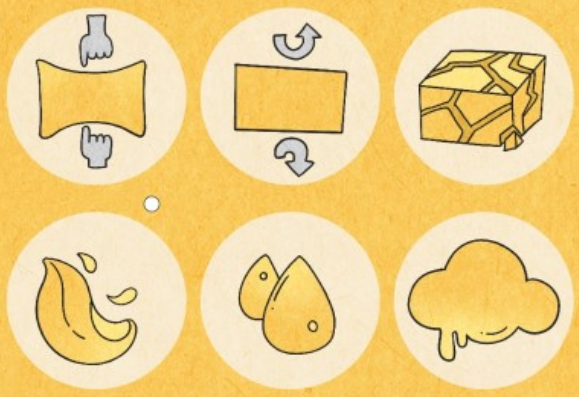
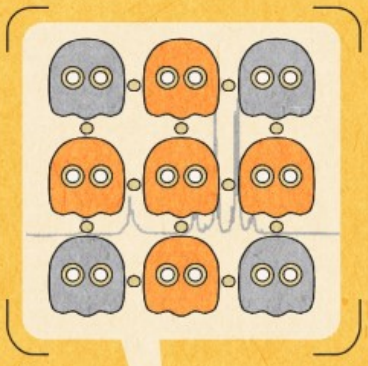
UNDERSTANDING CHEESE TEXTURE

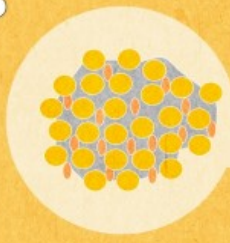
Role of casein hydrolysis and bolus properties

Huifang Cai









Propositions

1. Investigating the hydrolysis of α_{s1}-casein and β-casein provides more information on cheese texture development than determining the hydrolysis of total casein.
   (this thesis)

2. To extrapolate the findings obtained in model cheeses to commercial cheese varieties, the first step is to consider the composition.
   (this thesis)

3. The “echo chamber” has a negative effect for social media users unless personalized algorithms are reduced.

4. Designing healthy food for dysphagia patients and elderly also improves the quality of our own life.

5. The transition to a plant-based diet will be promoted by increasing the awareness of animal welfare.

6. Raising a kitten to be a cat helps to release stress.

Propositions belonging to the thesis, entitled

Understanding cheese texture: Role of casein hydrolysis and bolus properties

Huifang Cai

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UNDERSTANDING CHEESE TEXTURE

Role of casein hydrolysis and bolus properties

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UNDERSTANDING CHEESE TEXTURE

Role of casein hydrolysis and bolus properties

Huifang Cai

Thesis

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Table of contents


CHEESE

Chapter 1

General Introduction

1.1 Why studying cheese texture?

Cheese texture results from a combination of physical properties that are perceived by the senses of touch, sight and hearing during consumption (Brennan, 1984; Delahunty and Drake, 2004). The great diversity of cheese textures, including hard cheese (i.e. Cheddar, Gruyère, Emmentaler), semi-hard cheese (i.e. Gouda, Appenzeller, Maasdam) and soft cheese (i.e. Camembert, Cottage, Quarg), is highly valued by consumers. Although many recipes and cheese-making technologies exist, it is challenging to predict and control the properties of cheese texture due to its complex structure, arising from different interactions among various compounds, including its main component casein. Numerous studies have been carried out to reveal the role of different compositional properties in cheese texture, such as moisture, protein, fat, and salt content, and the pH of the cheese after production. However, the relation between textural properties and specific casein fractions is not well understood yet. Especially during ripening, the caseins are hydrolyzed by different proteases, which consequently changes the matrix structure. A better understanding of the relation between casein hydrolysis and cheese texture is needed to reveal the mechanisms related to texture development during ripening. The relation between structure, texture and sensory perception is still not completely understood. Most studies published in literature focused on the links between cheese properties and some simple texture attributes that perceived at the early stage of mastication, such as hardness, brittleness and elasticity. However, the product properties alone are not sufficient to explain complex texture attributes, such as smoothness, creaminess, and fattiness, since they are mostly perceived after bolus formation (Saint-Eve et al., 2015; Ningtyas et al., 2019; Sala and Scholten, 2022). Unfortunately, rare attempts have been made to link the bolus properties to the perception of cheese texture.

In this thesis, the relation between casein hydrolysis, cheese texture and sensory perception was explored. This chapter provides background information on cheese

composition, structure, texture and sensory perception. Lastly, the aim and outline of the thesis are given.

1.2 Cheese manufacture, composition and structure

1.2.1 Cheese production

In general, the production of most enzyme-coagulated types of cheese follows a similar process with five steps (Fig 1.1). The first four steps are considered as belonging to the dehydration process (Fox and McSweeney, 2004). The fat and casein in milk are concentrated 6-12 fold by a combination of several events: selection, pre-treatment and standardization of cheese milk (step 1), addition of rennet and starter culture to coagulate the milk (step 2), cutting and stirring of the coagulum to enhance whey drainage (step 3), and molding and pressing to shape the curd (step 4). The characteristics and quality of cheese largely depend on this dehydration process, whose degree can be regulated using different techniques. In addition, variations in the composition of the used milk can also be used. During the ripening step (step 5), different biochemical and microbiological events occur and consequently change the flavor, aroma, texture and functionality of the final product. Thus, even though the final composition is already largely determined by the first 4 steps, the flavor and textural characteristics further develop during the ripening period. A specific type of cheese with the desired characteristics (i.e. texture, flavor, functionality) can be produced by applying different cheese-making technologies in different steps. For example, a cooking process (53-55 °C) in Step 3 is usually used for the manufacture of Emmental and Reggiano cheese to generate its unique flavors and texture.

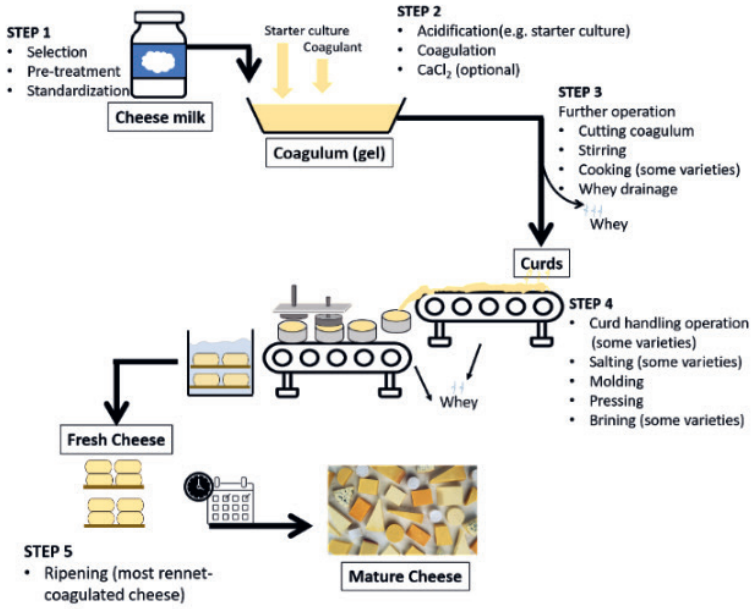

Figure 1.1 Illustrative description of general cheese manufacturing (based on Guinee (2016); Fox et al. (2017b) and Khattab et al. (2019)).

1.2.2 Cheese composition and structure

In general, the cheese composition varies according to the specific type, and the major components are fat, protein and water. After the formation of a coagulum by adding starter culture and rennet into liquid milk, most of the whey protein in milk is expelled during whey drainage step. As shown in Fig 1.2, casein is the main structural component of cheese and is present in the form of a particle network, in which the fat globules, water and other components such as minerals, bacteria, lactose, and peptides are all interspersed (Luyten et al., 1991; Lamichhane et al., 2018). There are four individual types of casein (CN) present in cheese, as α_{s1}-, α_{s2}-, β-, and κ-CN, and α_{s1}- and β-CN represent the majority (> 80%) of the casein fractions (Dalgleish and Corredig, 2012). The spatial arrangement of all the cheese components and the interactions among them determines the structure of cheese, which is influenced by the relative volume fractions of each component, cheese manufacturing procedures,

ripening conditions, and the internal environment of the cheese (e.g., pH, and ionic strength) (Lucey et al., 2003; Lamichhane et al., 2018).

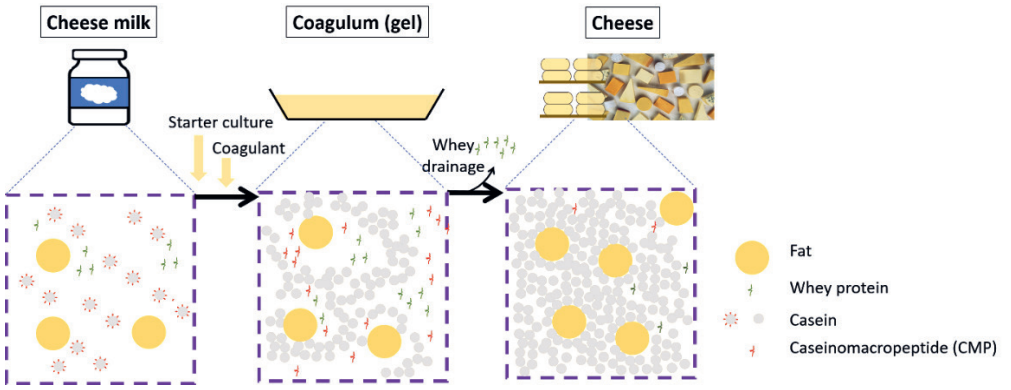

Figure 1.2. Schematic representation of the composition and structure at various stages involved in the production of cheese from milk.

The composition and structure of cheese determine its physical properties (i.e. texture). An important compositional factor is the moisture content on fat-free basis (MFFB) (IDF, 2021), as this distinguishes different cheese varieties based on their firmness: extra hard cheese (MFFB around 30%), hard cheese (MFFB: 49-56%), semihard cheese (MFFB: 54-69%) and soft cheese (MFFB > 67%). Many researches have been carried out to study the physical properties of cheese in relation to the compositional properties, such as moisture content (Hennelly et al., 2005; Everard et al., 2006), protein content (Green et al., 1981; Soodam et al., 2014), fat content (Bryant et al., 1995; Rogers et al., 2010), salt content (Barbano et al., 1994; Sheibani et al., 2015) and pH (Ong et al., 2012). However, knowledge on the role of individual caseins, the main components in the skeleton of the cheese matrix, on cheese texture is still limited.

1.3 Proteolytic enzymes in cheese

During cheese ripening, the occurrence of proteolysis is caused by enzymes from (1) milk (plasmin and other proteinases), (2) coagulant (i.e. chymosin, pepsin, or other

types), (3) other proteinases from starter, non-starter, or secondary cultures and other exogenous proteinases (if applied). A schematic presentation of these proteolytic enzymes involved during cheese production is shown in Fig 1.3. For many cheese varieties, the breakdown of caseins during ripening is mainly induced by coagulant and plasmin (Fox and McSweeney, 1996; Sousa et al., 2001), resulting in the formation of large (water-insoluble) and intermediate-sized (water-soluble) peptides. Subsequently, these products are degraded further by the proteinases from starter and non-starter culture to shorter peptides and amino acids (Sousa et al., 2001; Upadhyay et al., 2004). The initial proteolysis plays an important role in the texture development of cheese, due to the breakdown of the protein network and the structural alterations in the cheese matrix, while the proteolysis at a later stage mainly affects the formation of flavor by releasing peptides and free amino acids. The relation between proteolysis and texture development still remains unclear due to the complexity of the composition and the structure of the cheese matrix. This thesis focused on the effect of two types of proteinases that are related to the development of cheese texture: plasmin and different coagulants. To exclude the possible influence of other exogenous proteases, starter and secondary cultures were not applied in our work.

1.3.1 Indigenous milk proteinases

Plasmin (EC 3.4.21.7), the main indigenous milk proteinase, is predominantly bound to the lysine residues in the molecules of the casein fractions (Grufferty and Fox, 1988) and is mostly (~90%) present as an inactive zymogen, plasminogen (Barrett et al., 1999). Plasmin shows a preference for hydrolyzing β-CN and α_{s2}-CN during cheese ripening (Korycha-Dahl et al., 1983; Bastian and Brown, 1996), as shown in Fig 1.3. Plasmin activity substantially varies between cheese varieties. For instance, plasmin activity is relatively high in cheeses with high curd cooking temperature (53-56 °C), such as Swiss, Parmesan and Emmental cheese (Richardson and Pearce, 1981; Qian and Burbank, 2007; Ardö et al., 2017). This is the result of the low heat resistance of the inhibitor of the plasminogen activator that is also naturally present in milk, and thus the

conversion of plasminogen to plasmin is enhanced (Ollikainen, 1990; Gagnaire et al., 2001; Somers and Kelly, 2002; Fox and Kelly, 2006; Prado et al., 2006). Due to the significance of plasmin in the ripening of many cheese varieties, a number of researches investigated the effect of varying plasmin activity on proteolysis and how this affected cheese functionality (i.e. stretchability and meltability) and flavor development (Farkye and Fox, 1991; Farkye and Fox, 1992; Bastian et al., 1997; Fiona M et al., 1999; O'Farrell et al., 2002; Somers et al., 2002). However, there is limited knowledge on the relation between plasmin-induced hydrolysis of casein and textural changes of the cheese during ripening. A better understanding of this relation is needed to reveal the mechanisms related to texture development during ripening.

Other indigenous milk proteinases, such as cathepsins D, have been widely studied in milk (Hurley et al., 2000; Kelly et al., 2006). These enzymes were reported having a minor proteolytic role in the cheese when compared to plasmin (Larsen et al., 2000; Hayes et al., 2001). In this thesis, we therefore focused only on how plasmin affects the hydrolysis of casein and subsequently the texture of cheese.

1.3.2 Coagulants

The traditional coagulant for cheese production, calf rennet, is obtained from the abomasum of recently born calves, and consists of chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1) (Liburdi et al., 2018; Andrén, 2021). The principal role of the coagulant in milk coagulation is to cleave the C-terminal region from κ-CN at the Phe_{105}-Met_{106} bond. As a result, the net negative charge and steric repulsion of the casein micelles decrease, which impairs the colloidal stability of the micelles (Walstra, 1990; Lucey, 2002), thus leading to aggregation of the casein micelles and eventually the formation of a curd (Dalgleish and Corredig, 2012). Although both chymosin and pepsin have the ability to hydrolyze κ-CN, chymosin shows a specificity for the cleavage at the Phe_{105}-Met_{106} bond higher than pepsin (Figure 1.3). After cheese manufacture, most of the coagulant added to the milk is lost in the whey as a result of syneresis; less than 15% of the

coagulant remains in the curd (Guinee and Wilkinson, 1992). The residual coagulant in the cheese plays a critical role in proteolysis during ripening for most cheese varieties.

The extent and pattern of proteolysis highly depend on the composition and properties of the cheese (e.g. pH, moisture content) and other factors, including the type and concentration of coagulant and the ratio between different proteinases (i.e. chymosin and pepsin) in the coagulant (Exterkate et al., 1997; Michaelidou et al., 1998; Sousa et al., 2001). This is due to the fact that different enzymes have a preference to hydrolyze different caseins, or at different positions within a casein. As is shown in Fig 1.3, chymosin has a preference for hydrolyzing α_{s1}-CN, and to a significantly lesser extent β-CN, while α_{s2}-CN appears to be relatively resistant to proteolysis by chymosin (Sousa et al., 2001; Uniacke-Lowe and Fox, 2017). Pepsin is also known to hydrolyze α_{s1}- and β-CN, but with more cleavage sites than chymosin (Ardö et al., 2017).

Many studies have been carried out to investigate the effect of chymosin-induced hydrolysis on the mechanical properties during cheese ripening (Lane et al., 1997; Watkinson et al., 2001; Bijl et al., 2014; McCarthy et al., 2017). Changes in cheese texture, such as a decrease in hardness, toughness and Young's modulus, have usually been attributed to the hydrolysis of casein (mainly α_{s1}-CN). However, the role of the accompanying occurrence of plasmin-induced hydrolysis on β-CN has been less studied. More knowledge on the link between the mechanical properties and the hydrolysis of individual caseins from both chymosin and plasmin is thus needed.

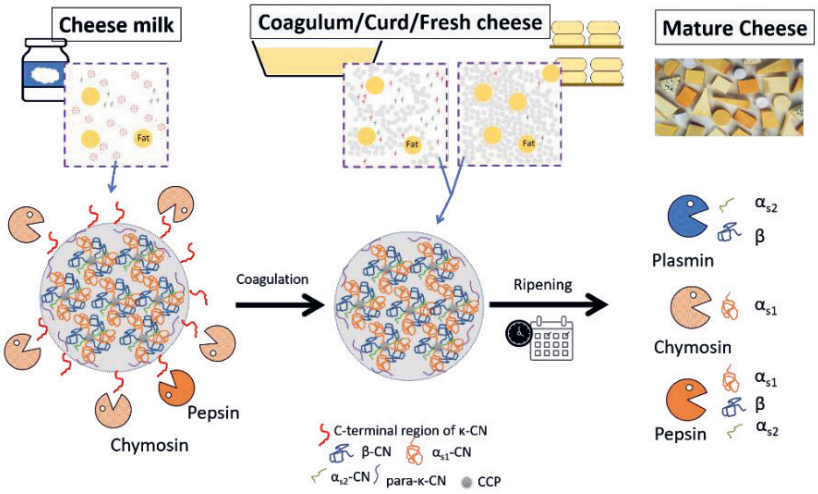

Figure 1.3. Schematic of the hydrolysis of caseins by coagulant (chymosin and pepsin) and plasmin.

1.3.3 Commercial rennet

In the 1950's, cheese consumption increased while the availability of calf rennet decreased, which was partly related to restrictive ethical concerns on the extraction of rennet from young calves (Garg and Johri, 1994; Sousa et al., 2001). Thus, several proteinase from other animals, plants, microbial sources and recombinant rennet have been investigated as potential substitutes (Garg and Johri, 1994; Broome and Limsowtin, 1998). Today, the most used rennet substitute is recombinant rennet (Andrén, 2021). Recombinant rennet is produced via fermentation, by the use of cloning and the expression of genes in bacteria, and recombinant rennet is currently responsible for 55-60% of the global coagulants present in the market (Johnson and Lucey, 2006; Yegin and Dekker, 2013; Andrén, 2021). Meanwhile, traditional calf rennet is still the majority coagulant for most European countries such as Germany, the Netherlands, and France, due to stringent regulations towards genetically engineered foods (Egito et al., 2007).

Nowadays, commercial calf rennet used for milk clotting is extracted from a mixture of grinded abomasum tissues from both calf and adult bovines (Andrén, 2021). This rennet contains 50 - 95 % chymosin and 5% - 50% pepsin (Winwood, 2007; Jacob et al., 2011). To our knowledge, limited studies have been carried out yet to gain insights on the relation between casein hydrolysis by coagulants containing different chymosin/pepsin ratios and the mechanical properties of cheese during ripening. How the chymosin/pepsin ratio influences casein hydrolysis, specifically the hydrolysis of different caseins, and its relation with texture still needs further investigation.

1.4 Cheese texture

We mentioned that cheese texture is highly related to the composition and the structure of cheese. To reveal the mechanisms responsible for the changes in composition and subsequent textural properties, we need to understand how the components within the cheese matrix and the interactions among them affect both the structural organization of cheese on a small length scale and its physical properties on a larger length scale. These properties can be measured by small strain rheology tests and large strain compression tests. The parameters obtained from a compression test are known to better relate to sensory attributes. A better understanding on cheese texture would provide a guideline of how we can control the composition or other characteristics of cheese to produce a desired product, and how we can control the specific sensory profile of cheese as well.

The rheological properties of cheese can be measured instrumentally by the application of a small strain or stress (Fox et al., 2017a). The output variables from these tests (e.g. small amplitude oscillatory shear and creep tests) provide information on how the structural elements respond to an applied strain/stress. Small amplitude oscillatory shear (SAOS) tests give information on the critical strain at which permanent damage or fracturing of the microstructure starts (Fox et al., 2017a). The

storage modulus (G') at the critical strain represents the rigidity of the network at the end of the linear regime. The creep test is usually performed to obtain information on the time-dependent rheological behavior of a material. For this test, a low stress that is insufficient to induce permanent damage or fracture (breaking of bonds between the structural elements) of the microstructure in a short time is applied. When this low stress is applied over a relatively long time, it results in an increasing strain, indicating a gradual failure of the structure. Practical examples of creep occur when curd or cheese is gradually compressed under its own weight, pressed or stacked, e.g. during retailing (O'Callaghan and Guinee, 2004; Ipate et al., 2019).

Texture evaluation based on compression tests are designed to simulate the compression of food between the molars during mastication. The most commonly used methods are the large deformation test and the texture profile analysis (TPA) test (Fox et al., 2017a). The typical stress-strain curve obtained from a large deformation test and the force-time curve from a TPA test are shown in Fig 1.4. Large deformation tests provide information on the Young's modulus (stiffness) of the cheese in the linear region (A-B), and on its fracture strain (brittleness, ε_{fr}) and fracture stress (hardness/firmness, σ_{fr}) at point C. The area underneath the stress-strain curve before point C is also used to indicate the toughness of cheese. In addition, the structural elements in the cheese matrix (e.g. intact caseins and peptides) start to move and rearrange to resist further deformation (Huc et al., 2014; Joyner et al., 2018), leading to strengthening of the material, known as strain hardening behavior, seen as a more than linear increase in the stress as a function of strain (Bast et al., 2015; Sharma et al., 2018). In this case, the strain hardening index (SHI) is used to describe the rate of the rearrangements of structural elements occurring outside the linear region. Strain hardening index (SHI) is calculated according to the empirical equation (Kokelaar et al., 1996; van Vliet, 2008) as:

$$\sigma = a \cdot \varepsilon^{b} \quad (1)$$

where σ is the stress, a is the strength coefficient (Pa), ε is the deformation strain (-), and b is the strain hardening index (-). In this thesis, Eqn. 1 was fitted to the stress-strain data over the strain range before fracture, which was from 0.02 to 0.6.

The TPA test gives information on the response of cheese to two-bite deformation, and also allows a description of parameters that are important during mastication. Based on the force-time curve from a TPA test (Fig 1.4b), we can obtain different parameters, as hardness (the maximum force of the 1st compression), resilience (A1/A2), cohesion ((A4 + A5) / (A1 + A2)), adhesiveness (A3), springiness (distance 2/distance 1), gumminess (hardness × cohesiveness) and chewiness (hardness × cohesiveness × springiness), which usually correlate to the sensory attributes with the same name.

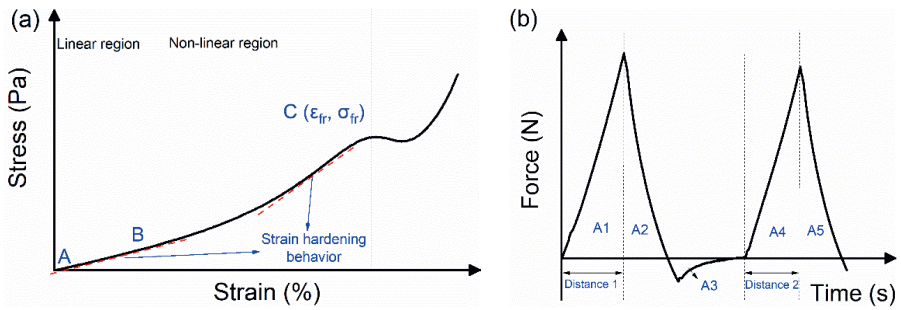

Figure 1.4. Examples of the stress-strain curve from a large compression test (a) and the force-time curve from a TPA test (b).

1.5 Sensory perception

Clear correlations between instrumental physical properties and sensory attributes seem to be limited to the attributes perceived at the first bite (Foegeding and Drake, 2007). In general, attributes as hardness and firmness have been linked to mechanical properties such as fracture stress, fracture strain, Young's modulus and work to fracture (Foegeding et al., 2003; Everard et al., 2006). These attributes have also been linked to the compositional properties of cheese (Brown et al., 2003; Everard et al., 2006; Chen and Opara, 2013). For example, it has been widely reported that a higher

dry matter content results in a stiffer and harder cheese, which also leads to higher perceived hardness and firmness (Xiong et al., 2002; Adhikari et al., 2003). Such a high dry matter content often leads to a lower fracture strain as well, which is linked to a more brittle sensation. However, for many foods, the mechanical properties are not sufficient to explain more complex texture attributes such as smoothness, creaminess and fattiness (Saint-Eve et al., 2015; Ningtyas et al., 2019). The main reason is that these attributes are perceived at later stages of oral processing, during the chew down and swallowing phase (Foegeding et al., 2015; Devezeaux de Lavergne et al., 2017).

1.5.1 Oral processing

Oral processing transforms the food into a bolus, which is accompanied by various modifications of the food properties. For example, the structure is broken down by mastication and saliva is incorporated. In the case of cheese, fat is also released from the cheese matrix with changes in temperature (Chen, 2009; Foegeding et al., 2011). These modifications affect the physical properties of the food. Texture, therefore, is not simply related to the initial properties of a product, but also to the properties of the food bolus. Recently, many studies have been performed to link sensory perception to the properties of the bolus. Seo et al. (2007) indicated that the texture attribute slipperiness of liquid products, such as yogurt, tomato juice, ketchup and mustard, was related to the rheological properties of the bolus during swallow. In the case of emulsion-filled gels, Devezeaux de Lavergne et al. (2015) found that these gels were perceived as either creamy or grainy at the end of oral processing depending on the specific break down pattern of the gel. The attribute creaminess was associated with a high bolus flowability, while graininess could be explained by a high number of particles within the bolus. For solid foods, Rizo et al. (2019) reported that the fibrousness of commercial cooked ham was linked to the number of particles in the bolus, which was used to represent the degree of fragmentation during mastication. These studies have delivered important information that complex texture attributes are more related to the properties of the bolus than to the properties of the food itself.

However, there is little knowledge about the effect of oral processing on the perception of cheese texture and to what extent the bolus properties are related to different complex texture attributes. To fully understand how the structural changes in the cheese arising from mastication affect the specific sensory profile, further research with consideration of oral processing and bolus properties is required.

1.5.2 Bolus lubrication

In the last decades, the role of lubrication on sensory perception of complex texture attributes has been highlighted. For example, it has been reported that low friction coefficients of food were related to higher scores for creamy, fatty and slippery in different products such as milk, mayonnaise, yogurt and food gels (Malone et al., 2003; Weenen et al., 2003; Tournier et al., 2007; Chojnicka-Paszun et al., 2012; Liu et al., 2016). Also in the case of the bolus, lubrication plays an important role. The degree of lubrication is described as one of the main parameters contributing to bolus properties (Hutchings and Lillford, 1988). Commonly, the degree of lubrication of the bolus increases during oral processing, with incorporation of saliva. Saliva shows good lubrication due to the presence of salivary proteins (Bongaerts et al., 2007). Besides saliva incorporation, bolus lubrication can also be increased by the release of fluids, such as oils, from the food matrix during oral processing. This was observed in model dairy products and emulsion-filled gels where high fat/serum release showed a lower friction (higher lubrication) of the bolus (Drago et al., 2011; Devezeaux de Lavergne et al., 2016). Saliva and other fluids glue the particles in the bolus together, which is required before swallowing. The number, size, shape and deformability of particles in the bolus also play an important role in the bolus properties (Chojnicka et al., 2009; Fuhrmann et al., 2020). Especially the number and size of particles determines the amount of fluid that required to glue the particles and to form safe-to swallow bolus (Devezeaux de Lavergne et al., 2017; Gray‐Stuart et al., 2017).

Therefore, even though food properties are able to provide information on the perception of simple texture attributes, understanding the bolus properties enables to

unravel insights into complex texture attributes that are perceived at later stages of mastication, such as creaminess, smoothness and fattiness. It is worthwhile to understand the mechanisms behind the perception of cheese texture, which is beneficial for the improvement of existing products and the development of new products.

1.6 Aims and outline of the thesis

Cheese texture highly depends on the composition and structure of the cheese matrix. However, there is still limited knowledge on how the hydrolysis of specific casein fractions by different proteolytic enzymes affect the structure of cheese and its physical properties, and how the corresponding textural attributes affect sensory perception. In this thesis, we **aimed** to gain insights into the effect of structural changes arising from proteolysis of specific casein fractions on texture development and the role of bolus formation during oral processing in the perception of complex texture attributes. The thesis outline is given below and schematically presented in Fig 1.4.

To focus on the correlations between the casein hydrolysis and cheese texture, our studies were performed in non-fat model cheese to exclude the possible extra effects from fat variations (Chapters 2-4). In **Chapter 2**, we investigated the effect of added plasmin on casein hydrolysis and the subsequent effect on the textural properties. A relation between plasmin-induced proteolysis and textural changes was established. In **Chapter 3**, we assessed the role of plasmin and chymosin in the textural changes during model cheese ripening. To gain information on structural properties, different rheology measurements were performed as well. In **Chapter 4**, we studied the proteolysis as a result of coagulant addition with different chymosin/pepsin ratios and the effect on different physical properties . A traditional rennet was also assessed as comparison. These chapters together highlight how the hydrolysis of specific casein

leads to changes in structural and textural properties. Next, in **Chapter 5**, we investigated how certain textural properties affect different sensory attributes using commercial cheeses, taking into account the properties of the cheese bolus after oral processing. Correlations between bolus properties (composition and physical properties) and complex texture attributes were explored. In **Chapter 6**, a general discussion is provided to connect the main findings of Chapter 2-5 and to present a view on the field of cheese texture. The results of this thesis may guide scientists and cheese manufactures to better understand and further help to engineer cheese texture. For example by modifying the casein hydrolysis, which can be achieved by modulating the content and the ratio of different proteolytic enzymes. Additionally, this study advances the understanding on cheese texture perception, which is valuable for improving the properties of existing products and designing new products.

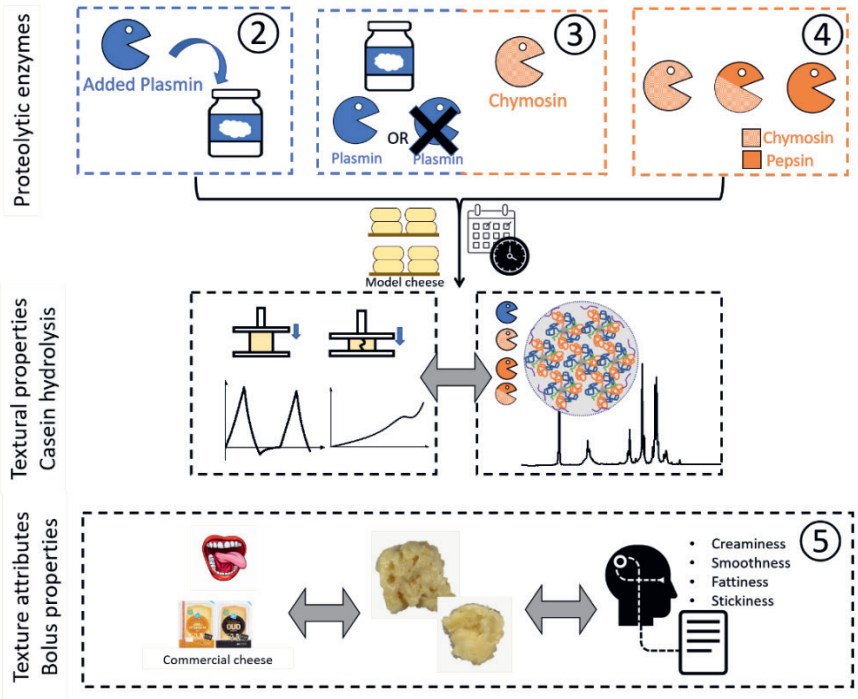

Figure 1.5. Schematic depiction of the outline of the chapters in this thesis. Numbers indicate corresponding chapters.

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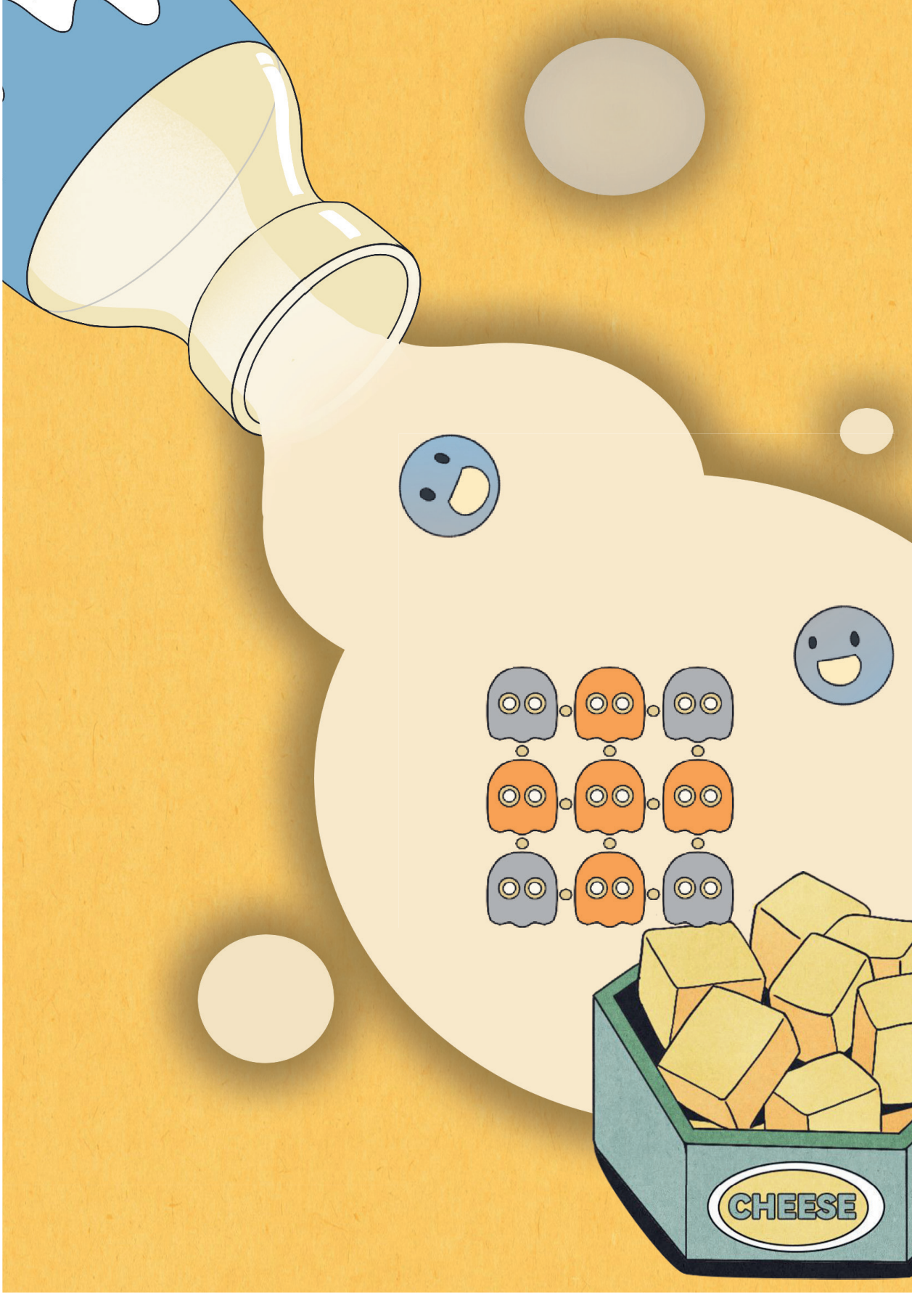
CHEESE

Chapter 2

Effect of plasmin on casein hydrolysis and textural properties of model cheeses

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ABSTRACT

The hydrolysis of casein influences the properties of casein network and the texture of final cheese product. Plasmin, a primary indigenous milk enzyme, plays an important role in the hydrolysis of casein and the texture development of many cheeses with a high cooking temperature, such as Parmesan and Emmental. However, there is still limited information about the relation between plasmin-induced casein hydrolysis and the texture development of cheese. The aims of this study were (1) to study the influence of the added plasmin on casein hydrolysis of rennet-induced model cheeses, and (2) to relate casein hydrolysis to textural changes. Four batches of model cheese with different concentrations of added plasmin (0-1 μL/g milk) were prepared, which were stored for 12 weeks at 16 ˚C.

Our results show that the addition of plasmin had significant effect on the degree of casein hydrolysis. As a result, cheeses with different addition of plasmin showed different textural properties. With increased plasmin concentration, Young's modulus, hardness, resilience and cohesion decreased, while brittleness increased. All textural properties showed linear correlations with the degree of casein hydrolysis, and logarithmic correlations with the percentage of intact casein fractions. At the beginning of proteolysis, only slight changes on textural properties were found, although a substantial part (40-60%) of the casein fractions was already broken down. When proteolysis progressed, the protein network significantly became weaker and consequently led to noticeable textural changes. Model cheese became softer, more brittle and less elastic. The knowledge gained from this study represents a potential tool to control cheese texture by modifications of the activity of the enzymes present in the curd, regulating the extent and the pattern of casein hydrolysis. This would allow the optimization of existing products and the creation of new ones.

2.1 INTRODUCTION

Plasmin, a primary indigenous enzyme in milk (Fox and Kelly, 2006), is predominantly bound to the lysine residues in the molecules of the casein fractions (Grufferty and Fox, 1988) and is mostly (~90%) present as inactive zymogen, plasminogen (Barrett et al., 1999). The transformation from plasminogen into active plasmin occurs with the cleavage of specific peptide bonds by plasminogen activators (PA's), which are present in many animal tissues and fluids, including the mammary gland and milk (Korycha-Dahl et al., 1983). Plasmin concentration in bovine milk can vary due to several factors, including cow breed, season, feed, duration of dry periods, lactation stage and mastitis infections (Benslimane et al., 1990; Bastian and Brown, 1996; de Vries et al., 2015; Guerrero et al., 2015; de Vries et al., 2016). Such natural variations in concentration have been suggested to influence the milk renneting properties and the characteristics of the curd, since plasmin hydrolyzes a certain amount of casein in the milk (O'Keeffe et al., 1982; Okigbo et al., 1985). Mara et al. (1998) showed for instance that extensive casein hydrolysis by plasmin significantly increased milk coagulation time and reduced final curd firmness. Srinivasan and Lucey (2002) found similar results and demonstrated that the microstructure of rennet-induced gels was drastically altered with a high casein degradation (>40%) by plasmin.

During cheese ripening, caseins are hydrolyzed in a preferential way by different proteases, which influences both casein network rearrangements and related textural changes (Fox and Stepaniak, 1993; Sousa et al., 2001; Tovar et al., 2004). Plasmin is one of these proteases and readily hydrolyzes β- and α_{s2}-casein, while slowly attacking α_{s1}-casein (Farkye, 1995). Some studies have shown that plasmin activity in cheese highly depends on the processing steps during cheese-making, such as milk heat treatment, curd cooking and curd washing (Voigt et al., 2012; Vélez et al., 2015). Benfeldt et al. (1997) showed that inactivation of plasmin by heat treatment of the cheese-milk at temperature higher than 80 °C resulted in a reduced degradation of β-casein and α_{s2}-casein during the ripening of semi-hard cheese. In many cheeses with

high curd cooking temperature (53-56 °C), such as Parmesan and Emmental (Fox, 1993; Qian and Burbank, 2007), plasmin plays a relatively important role in casein degradation and texture changes due to the extensive denaturation of chymosin. The role of plasmin in the ripening of these cheeses is also the result of the different heat resistance of plasmin, plasmin inhibitor and plasminogen activators (Ollikainen, 1990; Gagnaire et al., 2001; Somers and Kelly, 2002; Fox and Kelly, 2006; Prado et al., 2006). Vélez et al. (2015) found that curd washing in semi-hard Pategrás cheese could eliminate the inhibitors of plasminogen activators, as those activators are present in the serum, while the plasminogen activators are associated with casein micelle (McSweeney, 2004). Thus, plasminogen activation was enhanced, resulting in a high plasmin activity. This study did not provide information on specific caseins hydrolysis and textural development. The researches mentioned here above have clearly demonstrated that plasmin activity in cheeses is important for the hydrolysis of casein. However, there is still limited knowledge on the relation between plasmin-induced hydrolysis of casein and textural changes of the cheese. A better understanding of this relation can be used as a tool to control and optimize cheese texture by modifying plasmin-induced hydrolysis.

The objective of this study was (1) to study the influence of the concentration of added plasmin on casein hydrolysis of a rennet-induced model cheese and (2) to relate casein hydrolysis to the changes in cheese textural properties. To vary plasmin concentration, we added purified plasmin to the milk to prepare the model cheeses. During 12 weeks of storage, plasmin activity, casein hydrolysis (protein fraction soluble at pH 4.6 and intact casein fractions), textural properties (Young's modulus, hardness, fracture strain, resilience and cohesion) and other compositional parameters (pH and dry matter) of the model cheeses were studied. We used skim milk to eliminate any additional effect of fat on texture, and a high pH of 6.2 was chosen, as it was a relatively more favorable for plasmin activity than for chymosin activity.

2.2 MATERIALS AND METHODS

2.2.1 Materials

All experiments were carried out using the same batch of pasteurized skimmed milk purchased from a local supermarket ('magere melk', Jumbo brand, the Netherlands), which according to the supplier contained 3.6% protein and less than 0.1% fat. Commercial salt (Salina Salt, the Netherlands) was used to brine the model cheese. CHY-MAX® M 1000 (1000 IMCU per mL; Chr. Hansen, Denmark) chymosin was used for milk renneting. Bovine plasmin (EC 3.4.21.7, Roche 1060237001, with an activity of 5 U/mL) and protein standards (β-CN and α_S-CN) were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Biophen CS-41(03) chromogenic substrate (Hyphen biomed 229041; Hyphen Biomed, USA) was used for plasmin activity determination. All other chemicals were obtained from Sigma Aldrich (Sigma-Aldrich, USA). Milli-Q water (water purified with an ultrapure water system, PURELAB Ultra, ELGA LabWater, High Wycombe, UK) was used for all solution preparations.

2.2.2 Model cheese preparation

Model cheeses with added bovine plasmin (0 µL, 0.4 µL, 0.6 µL, 1.0 µL /g milk) were produced. The procedure to prepare the model cheeses (Fig 2.1) was based on a general semi-hard cheese production process (O'Mahony et al., 2005; Cipolat-Gotet et al., 2013; Velázquez-Varela et al., 2018). The reproducibility of this procedure in terms of properties of the obtained model cheeses (moisture content, yield, pH and textural characteristics) was confirmed with several pre-tests. For each experimental batch, a reference model cheese (0 µL plasmin/g milk) was also made on the same day to evaluate the standard deviation caused by factors related to the cheesemaking process (Figure S2.1).

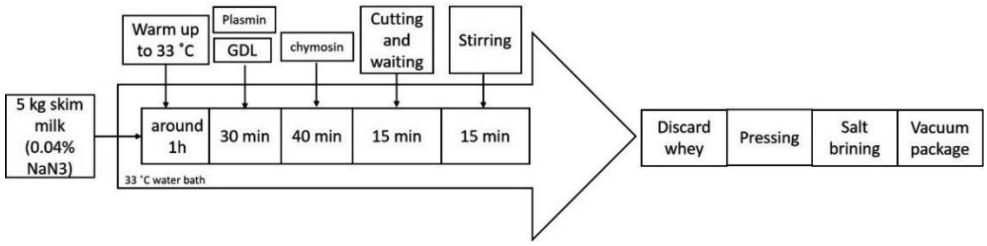

Figure 2.1. Graphic illustration of the production process used to prepare the model semi-hard cheeses.

Briefly, 5 kg skimmed milk (with 0.04 % NaN_3, w/w) was heated to 33 °C in a water bath. Plasmin was added to the milk and mixed thoroughly. Next, 0.3% (w/w) GDL (D-(+)-glucono-delta-lactone) was added to reduce the pH to 6.20-6.30 in 30 min. Then, chymosin (20 ICMU/kg milk) was added to the milk, and the renneting process was allowed to continue for 40 min. The curd was cut into 15×15×15 mm^3 cubes by using 3 custom-made knives, followed by a 15 min waiting step. Subsequently, the curd was gently stirred for 15 min by means of an overhead stirrer equipped with two square blades (66x66 mm) at 30 rpm. The curd was transferred into 3 cylindric cheese moulds (with a diameter of 8.5 cm and a height of 9.5 cm) for shaping and pressing. Each mould was filled with approximately 400 g curd. Each curd were first pressed with a weight of 1 kg for 2 h and then with a weight of 2 kg for another 1 h. The residual curd without pressing steps was collected for further analysis to check the degree of casein hydrolysis during cheese-making. After pressing, the separated fresh whey was collected for further analysis and the obtained 3 cheeses were immersed in a brine with 25 % (w/w) salt for 45 min. Then, each model cheese was wiped dry with lab paper and cut into 4 blocks. Each cheese block was vacuum packed in plastic bag and stored at 16 °C for a period of 12 weeks. Two bags of samples were randomly chosen and analyzed 1, 3, 5, 7, 9, 12 weeks after cheese preparation as described in the next sections. At each analysis time point, the separated whey was collected in a tube for analysis of plasmin activity.

2.2.3 Determination of plasmin activity, dry matter and pH

The determination of plasmin activity was carried out according to the spectrophotometric method from Rollema et al. (1983); Rauh et al. (2014). Milk and fresh whey were diluted in a ratio of 1:7 (v/v) with an assay buffer (pH 7.4) containing 0.08 mM tris-hydroxy-(methyl)-aminomethane, 0.06 mM KCl, 0.03 mM EDTA-2Na.2H_2O, 0.14 mM EACA (ε-aminocaproic acid), and mixed for 15 min to dissociate plasmin from the caseins. Additionally, whey separated from the cheeses during storage was diluted in a 1:70 (v/v) ratio with the same assay buffer, as the plasmin activity was higher. Solid samples (cheese/curd) were ground and then mixed with the same assay buffer (1:70, w/v) for 2 h at 40 ˚C. After incubation with the assay buffer, the solution was filtered through a 0.22 µm filter (Millipore Millex-GP Hydrophilic PES, Darmstadt, Germany). The filtrate were used for further determination of plasmin activity. All the filtrates were prepared in triplicates.

For determination the plasmin activity, duplicates 200 µL filtrates were pipetted into wells of the microtiter plate. The microtiter plate was then equilibrated at 37^{o}C for 15 minutes inside the VersaMax™ Microplate Reader (Molecular Devices LLC, San Jose, USA). After equilibration, 50 µL Biophen CS-41(03) (1mg/ml) was added to one well as a reaction substrate while 50 µL Milli-Q water was added to another well as a baseline. The absorbance (A) was recorded at 405 nm at 37 °C for 30 min. Plasmin activity was determined in triplicates and was calculated as the slope of the absorbance versus time for each measurement.

The dry matter content (0.3-0.4 g cheese, oven drying at 105 °C) and pH (1 cheese : 1 water) of the cheese samples were determined according to standard methods (Lynch et al., 1997; Patrignani et al., 2019). All the measurements were conducted in triplicate to obtain an average value with standard deviation.

2.2.4 Determination of casein hydrolysis

2.2.4.1 Degree of casein hydrolysis

Duplicate cheese samples (2.5 g) were ground after addition of 15 mL MilliQ water by means of a mortar and pestle. The mixture was kept at 40 °C for 1 h, then cooled to room temperature. When room temperature was reached, the pH of the mixture was adjusted slowly to 4.6 with a 1 M HCl solution. The mixture was then centrifuged at 4000 × g for 20 min according to a method described previously (Hynes et al., 2004). The supernatant was collected to determine the content of protein soluble at pH 4.6 by the Dumas method, using a factor of 6.38 to convert the estimated nitrogen to protein content. Total protein (TP) content in the cheese was also measured by the Dumas method. The degree of casein hydrolysis was expressed as protein fraction soluble at pH 4.6 and was obtained by equation (1),

$$\text{protein fraction soluble at pH 4.6} = \frac{\text{content of protein soluble at pH 4.6 (\%)} * \text{weight of the supernatant (g)}}{\text{total protein content in the cheese (\%)} * 2.5\text{g}} \quad (1)$$

2.2.4.2 Intact casein fraction by RP-HPLC

Reversed-phase high-performance liquid chromatography (RP-HPLC, Thermo Science^{TM} UltiMate 3000; Waltham, USA) was used to determine the peak areas of the intact casein fractions. Triplicate cheese samples (0.15 g each) were mixed with solution A (0.1 M Bis-Tris buffer, 8 M urea, 5.37mM sodium citrate and 19.5 mM DTT, pH 7.0) at a ratio of 1:30 (w/v) and incubated in a water bath for 2 h at 40°C. Each mixture was vortexed for 10 s every 30 min during incubation to ensure that the aggregates were well dispersed. Then, the mixtures were diluted with solution B (6 M urea in 0.1% TFA aqueous buffer, pH 2.0) at a ratio of 1:3 (v/v) at room temperature. The diluted mixture was vortexed for 10 s and filtered through a 0.2 µm RC filter. Next, RP-HPLC was performed to analyze the fractions of different types of intact caseins.

The chromatographic conditions and elution gradients followed the method described by de Vries et al. (2015), which was based on the method reported by Bobe et al. (1998) and Bonfatti et al. (2008). Casein standards (6 mg/ml β-CN and 6 mg/ml α_S-CN) were also analyzed to confirm the retention time of the different major casein fraction peaks.

An Aeris 3.6 µm Widepore XB-C18 column (250×4.6mm, Phenomenex, Utrecht, the Netherlands) was used for analysis. The chromatograms were analyzed with Chromeleon 7.1.2 software. The residual intact total casein fraction (%) at the different sampling moments was expressed as sum of total casein peak area divided by the total casein peak area of control cheese at week 1, which was set as 100%. To investigate the decrease of specific casein fractions during storage as result of hydrolysis, the relative fraction of intact α_{s2}-CN, α_{s1}-CN and β-CN over time was calculated based on the method described by Bijl et al. (2014). All the results of the intact casein fractions (%) were fixed based on the dry matter.

2.2.5 Texture analysis

For the characterization of the textural properties of cheese samples at different moments during storage, large deformation measurements and texture profile analyses (TPA) were carried out using a Stable Micro Systems TA-TX plus (Stable Micro Systems TA-TX plus, Godalming, United Kingdom) equipped with a 50 mm diameter cylindrical probe (perspex) and with a 5 kg load cell. Cylinders with a diameter and height of 1 cm were cut from the cheeses. For the compression test, the samples were compressed until a strain of 85% at a rate of 2 mm/s. The Young's modulus was defined from the linear region (1-3% strain) of stress-strain curves. From the fracture point, the fracture strain was extracted from week 1 to week 9. Data of fracture strain at week 12 were not determined as the strain-stress curves did not show a clear fracture point even though the samples were fractured after the measurement. The hardness could not be accurately determined by the fracture stress as the error bars were too large. Instead, hardness was determined as the stress at a strain of 40% (hardness_40%), which has also been used by others as a measure of cheese hardness (Ak and Sundaram, 1997; Alvarez et al., 2000). In addition, to mimic the first event of cheese chewing, a TPA test was performed at a rate of 2 mm/s with a strain of 20%. From the obtained TPA curves, resilience (upstroke peak area first compression/downstroke peak area first compression) and cohesion (ratio of the positive force area of the

second compression to that of the first compression) were extracted, since these parameter are particularly related to the sensory perception of cheese at the beginning of mastication (Foegeding et al., 2003; Saint-Eve et al., 2015; Ningtyas et al., 2019).

2.2.6 Statistic analysis

One-way analysis of variance (ANOVA) with Tukey Post Hoc test was used to evaluate the significant differences of obtained results among cheeses, using IBM SPSS Statistics 25 (IBM Corporation, NY, USA). The significance level was set at 0.05. Logarithmic transform functions ($P = a - b * \ln(fx + c)$) were used for correlation fittings between textural parameters (P) and casein fractions (fx), using Origin (Origin® 2018 Graphing & Analysis, Northampton, USA).

2.3 RESULTS AND DISCUSSION

2.3.1 Compositional properties of milk, whey and curd during cheese making

2.3.1.1 Plasmin activity in milk, whey and curd

To check the distribution of plasmin added to milk during preparation of the cheese samples, the activity of this enzyme was determined in milk (collected after adding plasmin and GDL), fresh whey (collected before pressing) and curd (collected before pressing) (Fig 2.2).

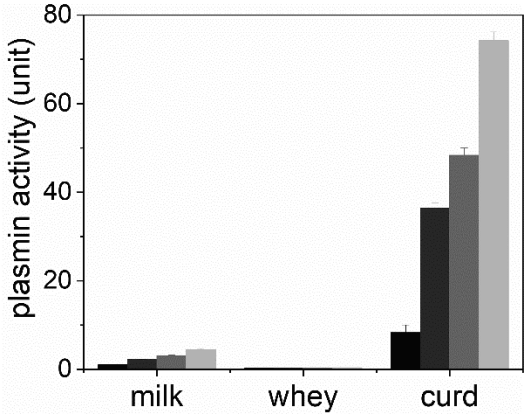

Figure 2.2. Plasmin activity in milk (per ml), whey (per ml), and curd (per g) collected during cheese manufacture. Samples were produced from milk containing 0 µL/g (■), 0.4 µL/g (●), 0.6 µL/g (▲), and 1.0 µL/g (▼) additional plasmin.

The plasmin activity in the commercial milk was 1.00 ± 0.08 unit per ml. As expected, plasmin activity was higher in milk samples with more added plasmin. The values of plasmin activity (unit per ml milk) in samples with 0.4, 0.6, 1.0 µL/g plasmin increased by 1.21, 2.04, 3.42 times, respectively, when compared to sample without plasmin addition. This result confirms that casein is able to bind more plasmin than the amount of plasmin naturally present in milk, as also described by Farkye and Fox (1992). For the sample without plasmin addition, the plasmin activity in the curd was 8.3 times higher than in milk. For the samples with added plasmin, the plasmin activity was measured to be 15.9-16.8 times higher than in the milk. For all cheeses, the same level

of plasmin was detected in the whey for all cheese samples, i.e. around 0.2-0.3 units per ml fresh whey. This loss of plasmin had an impact on the concentration of plasmin from milk to curd for the control sample (without plasmin addition), but had a negligible effect on samples with plasmin addition. Thus, a lower increase factor in plasmin activity was found for samples without plasmin addition compared to those with extra enzyme.

To conclude, the added plasmin tended to bind with casein in the milk. During cheese preparation, most plasmin was retained in the cheese curd, while a low amount of plasmin was lost into the whey. However, this loss was negligible for cheese sample with plasmin addition.

2.3.1.2 Casein hydrolysis and pH change during cheese preparation

As previously reported in literature, added plasmin is able to hydrolyze casein in milk during long incubation times, consequently forming a weak gel and reducing cheese yield (Mara et al., 1998; Srinivasan and Lucey, 2002). To check the occurrence of casein hydrolysis during the sample preparation, casein fraction profiles were analyzed by RP-HPLC in milk (collected after adding plasmin and GDL), fresh whey (collected after pressing) and curd (collected before pressing). Compared to studies in which high concentrations of plasmin (10 µg or 0.1-10 µg /ml milk) were added to milk and long incubation times (range 0.5-8 hours) were applied (Mara et al., 1998; Srinivasan and Lucey, 2002), in our study relatively low concentrations (0-1.0 µL/g milk) of plasmin and a short incubation time (30 min) were chosen. Thus, no extensive casein hydrolysis was observed in these samples during the initial steps of cheese-making (data not shown). The pH value was also recorded during the cheese making process. No significant difference in pH changes was found among the 4 batches of cheese (data not shown). These results indicate that the addition plasmin had no influence on the casein hydrolysis and pH changes during cheese preparation.

2.3.1 Plasmin activity and other compositional properties of model cheese during storage

2.3.1.1 pH and dry matter changes during cheese storage

Dry matter content and pH of the experimental model cheeses and references were monitored during storage. In general, the pH values did not show changes over time. They remained constant (around 6.20 ± 0.03) for all 8 cheese batches (see supplemental Table S2.1). As mentioned before, this pH value was chosen in the assumption that it would be more favorable for plasmin activity during cheese storage, leading to a plasmin-dominated proteolysis. No significant difference in dry matter (P=0.975) was observed among the 8 cheese batches during the whole storage period (see supplemental Table S2.2). The standard deviation of pH and dry matter caused by factors related to the cheesemaking process was negligible. No significant difference in pH (P = 0.517) and dry matter (P = 0.696) among the 4 reference samples). During weeks 1-3, the dry matter content of experimental model cheese increased from 35.3 ± 1.2% to 39.6 ± 1.0%, due to the occurrence of syneresis. After the 3rd week, the dry matter content remained constant, around 39.8 ± 0.7%. The model cheese optimized for our study had a relatively lower dry matter content compared to commercial cheese (such as Gouda or Cheddar cheese, which have a dry matter content around 45-57% (Fox et al., 2017). In general, the dry matter content is related to fat and protein content of the cheese. In this study, skimmed milk (with less than 0.1 % fat) was used to produce model cheese. This was chosen to exclude the effect of fat content and its variations on cheese texture, and thus gain more insight on the relation between casein fractions and textural parameters. The absence of fat led to a low dry matter content in the model cheese. The relatively high pH was another factor that could induce a high moisture content in the cheese samples, as the rate of whey drainage of skim milk gels is lower at a higher pH (van Vliet and Walstra, 1994). The fact that no cooking step was applied was also a factor that could induce a high moisture content. During storage, the high moisture content and the relatively high temperature (16 °C) subsequently enhanced the occurrence of syneresis.

2.3.1.2 Plasmin activity during storage

The experimental model cheeses were allowed to ripen for a period of 12 weeks, and plasmin activity in the cheese matrix was determined after 1, 3, 5, 7, 9 and 12 weeks. The references showed the same plasmin activity level (P = 0.356) as the control model cheese (without plasmin addition) during the whole storage period. Thus, the influence of factors related to the cheesemaking process on plasmin activity changes during storage was insignificant. As expected, cheese with a higher amount of added plasmin had a higher plasmin activity (Fig 2.3 (a)).

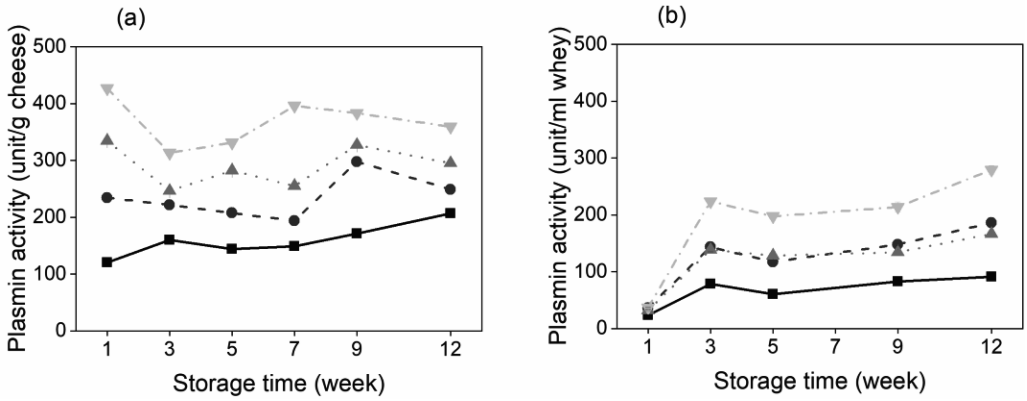

Figure 2.3. Plasmin activity in cheese (a) and whey (b) during 12 weeks of storage. Cheeses were produced from milk containing 0 µL/g (■), 0.4 µL/g (●), 0.6 µL/g (▲), and 1.0 µL/g (▼) added plasmin. Data of plasmin activity in the whey at week 7 are missing.'

Over time, from week 1 to week 3, plasmin activity in control cheese increased significantly (Fig 2.3a; P < 0.01), which can be attributed to the concentration of protein content in the samples resulting from syneresis. Afterwards, plasmin activity in control cheese slightly decreased until week 5 and then significantly increased from week 5 to week 12 (Fig 2.3a; P < 0.01). The later increase of plasmin activity was probably caused by the conversion of plasminogen to plasmin (Cortellino et al., 2006; Vélez et al., 2015). However, for cheeses with added plasmin, both increases and decreases in plasmin activity were observed during storage. This was likely caused by the competition between the activation of plasminogen and dissociation of additional plasmin from

casein micelles due to hydrolysis during cheese storage. To ascertain this, plasmin activity in the whey released from the cheese was also determined (Fig 2.3b). As already mentioned, plasmin activity was negligible in the fresh whey. However, it increased substantially during the first 3 weeks, and increased only slightly at later stages of storage until 12 weeks, for whey from both the control cheese and cheeses with added plasmin. The diffusion of salt from the surface into the interior might also have enhanced the release of plasmin into the whey. Besides the dissociation of additional plasmin into the whey, the autolysis of plasmin could also cause a decrease in plasmin activity during storage (Kelly et al., 2006; Gazi et al., 2014). Although the plasmin activity changed during the whole storage period, in general it was higher for cheese samples with more plasmin addition.

Overall, for the 4 cheeses with different amounts of added plasmin both pH and dry matter content were not significantly different, while plasmin activity was always higher in cheese samples with higher plasmin addition. The effect on casein hydrolysis and accompanying changes in textural properties related to the plasmin concentration will be discussed in the next sections.

2.3.2 Casein hydrolysis

2.3.2.1 Degree of casein hydrolysis

To understand the effect plasmin activity on casein degradation, the degree of casein hydrolysis was determined over time (Fig 2.4). For all cheeses, the degree of casein hydrolysis, expressed as protein fraction soluble at pH 4.6, increased gradually until week 7, and was always higher in samples with more plasmin addition. At week 12, the protein fraction soluble at pH 4.6 ranged from 14.2% (without plasmin addition) to 19.3% (with 1.0 μL/g plasmin addition). The addition of plasmin thus clearly changed the degree of casein hydrolysis at the end of storage (week 12). This change is in agreement with the study from Barrett et al. (1999), in which plasmin activity in Cheddar cheese was increased by adding urokinase. In their study, after 90 days of ripening, the protein fraction soluble at pH 4.6 was 5.9-6.1% higher in cheese with 5

U/ml urokinase addition than in control cheese without any urokinase addition. It was reported that higher plasmin activity in cheese effectively resulted in a higher production of γ-caseins, which are also known as β-CN(f106–209), β-CN (f29–209) and β-CN (f108–209). After primary proteolysis, γ-caseins will be further hydrolyzed into smaller peptides by plasmin, leading to a higher amount of protein soluble at pH 4.6 (Farkye and Fox, 1992; Barrett et al., 1999). This supports our observation that addition of plasmin increased the degree of casein hydrolysis with higher amount of hydrolysis products at the final storage stage. These hydrolysis products had different hydrophobic/hydrophilic properties when compared to the intact casein fractions, which can be derived from their position in the chromatograms. This might induce changes in hydrophobic and electrostatic interactions within the cheese matrix, which will have an influence on the final cheese textural properties (Guo and Kindstedt, 1995; Marchesseau and Cuq, 1995; McCarthy et al., 2016).

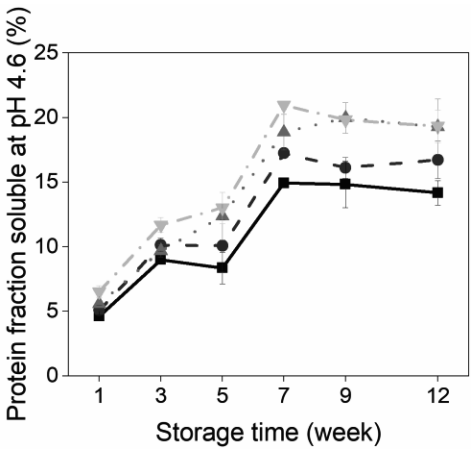

Figure 2.4. Degree of hydrolyzed casein, expressed as protein fraction soluble at pH4.6/total protein fraction, measured by DUMAS. Cheeses were produced from milk containing 0 µL/g (■), 0.4 µL/g (●), 0.6 µL/g (▲), and 1.0 µL/g (▼) added plasmin.

2.3.2.2 Residual intact casein fractions

To understand the effect of plasmin activity on the pattern of proteolysis in cheese during storage, the residual intact casein fractions were determined over time by RP-

HPLC. The results are shown in Fig 2.5 & 2.6. As the molecular properties of the different casein fractions (e.g. presence of phosphate groups, hydrophobicity) highly impact the formation of the protein network in the cheese matrix, differences in proteolytic pattern might have a strong effect on cheese texture.

At week 1, differences in intact casein fractions among different cheese samples were found. It has been mentioned that no casein hydrolysis was observed during the manufacture of the samples. The difference in intact casein fractions was caused by the hydrolysis related to different plasmin levels. The intact casein fractions continually decreased until week 9, and lower values were obtained for cheeses with more added plasmin (Fig 2.5&2.6). The intact total casein fraction reached levels between 2.5 and 6.7% at week 12. It was no surprise to find that cheese without plasmin addition already showed a high casein degradation after 9 weeks, as in our model cheeses the high moisture content and high storage temperature (16 °C) accelerated the proteolysis. The proteolytic enzymes (plasmin and chymosin) present in this cheese sample were able to hydrolyze most of the caseins after 9 weeks of storage.

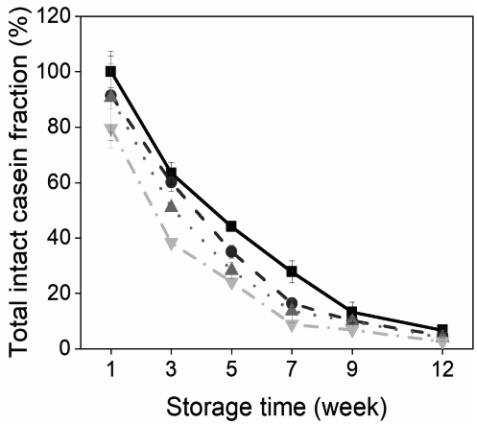

Figure 2.5. Total intact casein fraction in cheeses during 12 weeks of storage, determined by RP-HPLC. Cheeses were produced from milk containing 0 µL/g (■), 0.4 µL/g (●), 0.6 µL/g (▲), and 1.0 µL/g (▼) added plasmin. Total intact casein fraction (%) were calculated based on the peak area, the peak area of control cheese (without plasmin addition) at week 1 being 100%.

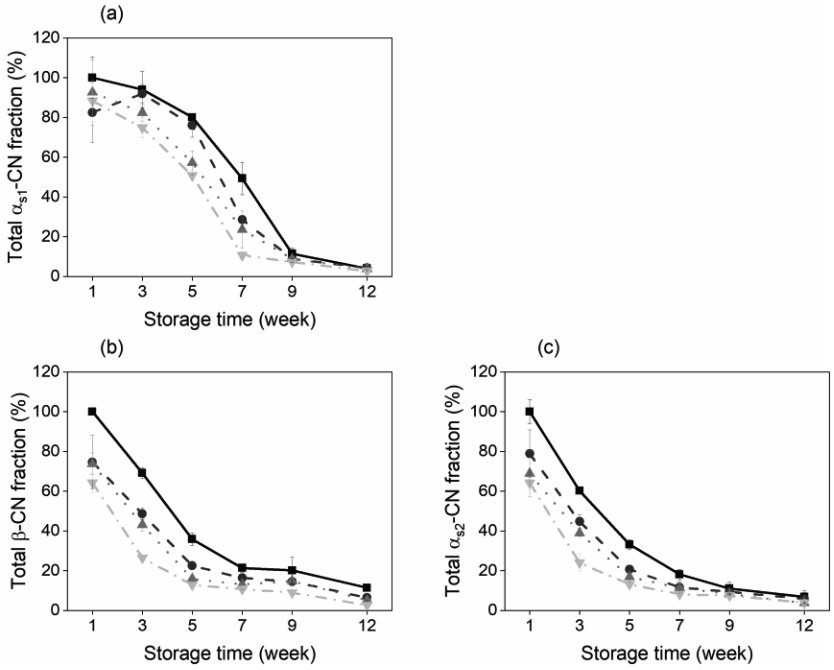

Figure 2.6. Intact casein fraction of α_{s1}-CN (a), β-CN (b) and α_{s2}-CN (c) as a result of protein hydrolysis in cheeses during 12 weeks of storage, determined by RP-HPLC. Cheeses were produced from milk containing 0 μL/g (■), 0.4 μL/g (●), 0.6 μL/g (▲), and 1.0 μL/g (▼) added plasmin. All the casein fractions were calculated based on the peak area, the peak area of control cheese (without plasmin addition) at week 1 being 100%.

In Fig 2.6b-c, it can be seen that both β-CN and α_{s2}-CN fractions rapidly decreased from week 1 to week 7. In contrast, the decrease of α_{s1}-CN was initially limited, and accelerated from week 5 to 12 (Fig 2.6a). This phenomenon was also noticed in cheese without plasmin addition (Fig 2.5&2.6). According to literature, plasmin predominantly breaks down β-CN by cleavage of Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}, yielding three γ-caseins, which are hydrophobic and insoluble at pH 4.6 (Sousa et al., 2001). At an early storage time, in the RP-HPLC chromatograms of our samples β-CN decreased and several peaks appeared at the right side. These peaks were confirmed as γ-caseins in a research in which a same chromatograms was shown by using a similar RP-HPLC method (Akkerman et al., 2021). This information indicates that at the beginning of the storage period, the casein hydrolysis in our model cheese was plasmin-dominated

while the effect of chymosin was shown at the later storage stage. However, in many cheese varieties, initial casein hydrolysis is dominated by the presence of chymosin, which is known to preferably hydrolyze α_{s1}-CN (Sousa et al., 2001). One of the reasons for the specific proteolytic pattern observed in our study is that the pH of the cheeses (~6.20) was relatively more favorable for plasmin activity than for chymosin activity. Furthermore, the chymosin used in this study (CHY-MAX® M 1000) was an enzyme obtained by recombinant technology, with high specificity in hydrolyzing κ-CN during milk coagulation, but low proteolytic activity on other caseins such as α_{s1}-CN (Jacob et al., 2011; Biswas and Metzger, 2016). Also, in the production of our model cheese only a low concentration of this chymosin (0.02 ml per kg milk) was used compared to the concentrations commonly chosen for this brand of chymosin (0.04-0.05 ml per kg milk) (Soodam et al., 2015; Mamo et al., 2020; Myagkonosov et al., 2021). After whey draining, we expected that a low concentration of chymosin was retained in the curd. Thus, for our samples, pH conditions, proteolytic activity and chymosin concentration limited the effect of chymosin at the initial storage time, and, therefore, the effect of plasmin was more pronounced. Plasmin activity showed a strong impact on β-CN, α_{s2}-CN and α_{s1}-CN degradation: the higher the plasmin activity, the more extensive the degradation of these casein fractions was.

The hydrolysis of casein by plasmin and chymosin during cheese ripening has been well documented. As a result of the hydrolysis of β-casein by plasmin, γ-caseins and the complementary peptides, β-CN (f1-28), (f1-105), (f1-107), (f29-105) and (f29-107), are liberated (Eigel et al., 1984). The peptides are part of the fraction soluble at pH 4.6, as shown in Fig 2.4. Plasmin can also hydrolyze the γ-caseins further at the Lys_{113}-Tyr_{114} and Arg_{183}-Asp_{184} cleavage sites (Ardö et al., 2017). Hydrolysis of these bonds results in release of additional peptides soluble at pH 4.6. Plasmin also cleaves α_{s2}-CN at 8 sites, producing around 14 peptides (Le Bars and Gripon, 1989), which are also soluble at pH 4.6. In addition, chymosin initially acts on α_{s1}-CN at Phe_{23}-Phe_{24}, producing the peptides α_{s1}-CN (f1–23) and α_{s1}-CN (f24–199) (McSweeney et al., 1993). Research

showed that at pH 4.6, α_{s1}-CN (f24–199) is insoluble, while α_{s1}-CN (f1–23) is soluble (Piraino et al., 2007).

Overall, the presented results show that cheese samples with higher plasmin activity showed higher degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and lower intact casein fractions (α_{s1}-, α_{s2}-, and β-CN). Most intact caseins were hydrolyzed within the 12 week of storage and the value of intact casein fractions ranged from 2.5 to 6.7%. The pattern of proteolysis was clearly dominated by plasmin. As the type and amount of hydrolysis products depended on the extent and pattern of proteolysis, the physical and chemical properties of these hydrolyzed products also varies during storage, which will be reflected in the structural organization of the protein network and textural properties of the cheese.

2.3.3 Changes of textural properties

To reveal how the extent and pattern of proteolysis affected the development of the textural properties, large strain compression measurements and TPA tests were carried out. The results are presented in Fig 2.7 and Fig 2.8.

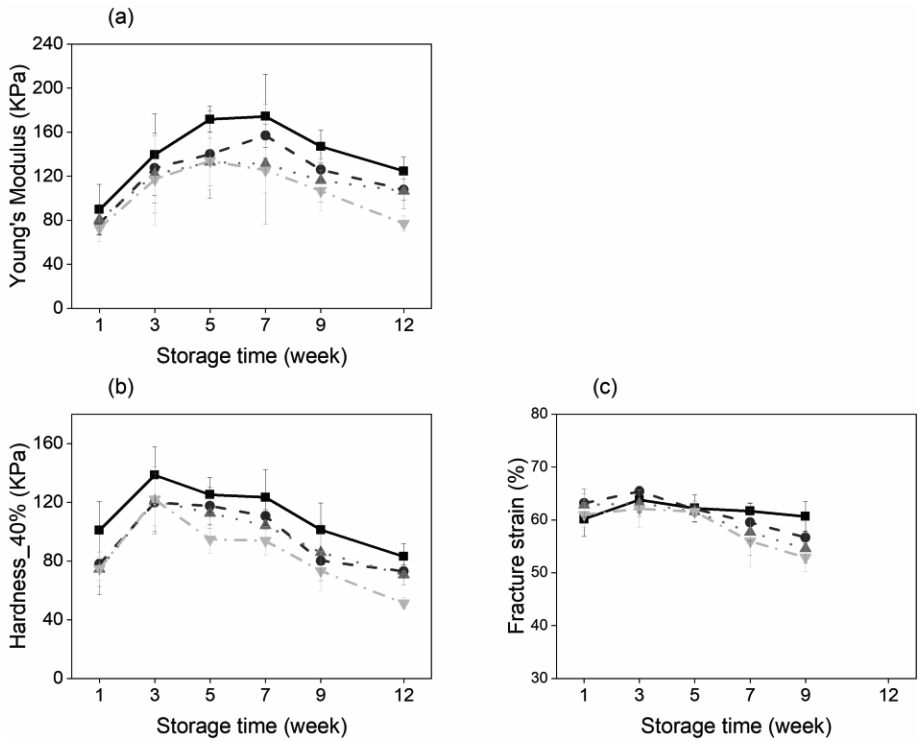

Figure 2.7. Young's modulus (a), hardness_40% (b) and fracture strain (c) of 4 cheeses with 4 different plasmin concentrations during 12 weeks of storage. Cheeses were produced from milk containing 0 μL/g (■), 0.4 μL/g (●), 0.6 μL/g (▲), and 1.0 μL/g (▼) additional plasmin. Data of brittleness at week 12 were not determined.

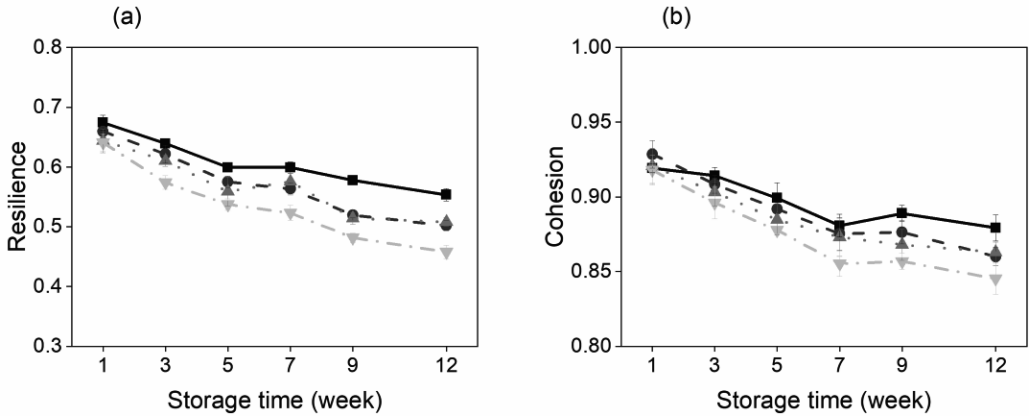

Figure 2.8. Resilience (a) and cohesion (b) of 4 cheeses with 4 different plasmin concentrations during 12 weeks of storage. Cheeses were produced from milk 0 μL/g (■), 0.4 μL/g (●), 0.6 μL/g (▲), and 1.0 μL/g (▼) additional plasmin.

As shown in Fig. 2.7(a), the Young's modulus significantly increased from week 1 to week 3, which was in principle associated to syneresis. Decreasing moisture content or decreasing the water/casein (non-fat components) ratio in cheese leads to an increase in the volume fraction of caseins (Lucey et al., 2003); more and stronger bonds among caseins are formed and the Young's modulus thus increases. The Young's modulus continually increased until week 5, even though the dry matter did not change remarkably after week 3. This change was thus not related to a further increase in dry matter, but probably related to other changes. Young's modulus indicates the stiffness of cheese and is known highly depends on the behavior and properties of the protein matrix (Walstra and van Vliet, 1982; Lucey et al., 2003), which is related to the number and the strength of intra- and intermolecular interactions among components. The increase in Young's modulus was probably caused by the rearrangements of the protein network and the formation of additional interactions. The rearrangements of the protein network was previously reported in to have influence on the structure of cheese matrix and the texture property (Irudayaraj et al., 1999; Lopez et al., 2007). It was shown in many researches that as a consequence of proteolysis the protein network in cheese started to rearrange and thus new bonds were formed among hydrolyzed products, such as hydrophobic interactions between large fragments (Watkinson et al., 2001; Karami et al., 2009; Piñeiro-Lago et al., 2020). With ongoing hydrolysis (after week 5), an increased casein degradation subsequently led to less interactions among intact caseins, which weakened the casein network. Thus the Young's modulus decreased. At the end of storage (12 weeks), the Young's modulus showed values similar to those at week 1 even though most caseins were hydrolyzed (Fig 2.5). As we mentioned above, the dry matter of the cheeses initially increased, which also led to an initial increase in Young's modulus. At later storage stages, proteolysis caused a softening of the samples. The similarity between the values at the beginning and at the end of storage was due to the combination of these two phenomena. As expected, the Young's modulus was lower for cheese samples with

higher plasmin activity during the whole storage period, indicating that a higher plasmin activity leads to less stiff cheese due to higher degree of casein degradation.

In many researches, cheese hardness was defined as the applied stress to fracture the cheese (Watkinson et al., 2001; Wium et al., 2003; Sayadi et al., 2013). As in this study the fracture stress showed large variations, hardness was defined instead as the stress required to deform the samples to a strain of 40% (i.e. lower than the fracture strain). Fig 2.7b shows that hardness_40% increased until week 3, which was also caused by syneresis. Afterwards, hardness_40% decreased as a result of proteolysis and reached values similar to or lower than those at week 1. In contrast to the Young's modulus (measured in the linear regime), hardness_40% started to decrease already from week 3. The interactions occurring as a result of rearrangements were insufficient to remain the strength of the protein network to be resistant to the deformations outside the linear regime. Hardness_40% was lower for cheese samples with higher plasmin activity during the whole storage time, as a result of a weaker casein network with higher casein degradation.

According to Fig 2.7c, fracture strain showed no significant change from week 1 to week 5. After week 5, the fracture strain decreased, indicating the cheeses became more brittle. That a higher plasmin activity led to a brittle cheese (lower fracture strain) was observed. The unvaried fracture strain from week 1 to week 5 could be explained by the syneresis and the newly formed bonds, including hydrophobic and electrostatic interactions among large peptides and casein fractions. As all these newly formed bonds contributed to the strength of the protein network, the brittleness (fracture strain) of cheese remained unchanged. With ongoing casein hydrolysis after week 5, the strong interactions among intact casein dramatically decreased and the protein network became weaker. The cheeses became more brittle and thus a low fracture strain was observed. That the lower fracture strain for cheese with a high plasmin seems also attributed from the weaker protein network.

TPA tests were performed to mimic the first two compressions occurring during consumption and to provide more information about possible relations between casein degradation and material properties. Resilience and cohesion were selected as two important attributes in this study. Other parameters in a typical TPA, such as gumminess and springiness, were not considered for further discussion due to the following reasons. Gumminess is calculated as hardness × cohesion. The indications of the data for hardness and cohesion can thus be considered to cover also gumminess. Springiness is used to describe how a product physically springs back between the first compression and the second compression, and resilience gives a similar indication.

As shown in Fig 2.8a, resilience kept decreasing for all cheeses, in spite of the syneresis and rearrangements occurring at the initial storage period. Our results indicate that changes in resilience during 12 weeks of storage were mainly related to proteolysis. With continuous proteolysis, the resistance of the protein network against deformation decreased, and thus the resilience kept decreasing. Fig 2.8b shows that cohesion continually decreased during 12 weeks of storage, indicating that also in this case the change of cohesion was dominated by the occurrence of proteolysis. The decreased cohesion was mainly related to the breakdown of protein. As proteins were hydrolyzed, the interactions within the protein matrix decreased, while the interactions among hydrolyzed products were insufficient for the mass to stick together and, consequently, the cheese became less cohesive.

2.3.4 Correlation between intact casein fractions and cheese texture

To study possible correlations between the texture properties of cheese and the degradation of the casein network, the values of the obtained textural parameters were plotted as a function of the degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and intact casein fractions (total casein fractions, and individual fractions, e.g. α_{s1}-, α_{s2}- and β-casein). The parameters of the curves fitting the experimental data points are shown in Fig 2.9. The square of the correlation coefficients, R^2 , and *p* values are shown in Table 2.1. In this analysis, to exclude the

general effect of syneresis during storage, the data collected at week 1 were excluded and the used data were standardized based on the dry matter of the samples.

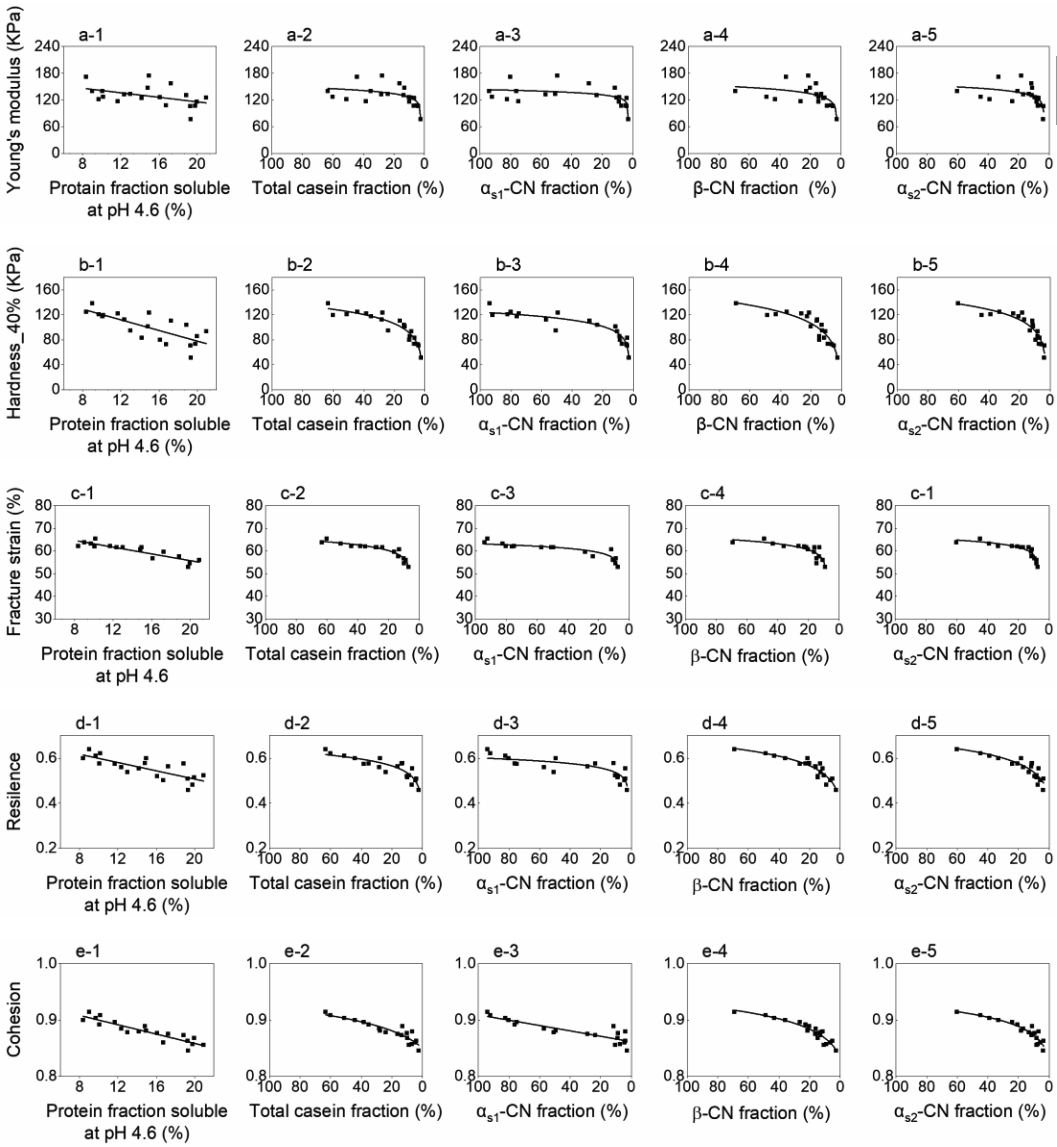

Figure 2.9. Young's modulus (a, 1-5), hardness (b, 1-5), fracture strain (c, 1-5), resilience (d, 1-5), cohesion (e, 1-5) as a function of the protein fraction soluble at pH 4.6 and the casein fractions. All fractions were calculated based on the peak area of control cheese at week 1, which was set as 100%. All data at week 1 were excluded and all intact casein fractions were recalculated based on the dry matter. The linear fittings are shown for the protein fraction soluble at pH 4.6. The curves for casein fractions are results of the best fit according to the equation $P = a - b * ln(fx + c)$.

Table 2.1. Correlation coefficients (R^2) between cheese texture parameters and indicators of casein hydrolysis (protein fraction soluble at pH 4.6 and intact casein fractions).

	Protein fraction soluble at pH 4.6[1]	Intact casein fractions[2]			
		Total casein	α_{S1}-CN	β-CN	α_{S2}-CN
Young's Modulus	0.212*	0.492**	0.496**	0.465**	0.398**
Hardness	0.576**	0.909**	0.881**	0.847**	0.875**
Brittleness	0.789**	0.915**	0.834**	0.706**	0.906**
Resilience	0.612**	0.801**	0.695**	0.853**	0.804**
Cohesion	0.827**	0.831**	0.763**	0.900**	0.860**

1. Linear fitting was used to correlate cheese texture parameters and protein fraction soluble at pH 4.6.
2. Logarithmic transform function ($P = a - b * ln(fx + c)$) was used to correlate cheese texture parameters (P) and intact casein fractions (fx).
** P<0.05, ** P< 0.01.*

As shown in Fig 2.9 and Table 2.1, the textural properties had good correlation with both the degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and the intact casein fractions (including intact total casein and the intact individual caseins), indicating that both parameters are important to explain the development of the textural properties. The total casein fractions showed a relation with the textural parameters similar to that of α_{S2}-CN and β-CN, while that of α_{S1}-CN slightly deviated. All textural properties (P) were related to the intact casein fractions according to the following equation (2),

$$P=a-b*\ln(fx+c) \quad (2)$$

in which fx refers to the different casein fractions, and a, b, and c are fitting parameters. The supplemental Table S2.3 provides the parameters (a, b, c) of the fitting curves relating cheese texture parameters and intact casein fractions.

It can be seen that the values of the fitting parameters of the total casein curves are similar to those of the curves of α_{S2}-CN and β-CN, confirming that the proteolysis in our model cheeses was plasmin-dominated, with preferential hydrolysis of α_{s2}-CN and β-CN by plasmin (Fig 2.6).

The degree of casein hydrolysis gives the proportion of cleaved peptide bonds. As the peptide bonds of casein and large fragments were broken, the protein network gradually weakened and the textural properties also changed. The textural properties showed a similar trend as the changes of the soluble peptides and intact caseins over time. The degree of casein hydrolysis showed a linear relation with the textural properties, while a logarithmic correlation was found to explain the link between the intact casein fractions and the textural properties. The largest changes in Young's modulus, hardness and brittleness were observed only when a certain amount of casein was hydrolyzed, i.e. later in the storage period (Fig 2.9). On the other hand, resilience and cohesion kept decreasing more gradually as proteolysis proceeded, as also reflected by the lower b values. A possible explanation for the slow change in textural parameters in the initial stage of casein hydrolysis is that although a substantial part (40-60%) of the casein fractions was already broken down after 3 weeks of storage (Fig 2.5), the main hydrolyzed products likely consisted of large fragments insoluble at pH 4.6. As a matter of fact, at this sampling moment low values of protein fraction soluble at pH 4.6 were obtained (9-12%, Fig 2.4). These large peptides could still be trapped in the casein matrix and interact with intact casein fractions, and thus contribute to the formation of the casein matrix and to cheese texture. Therefore, the effect of the initial cheese texture breakdown on texture was not evident at the early stage. However, a significant change in textural properties occurred when the intact casein fraction decreased beyond a certain level. For example, brittleness rapidly increased when the total intact casein fraction was less than 20%. Moreover, Young's modulus and hardness showed a sudden decrease when the total intact casein was less than 40%. Further hydrolysis of intact casein fractions and large peptide fragments led to the formation of smaller peptides, with greater changes in the protein network. For higher degree of hydrolysis, i.e. lower intact casein fractions and higher content of protein soluble at pH 4.6, the casein network became substantially weaker and noticeable changes in the texture attributes became visible.

To conclude, the changes in textural properties of cheese can be explained based on both the degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and the residual intact casein fractions. At the beginning of storage stage, proteolysis by plasmin and chymosin occurred and some hydrophobic fragments were released. A rearrangement of the protein network also took place, and, therefore, interactions between these hydrophobic fragments and casein micelles were enhanced. As a result, the number of internal bonds significantly increased. Consequently, at the beginning of storage only slight changes in cheese textural properties were found. Later, these fragments were further hydrolyzed by plasmin and chymosin, and more peptides were released. The impact of rearrangement on textural properties at this later stage was less notable than for the initial stage, as the hydrophobic interactions between large hydrolyzed fragments became less. The obtained knowledge represents a starting point for further studies about the possibility to optimize the properties of existing cheeses by adjusting the activity of the enzymes present in the curd, regulating the extent and the pattern of proteolysis during ripening. To investigate techniques to vary plasmin activity in cheese without additions, the option of producing cheese with specific processes (e.g. curd washing so as to eliminate the inhibitors of plasminogen activators) to active plasminogen could be examined. On the other hand, milk with varying plasmin activity can also be achieved by adding a stream of milk with a high or low plasmin content obtained from cows at a specific lactation stage to the bulk cheese milk. The absence of fat and the relatively high pH (6.2) of the model cheeses developed for the present study might limit the direct application of our findings to most cheese types. Further research is needed to confirm the indications reported in this paper for cheese with fat and a pH closer to that of most semi-hard cheese varieties.

2.4 CONCLUSION

In this study, we investigated how plasmin activity affects caseins hydrolysis and cheese textural properties in a model system representative of semi-hard cheese. Our results show that casein hydrolysis was accelerated with increased plasmin activity and led to textural variations during 12 weeks of storage. Model cheese with more plasmin was softer, more brittle and less elastic. All textural properties showed good linear correlation with the degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and a logarithmic correlation with intact total casein fractions. This indicates that both the degree of casein hydrolysis and intact total casein fractions are important to explain the changes in textural properties of cheese. The potential to control cheese texture by adjusting the activity of the enzymes present in the curd is of great importance for the cheese industry. Further researches on cheese with fat and a pH closer to most semi-hard cheese varieties should be done to confirm the indications reported in this paper.

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SUPPLEMENTARY INFORMATION

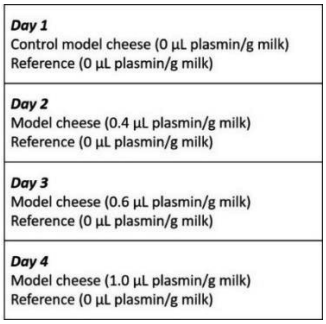

Figure S2.1. Experimental design of cheese trials.

Table S2.1. Changes of pH during 12 weeks of storage. Experimental model cheeses were produced from milk containing 0 µL/g, 0.4 µL/g, 0.6 µL/g, and 1.0 µL/g added plasmin. References were produced from the same batches of milk without plasmin addition.

Plasmin (µL/g)		Storage time (week)					
		1	3	5	7	9	12
Model cheese	0	6.19±0.09	6.35±0.00	6.23±0.01	6.16±0.01	6.16±0.06	6.22±0.01
	0.4	6.12±0.10	6.29±0.00	6.25±0.05	6.16±0.05	6.18±0.03	6.23±0.00
	0.6	6.18±0.02	6.25±0.04	6.22±0.00	6.17±0.00	6.18±0.01	6.22±0.00
	1.0	6.18±0.02	6.30±0.07	6.17±0.05	6.20±0.05	6.19±0.04	6.21±0.01
References	0	6.24±0.02	6.17±0.00	6.23±0.02	6.21±0.00	6.27±0.05	6.27±0.01
	0	6.24±0.03	6.28±0.00	6.25±0.01	6.18±0.02	6.17±0.01	6.24±0.06
	0	6.14±0.01	6.24±0.02	6.22±0.01	6.17±0.03	6.18±0.00	6.23±0.00
	0	6.14±0.00	6.33±0.04	6.15±0.03	6.21±0.02	6.17±0.02	6.21±0.01

Table S2.2. Changes of dry matter (%) during 12 weeks of storage. Experimental model cheeses were produced from milk containing 0 µL/g, 0.4 µL/g, 0.6 µL/g, and 1.0 µL/g added plasmin. References were produced from the same batches of milk without plasmin addition.

Plasmin (µL/g)		Storage time (week)					
		1	3	5	7	9	12
Model cheese	0	36.9±0.3	40.7±0.8	40.3±1.1	39.4±1.8	39.5±2.1	39.5±0.4
	0.4	34.2±0.3	38.3±0.8	40.9±0.7	41.1±0.3	39.5±2.8	40.0±1.6
	0.6	34.7±0.1	39.2±1.1	39.4±0.4	41.1±0.9	39.5±0.7	38.5±0.6
	1.0	35.5±0.9	39.9±0.4	39.6±0.6	39.9±1.2	40.0±1.0	39.9±1.0
References	0	35.1±2.0	39.7±1.0	39.0±1.9	41.0±0.6	39.3±0.6	39.4±1.5
	0	37.0±0.2	39.1±0.6	39.3±1.5	41.8±1.6	38.7±1.1	39.6±1.2
	0	36.2±2.5	37.6±1.4	40.2±0.4	40.4±1.2	38.8±0.5	38.9±1.3
	0	36.5±0.5	40.4±0.5	40.3±0.1	41.1±0.6	41.0±0.2	39.5±1.4

Table S2.3. Changes of protein content (%) during 12 weeks of storage. Cheeses were produced from milk containing 0 µL/g, 0.4 µL/g, 0.6 µL/g, and 1.0 µL/g added plasmin.

Plasmin (µL/g)		Storage time (week)					
		1	3	5	7	9	12
Model cheese	0	29.0±0.5	35.4±1.5	33.6±0.4	32.8±1.0	33.4±1.6	33.8±1.7
	0.4	29.5±0.9	34.3±1.6	33.2±1.3	33.2±0.5	33.0±1.3	34.7±0.3
	0.6	30.0±1.3	35.3±1.6	32.7±1.4	35.3±0.9	33.4±1.4	33.6±1.3
	1.0	31.0±0.9	35.1±1.3	34.3±1.4	34.9±0.9	34.8±1.3	34.2±1.0
References	0	30.0±1.7	32.5±0.2	33.4±0.3	34.0±1.0	33.1±0.7	33.9±0.9
	0	29.3±1.3	33.1±1.5	31.9±1.5	31.9±2.6	32.0±0.7	33.0±3.8
	0	30.8±1.1	33.5±0.5	34.0±0.6	33.6±0.3	33.1±0.4	ND
	0	30.6±2.1	34.1±1.1	32.1±1.3	35.2±2.4	33.7±0.5	ND

ND = not determined as device errors.

Table S2.4. Parameters (a, b, c) of the fitting curves of the correlation plots between cheese texture parameters and intact casein fractions.

		Intact casein fractions			
		Total casein	α_{S2}-CN	β-CN	α_{S1}-CN
Young's Modulus	a	150.575	155.15	154.68	143.78
	b	-9.42	-9.97	-11.73	-6.85
	c	-0.02	-0.04	-0.03	-0.03
Hardness	a	139.78	149.60	149.30	124.85
	b	-20.14	-21.57	-26.38	-13.46
	c	-0.01	-0.02	0.00	-0.02
Brittleness	a	65.84	66.27	66.31	63.36
	b	-2.91	-2.24	-2.69	-1.97
	c	-0.06	-0.07	-0.08	-0.07
Resilience	a	0.64	0.67	0.67	0.60
	b	-0.04	-0.06	-0.06	-0.03
	c	-0.01	0.02	0.01	-0.02
Cohesion	a	0.92	0.93	0.93	-3.14
	b	-0.03	-0.03	-0.03	-1.24
	c	0.13	0.02	0.05	25.44

Logarithmic transform function (P=a-b*ln(fx+c)) was used to correlate cheese texture parameters (P) and intact casein fractions (fx).


CHEESE

Chapter 3

Linking casein hydrolysis by chymosin and plasmin to the physical properties of model cheeses

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ABSTRACT

Cheese texture, which largely determines the overall cheese quality and the preference of consumers, develops during ripening due to the enzymatic degradation of casein. However, the influence of the hydrolysis of individual casein fractions (α_{s1}- and β-CN) on specific textural properties remains unclear. In this study, we aimed to link the breakdown of individual casein fractions by chymosin or plasmin to different physical properties used to characterize cheese texture. Model cheeses with two plasmin levels (active and inactive) and three chymosin levels (20, 50, and 80 µl/kg milk) were prepared. During a storage period of 7 weeks, dry matter content, pH, degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6), intact casein (α_{s1}- and β-CN) fractions, rheological properties (critical strain and storage modulus) and textural properties (Young's modulus, resilience, cohesion, adhesiveness, hardness_40%, fracture strain and stress, and strain hardening index) of the model cheeses were determined.

Our results showed that the hydrolysis of the specific casein fractions (α_{s1}- and β-CN) by chymosin and plasmin played different roles in the rheological and textural properties of the samples. The hydrolysis of α_{s1}-CN predominantly led to a decrease in G' and Young's modulus, parameters related to the strength of the protein network. Hydrolysis of β-CN was more associated with changes in critical strain, resilience and cohesion, which were related to rearrangements within the protein network resulting from hydrophobic interactions among hydrolyzed products. Hardness_40% (stress at a strain of 40%) was related to hydrolysis of both α_{s1}-CN and β-CN, although the effect of α_{s1}-CN degradation seemed more pronounced. The obtained knowledge offers new insights into the mechanisms behind cheese texture development. These insights may help to control cheese texture and to design new products with desired textures by tailoring the hydrolysis of different casein fractions. This could be achieved by adjusting the activity of proteolytic enzymes, such as chymosin, plasmin or both.

3.1 INTRODUCTION

Cheese texture is one of the major factors determining the overall quality of cheese and the preference of consumers (Guinard and Mazzucchelli, 1996). The textural properties of cheese highly depend on its composition, such as dry matter, fat and protein (mainly casein) content, and the structure of the cheese matrix, as affected by pH and ionic strength (Guinee, 2016). The intact casein fractions, especially α_{s1}-casein (α_{s1}-CN) and β-casein (β-CN), are hydrolyzed during ripening, which consequently influences the structure of the protein network in the cheese matrix and thus affects cheese texture (Fox and McSweeney, 1996). The breakdown of casein fractions is mainly induced by chymosin from the coagulant and by plasmin present in milk (Fox and McSweeney, 1996; Sousa et al., 2001). According to previous research, chymosin has a preference for hydrolyzing α_{s1}-CN, and to a significantly lesser extent, β-CN, while α_{s2}-casein appears to be relatively resistant to proteolysis by chymosin (Sousa et al., 2001; Uniacke-Lowe and Fox, 2017). In contrast, plasmin has a preference to hydrolyze β-CN and α_{s2}-CN, while α_{s1}-CN is hydrolyzed more slowly (Korycha-Dahl et al., 1983; Bastian and Brown, 1996). The extent and the pattern of hydrolysis of the different casein fractions thus depend on the specific enzymes present in the curd after cheese manufacture.

A number of studies have investigated the role of individual enzymes (either chymosin or plasmin) in casein hydrolysis and cheese texture. The effect of chymosin-induced proteolysis during ripening on the development of cheese texture has been extensively researched (Creamer and Olson, 1982; Wium et al., 1998; Hynes et al., 2001; Dave et al., 2003; Francisco-José et al., 2010; Lamichhane et al., 2019). Creamer and Olson (1982) reported that the initial chymosin-mediated hydrolysis of α_{s1}-CN is responsible for the softening (decreased elasticity and hardness) in Cheddar cheese. They hypothesized that the cleavage at the Phe_{23}-Phe_{24} position leads to the loss of α_{s1}-CN f(1-23). As this fragment contains a hydrophobic interaction site between residues 14 and 24, its loss in the serum would lead to a softer cheese. Many studies showed that

that an increase in the level of added chymosin results in a softer cheese after ripening (Prasad and Alvarez, 1999; Moynihan et al., 2014; Alinovi et al., 2018). A more extensive hydrolysis of α_{s1}-CN by chymosin was given as the main reason. Lamichhane et al. (2019) reported that the fracture stress (index of hardness) was lower in semi-hard model cheeses with lower levels of intact casein fractions, primarily α_{S1}-CN. However, the breakdown of α_{S1}-CN by chymosin had no pronounced influence on the fracture strain (index of brittleness). Although chymosin-induced hydrolysis of α_{s1}-CN has always been emphasized as the main cause for the changes in mechanical properties during ripening, the correlation between the hydrolysis of α_{s1}-CN and specific textural properties still needs to be further clarified.

Next to the studies on chymosin, studies on how plasmin-induced hydrolysis affects cheese properties have also been carried out. However, these works focused more on cheese functionality (i.e. stretchability and meltability) and flavor development (Farkye and Fox, 1991; Farkye and Fox, 1992; Bastian et al., 1997; Fiona M et al., 1999; O'Farrell et al., 2002; Somers et al., 2002) than on texture. Lamichhane et al. (2019) reported that the decrease in intact β-CN fraction caused by plasmin was negatively associated with a decrease in fracture strain (index of brittleness). Our previous study showed that textural properties (e.g. hardness, resilience, cohesion and brittleness) were highly correlated to the proportion of intact casein fractions, both α_{s1}-CN and β-CN, resulting from plasmin-induced hydrolysis (Chapter 2). Although the role of individual enzymes (either chymosin or plasmin) in cheese properties has been investigated, a limited number of studies have focused on the effect of a combination of enzymes on cheese texture. Looking at both plasmin and chymosin, the role of hydrolysis of the individual casein fractions α_{s1}-CN and β-CN in the changes of different textural properties will become more clear. This will provide relevant insights to engineer the textural properties of many cheese varieties using targeted enzymatic hydrolysis.

The objective of this study was to link the hydrolysis of individual casein fractions (α_{s1}- and β- CN) by chymosin or plasmin to the rheological and textural properties of model cheese. To assess this, three levels of recombinant chymosin (20, 50 and 80 µl/kg milk) were tested. Aprotinin, a serine protease inhibitor able to inhibit the activity of plasmin (Baer et al., 1994; Bijl et al., 2014), was used to obtain two groups of samples: one in which plasmin was active and one in which it was inactive. To eliminate any possible effect of fat on texture development, skim milk was used. Also, no starter culture was used, as bacterial enzymes would lead to additional changes in textural properties. To control the pH of the systems, we used D-(+)-glucono-delta-lactone (GDL) to reach two pH levels, 5.9 and 6.2. During 7 weeks of storage, dry matter content, pH, degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6), intact casein (α_{s1}- and β-CN) fractions, rheological properties (critical strain and storage modulus) and textural properties (Young's modulus, resilience, cohesion, adhesiveness, hardness_40%, fracture strain and stress, and strain hardening index) of the model cheeses were monitored.

3.2 MATERIALS AND METHODS

3.2.1 Materials

All model cheeses were made using the same batch of pasteurized skim milk (Jumbo, the Netherlands), which contained 3.6% protein and less than 0.1% fat. GDL, aprotinin from bovine lung (A1135, 3-8 TIU/mg), protein standards (β-CN and α_S-CN) and other chemicals for RP-HPLC measurements were obtained from Sigma Aldrich (Sigma-Aldrich, USA). Recombinant chymosin (CHY-MAX® M 1000, 1000 IMCU/ml; Chr. Hansen, Denmark) was used to coagulate the model cheese.

3.2.2 Determination of cutting time

During the preparation of the model cheese, the curd was cut when a certain value of the storage modulus (100 Pa) was reached to exclude the potential effect of

differences in curd rheology on syneresis during the cheese making process and on the texture of the final products. To determine the curd cutting time, the storage modulus of milk curd during coagulation was measured using a MCR501 Rheometer (Anton Paar, Graz, Austria) equipped with a measuring cup (CC17/Ti-3677) and a concentric cylinder geometry (CC17/Ti-3955). The milk was first heated to 33 °C and then 0.3% or 0.6% (w/w) GDL was added to reduce the pH to 6.2 or 5.9. After 30 min, the milk reached the target pH and chymosin was added. 4.7 ml milk sample was immediately transferred to the measuring cup, and the storage modulus was monitored at an applied constant frequency of 1 Hz and a strain of 1%.

3.2.3 Model cheese production

Twelve model cheese samples were made in duplicate based on the cheese production process reported in Chapter 2, with modifications of the stirring and pressing steps. Detailed information on the 12 samples is shown in Table 3.1. Two pH values (5.9 and 6.2) were chosen to vary the hydrolysis of different casein fractions, as chymosin and plasmin have different optimum pH values.

Table 3.1 Detailed information on the 12 studied model cheese samples.

Sample No.	pH[1]	Aprotinin (mg/kg milk)	Chymosin (µl/kg milk)	Cutting[2] time (min)
1	6.2	0	20	60
2	6.2	0	50	15
3	6.2	0	80	10
4	6.2	1.67	20	60
5	6.2	1.67	50	15
6	6.2	1.67	80	10
7	5.9	0	20	19
8	5.9	0	50	11
9	5.9	0	80	9
10	5.9	1.67	20	19
11	5.9	1.67	50	11
12	5.9	1.67	80	9

[1] The pH of cheese milk was lowered to 6.2 and 5.9 by adding 3% and 6% GDL respectively.
[2] Cutting time was defined as the time at which the storage modulus reached 100 Pa.

Briefly, 5 kg skimmed milk (with 0.04 % NaN_3, w/w) was heated to 33 °C in a water bath. Aprotinin (0 or 1.67 mg/kg milk) was added to the milk and mixed thoroughly for 20 s. Next, 3 or 6% (w/w) GDL was added to the milk to reduce the pH to 6.2 or 5.9. Thirty minutes after addition of GDL, chymosin was added to coagulate the milk. At the cutting time, curd was cut into 15×15×15 mm^3 cubes by using 3 custom-made knives, followed by a 15 min waiting step. Subsequently, the curd was gently stirred for 30 min. The curd was then transferred into 3 cylindric cheese moulds (with a diameter of 8.5 cm and a height of 9.5 cm) for shaping and pressing. Each mould was filled with approximately 400 g curd. Each curd in the mould was pressed with a weight of 2 kg for 3 h. After pressing, the obtained three cheeses were immersed in a brine with 25 % (w/w) salt for 45 min. Then, each cheese was wiped dry with lab paper and cut into 2 blocks. Each cheese block was vacuum packed in a plastic bag and stored at 16 °C for a period of 7 weeks. Two samples were randomly chosen and analyzed 1, 3, 5, and 7 weeks after cheese preparation as described in the next sections.

3.2.3 Chemical properties of model cheese during storage

3.2.3.1 Determination of dry matter and pH

The dry matter content and the pH of the model cheeses were determined according to methods described earlier (Lynch et al., 1997; Patrignani et al., 2019). All the measurements were performed in triplicate.

3.2.3.2 Casein hydrolysis during storage

3.2.3.2.1 Degree of casein hydrolysis

The degree of casein hydrolysis was determined as described in Chapter 2. The degree of casein hydrolysis was expressed as protein fraction soluble at pH 4.6.

3.2.3.2.2 Intact casein fraction by RP-HPLC

The fractions of intact casein during storage were determined in triplicate using reversed-phase high-performance liquid chromatography (RP-HPLC, Thermo ScienceTM UltiMate 3000; USA). Sample preparation and chromatographic conditions were the same as described in Chapter 2. Protein standards (β-CN and α_S-CN) were

also injected to confirm the retention time of specific casein fractions. An Aeris 3.6 µm Widepore XB-C18 column (250×4.6mm, Phenomenex, the Netherlands) was used for analysis. The chromatograms were analyzed with Chromeleon 7.1.2 software. The intact casein fraction (%) at the different sampling moments was expressed as the casein peak area divided by the casein peak area of the cheese after 1 week of storage. All reported values for the different casein fractions are thus relative, and are not based on absolute values.

3.2.4 Rheological and textural properties of model cheese during storage

3.2.4.1 Determination of rheological properties

Vacuum sealed cheese was taken out of the incubator (16 °C) and was equilibrated at room temperature for 1 h. Then the cheese was cut into specimens with cylindrical shape (diameter of 25 mm, height of 5 mm). The rheological properties were measured with a MCR501 Rheometer equipped with a parallel-plate geometry with a smooth stainless steel plate (diameter of 25 mm, code: PP25/P2/SS). A small amplitude oscillatory shear (SAOS) test was carried out in duplicate at a frequency of 1 Hz with a logarithmic increase of the strain amplitude from 0.01 to 100%. The normal force was set as 0.25 N and the gap size was set as 5 mm. The measuring temperature was set at 20 °C. From the SAOS test we extracted the critical strain and the storage modulus (G') at this strain. Critical strain was defined as the strain at which the storage modulus (G') decreased by more than 5% from the value in the linear regime.

3.2.4.2 Determination of textural properties

One hour after taking the samples out of the incubator (16 °C), the cheese was cut into specimens with cylindrical shape (diameter and height of 1 cm). Texture profile analyses (TPA) and large deformation measurements in compression were carried out at least in triplicate using a texture analyzer TA-TX Plus (Stable Micro Systems Ltd., United Kingdom), which was equipped with a 50 mm diameter cylindrical probe (perspex) and a 5 kg load cell. The TPA (double compression) test was performed at a rate of 2 mm/s with a strain of 20%. From the obtained TPA curves, resilience (upstroke

peak area of first compression/downstroke peak area of first compression), adhesiveness (the negative peak area between two compressions), and cohesion (ratio of the positive force area of the second compression to that of the first compression) were extracted. The large deformation test was carried out with a compression until a strain of 85% at a rate of 2mm/s. The Young's modulus was extracted as the slop of stress-strain curves from the linear region (1-3% strain). From the fracture point, the fracture strain and fracture stress were also extracted. Even though the fracture stress is well known as an index of cheese hardness, we could not accurately determined the hardness by the fracture stress as the error bars were too large. Thus, to indicate the cheese hardness we also determined the stress at a strain of 40% (hardness_40%). This parameter has also been used by others as a measure of cheese hardness (Ak and Sundaram, 1997; Alvarez et al., 2000). The strain hardening index (SHI) was calculated according to the empirical equation suggested by Kokelaar et al. (1996) and van Vliet (2008) as:

$$\sigma = a \cdot \varepsilon^{b} \quad (1)$$

where σ is the stress, a is the strength coefficient (Pa), ε is the deformation strain (-), and b is the strain hardening index (-). Eqn 1 was fitted (R^2~0.98–0.99) to stress-strain data over the strain range before fracture, which was from 0.02 to 0.6.

3.2.5 Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey Post Hoc test was used to evaluate significant differences of the obtained parameters among cheeses and among storage weeks, using IBM SPSS Statistics 25 (IBM Corporation, NY, USA). The significance level was set at 0.05.

3.3. RESULTS AND DISCUSSION

3.3.1 pH and dry matter of model cheese during storage

Dry matter and pH of the model cheeses were monitored throughout the storage period as changes in their values can strongly impact the development of textural properties. The results are shown in Table S3.1 and S3.2 of the supplementary information. No significant difference in pH was found among cheeses, both in the pH 5.9 group and the pH 6.2 group. During the whole storage period, the pH values did not change significantly. Concerning dry matter content, only the 3 cheeses with inactivated plasmin at pH 6.2 showed a slight increase, from 36.7 ± 0.8% in week 1 to 37.6 ± 0.8% in week 3 ($P < 0.05$). Afterwards, the dry matter stayed constant and was similar to the levels found in the other model cheeses during the whole storage period (37.9 ± 0.4%).

The model cheeses prepared in this study had higher moisture content than that of commercial semi-hard cheese, such as Gouda cheese and Cheddar cheese, which normally contain around 43-55% dry matter (Fox et al., 2017b). As discussed in in Chapter 2, the absence of fat, the relatively high pH and the fact that no cooking step was applied were the three main factors responsible for the high moisture content in our model cheeses. However, unlike naturally ripened commercial cheeses in which the moisture content gradually decreases during ripening, the moisture content of our model cheeses showed limited changes during 7 weeks of storage. This allowed to exclude the extra effect of moisture loss on texture development, and to focus on the effect of casein hydrolysis.

3.3.2 Casein hydrolysis

3.3.2.1 Degree of casein hydrolysis

To unveil how plasmin and chymosin influenced the degree of casein hydrolysis, the protein fraction soluble at pH 4.6 was determined over time. The results are shown in Fig 3.1.

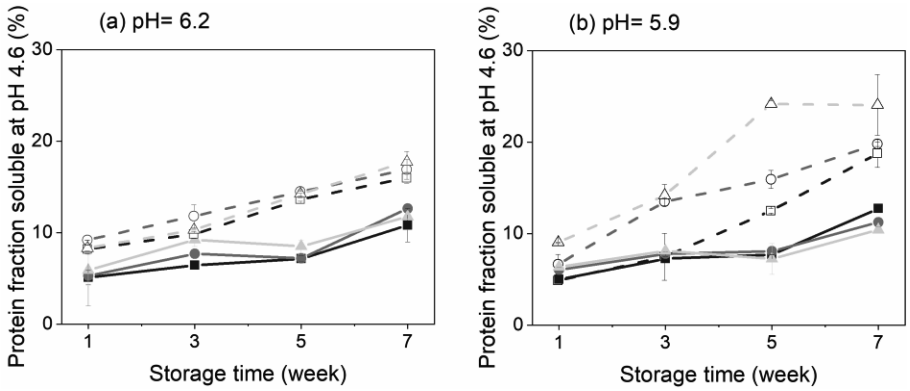

Figure 3.1. Protein fraction soluble at pH 4.6 as a result of protein hydrolysis at pH 6.2 (a) and pH 5.9 (b) in model cheeses during 7 weeks of storage, determined by DUMAS. Cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 µl/kg milk: ■, □; 50 µl/kg milk: ●, ○; 80 µl/kg milk: ▲, △) were added.

Independently of pH and chymosin activity, cheeses with inactive plasmin showed a lower increase in the protein fraction soluble at pH 4.6, with values starting at 4.9-6.3% in week 1 and increasing to 10.4-12.7% at week 7 (solid lines). When plasmin was active, higher values for pH 4.6 soluble protein were found (dashed lines). These results indicate that in our study the hydrolysis of casein by chymosin had less influence on the release of small (water-soluble) fragments, while the plasmin had a larger effect. According to literature, the hydrolyzed products from the initial hydrolysis of α_{s1}-CN by chymosin are a combination of large (non-soluble) fragments, α_{s1}-CN (f24–199) and small (soluble) peptides, α_{s1}-CN (f1–23) (McSweeney and Fox, 1993; Piraino et al., 2007). In our study, the small peptides only had limited contribution to the protein fraction soluble at pH 4.6. It was reported that three bonds (Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}) of β-CN are cleaved by plasmin, resulting in the liberation of some hydrophobic ϒ-CNs (water insoluble), but a larger amount of complementary water soluble peptides, as β-CN f(1-28), f(1-105), f(1-107), f(29-105) and f(29-107) (Eigel et al., 1984; Farkye, 1995; Exterkate et al., 1997; Møller et al., 2012). Thus, in our study, the larger amount of soluble peptides formed by β-CN by plasmin had a larger effect

on the pH 4.6 soluble protein fraction. It should be noted that in our study we used recombinant chymosin (CHY-MAX® M 1000) known to have a high specificity for hydrolyzing κ-CN during milk coagulation (Jacob et al., 2011; Biswas and Metzger, 2016). The required amount of chymosin for milk coagulation was lower (0.02-0.08 ml per kg milk milk) when compared to the concentrations commonly used for other commercial calf rennet (around 0.3 ml per kg milk) (Vicente et al., 2001; Irigoyen et al., 2002). Thus, a very low concentration of chymosin was retained in the curd after whey draining. This also explains why in our samples casein hydrolysis was less influenced by chymosin.

Interestingly, in cheese with active plasmin the chymosin content showed a significant impact on the degree of casein hydrolysis at pH 5.9 ($P < 0.01$), while this phenomenon was not observed at pH 6.2 ($P = 0.901$). Presumably, more small (water-soluble) hydrolyzed products were produced with higher chymosin content, which was the case only when plasmin was active. This suggests a synergistic effect between chymosin and plasmin on the formation of water soluble hydrolysis products. To further explain this, the pattern of casein hydrolysis was investigated.

3.3.2.2 Pattern of casein hydrolysis

To understand the effect of plasmin and chymosin on the breakdown of casein fractions during storage, the residual intact casein fractions were determined over time by RP-HPLC. The results are shown in Fig 3.2.

Intact α_{s1}-CN gradually decreased during the whole storage period in all cheese samples, as can be seen in Fig 3.2a&b. This is in line with the fact that chymosin is more active in hydrolyzing α_{s1}-CN (McSweeney et al., 1993). Although the difference in pH (5.9 and 6.2) was small, significant differences in hydrolysis of α_{s1}-CN were found between cheeses with different pH. First of all, intact α_{s1}-CN fractions decreased significantly faster at a pH of 5.9, as chymosin is more active at a lower pH. After 7 weeks of storage, around 40-60% of α_{s1}-CN was hydrolyzed at pH 6.2, and around 70-

90% at pH 5.9. Secondly, chymosin content showed a more significant influence on the α_{s1}-CN fraction at pH 5.9 than at pH 6.2. This also confirms that chymosin had a higher activity at pH 5.9. In general, plasmin had no significant influence on the breakdown of α_{s1}-CN, independently of the pH, though a slight difference was found at pH 5.9 after 3 weeks of storage (Fig 3.2b). The differences between inactive and active plasmin systems were actually limited at longer storage time.

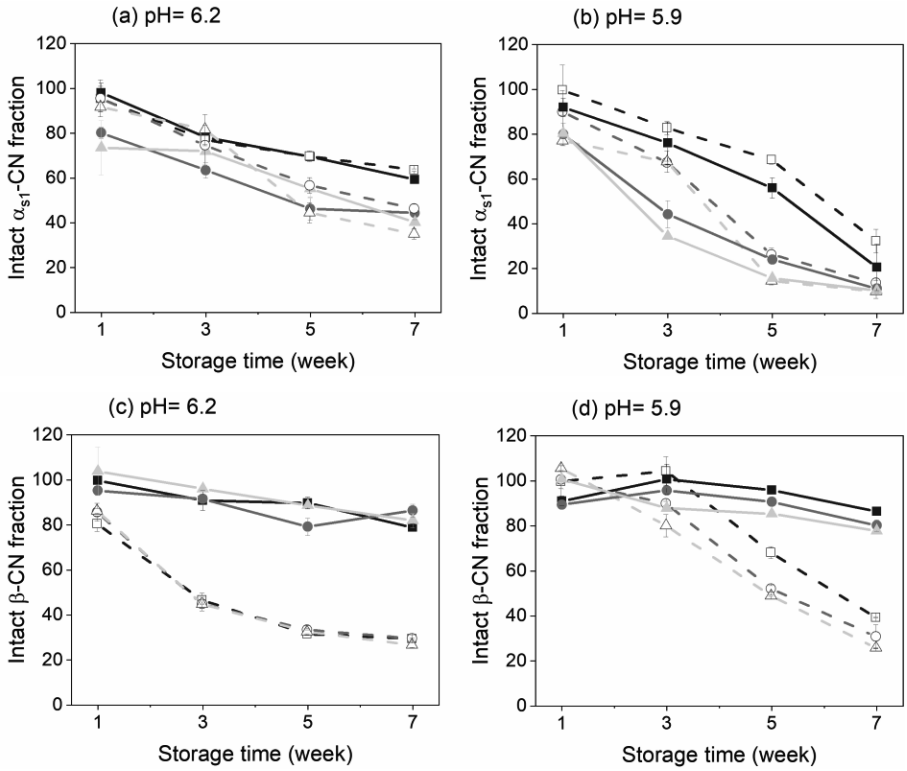

Figure 3.2. Intact α_{s1}-CN (a, b) and β-CN (c, d) as a result of protein hydrolysis at different pH (left: 6.2, right: 5.9) in model cheeses during 7 weeks of storage, determined by RP-HPLC. Cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 µl/kg milk: ■, □; 50 µl/kg milk: ●, ○; 80 µl/kg milk: ▲, △) were added.

For cheeses with inactive plasmin, β-CN stayed mostly intact both at pH 6.2 and pH 5.9 (solid lines in Fig 3.2c&d). As expected, intact β-CN fractions rapidly decreased when plasmin was active in the cheese (dashed lines in Fig 3.2c&d). The degradation of β-CN

was affected by the small difference in pH: for a higher pH (6.2), a faster degradation of the β-CN fraction was found, due to the higher activity of plasmin at this pH. However, after 7 weeks of storage, the degradation of β-CN was similar at pH 6.2 and 5.9, with values of 70-73% and 61-73%, respectively. We also found a synergistic effect between chymosin and plasmin on the breakdown of β-CN at pH 5.9, which is in line with the previous results on the protein fraction soluble at pH 4.6 (Fig 3.1b). According to literature, the cleavage site that is sensitive to chymosin, Leu_{192}–Tyr_{193}, is located at the hydrophobic C-terminal region of β-CN (Møller et al., 2012). Two of the released ϒ-CNs, β-CN f(106-209) and f(108-209), exposed the C-terminal region, which might enhance the accessibility of chymosin to the bonds of Leu_{192}–Tyr_{193} on β-CN. As a result, a higher chymosin content was able to provide a higher degradation of β-CN and a higher amount of water soluble protein fraction at pH 4.6 when plasmin was also active in cheese at pH 5.9.

Overall, the results showed that the hydrolysis of β-CN and α_{s1}-CN in our model cheese during storage depended on the activity of both plasmin and chymosin, which for both enzymes was influenced by pH. At pH 5.9, a synergistic effect of chymosin and plasmin on the hydrolysis of β-CN was seen. The released small and intermediate-sized fragments, known as the protein fraction soluble at pH 4.6, were mainly resulting from the hydrolysis of β-CN by plasmin. As the breakdown of intact casein fractions at different storage time changed the structural organization of the protein network, this was also expected to influence the rheological and textural properties of the model cheeses.

3.3.3 Rheological and textural properties of cheese during storage

3.3.3.1 Rheological properties

To reveal how the properties of the protein network changed during storage, small amplitude oscillatory shear (SAOS) tests were carried out. The critical strain and the storage modulus (G') at the critical strain of model cheeses are shown in Fig 3.3. The critical strain refers to the strain at which permanent damage or fracturing of the

microstructure starts to occur (Fox et al., 2017a), whereas the storage modulus represents a measure for the rigidity of the network.

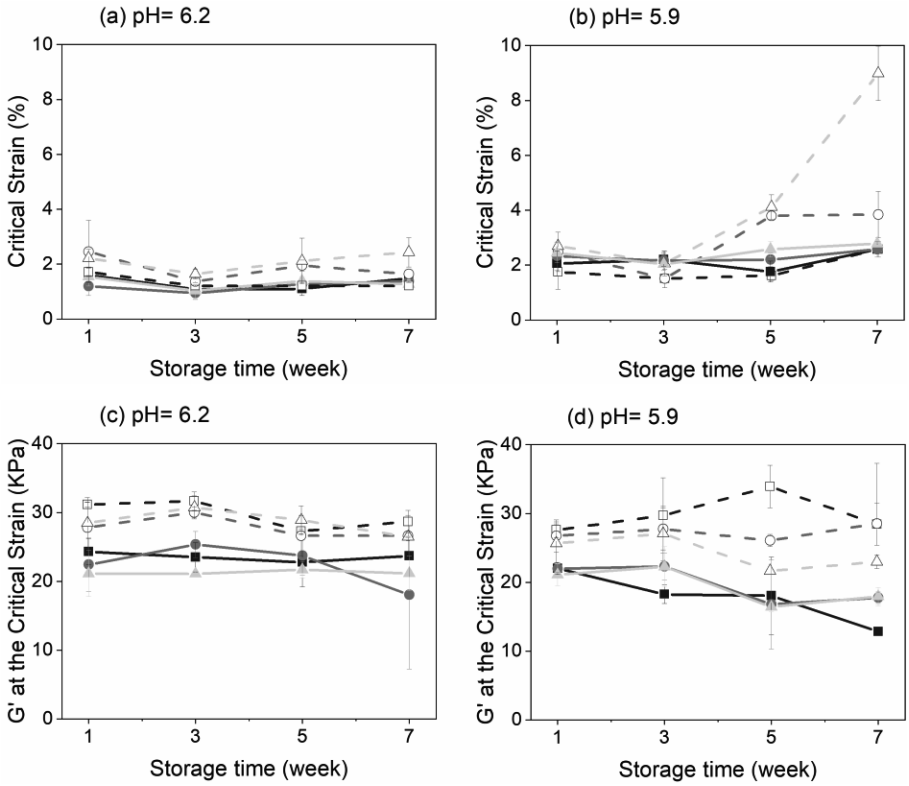

Figure 3.3. Critical strain (a, b) and G' at the critical strain (c, d) of model cheeses during 7 weeks of storage, determined by strain sweep test. Left: pH=6.2, right: pH=5.9. Cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 µl/kg milk: ■, □; 50 µl/kg milk: ●, ○; 80 µl/kg milk: ▲, △) were added.

Our results showed that at pH 6.2, when plasmin was inactive, critical strain and G' at the critical strain remained constant (solid lines in Fig 3.3a&c). With active plasmin, the G' were slightly higher (dashed lines in Fig 3.3c), indicating a stronger network compare to the network of cheese with inactive plasmin. This will be discussed in more detail in the next section. At pH 5.9 (higher activity of chymosin), when plasmin was inactive, a slight decrease in G' with ongoing storage time was observed (solid lines, Fig 3.2d).

This indicates that a higher chymosin activity slightly reduced the strength of the protein network. At pH 5.9, also a synergistic effect between plasmin and chymosin on the protein network was clearly shown (see dashed lines in Fig 3.2b). A significant influence of chymosin content on the critical strain was shown only when plasmin was active, as in this case a higher critical strain was seen for higher chymosin content. This may be associated with the synergistic effect of plasmin and chymosin on the hydrolysis of β-CN. This will be discussed in more detail in the next section.

3.3.3.2 Textural properties

To further reveal changes in textural properties during storage, the results of large deformation compression test and TPA are given in this section. The results are shown in Fig 3.4-3.6.

3.3.3.2.1 Textural properties from large deformation compression test

Similar to the results of G', at pH 6.2 (lower activity of chymosin) the Young's modulus remained constant, while at pH 5.9 (higher activity of chymosin) a decrease in the Young's modulus was shown during storage. Although cheese with active plasmin had a stronger network (higher G' in Figure 3.3), this was not seen form the result of Young's modulus, as the cheese with active and inactive plasmin had similar values for the Young's modulus, both at pH 5.9 (Fig 3.4a) and 6.2 (Fig 3.4b). Likely, chymosin dominated the changes of the Young's modulus.

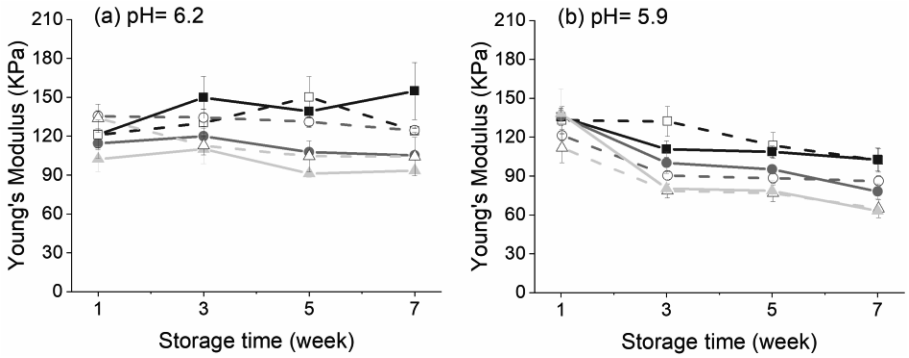

Figure 3.4. Young's Modulus of model cheeses during 7 weeks of storage at pH 5.9 (a) and at pH 6.2 (b), determined by a compression test. Cheeses were made with (inactive plasmin, solid

line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 µl/kg milk: ■, □; 50 µl/kg milk: ●, ○; 80 µl/kg milk: ▲, △) were added.

When the cheese was subjected to a large strain outside the linear range, the bonds among structural elements in the cheese matrix (e.g. intact casein fractions and peptides) were extensively broken. One of the parameters that describe the properties in this range was the stress at a strain of 40%, which we define here as the hardness. The results are shown in Fig 3.5. At pH 6.2, hardness_40% was higher in cheese with inactive plasmin (solid lines, Fig 3.5a) and decreased with increasing chymosin content (dashed lines, Fig 3.5a). At pH 5.9, when chymosin was more active, hardness_40% rapidly decreased during storage, and the influence of plasmin became less significant, as the difference in hardness_40% became less evident between cheese with inactive (solid lines, Fig 3.5b) and active (dashed lines, Fig 3.5b) plasmin. These results show that both plasmin and chymosin affected hardness_40%, but that chymosin played a more pronounced role.

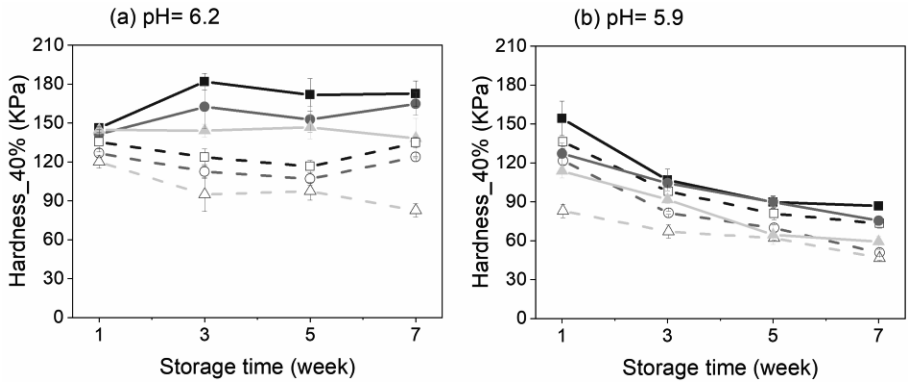

Figure 3.5. Hardness_40% of model cheeses during 7 weeks of storage determined by a compression test. (a) pH=6.2, (b) pH=5.9. Cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 µl/kg milk: ■, □; 50 µl/kg milk: ●, ○; 80 µl/kg milk: ▲, △) were added.

It has been discussed in literature that the structural elements in the cheese matrix (e.g. intact casein fractions and hydrolyzed products) start to move and rearrange to resist further deformation (Huc et al., 2014; Joyner et al., 2018). This can lead to strengthening/softening of the material, known as strain hardening/softening

behavior (Bast et al., 2015; Sharma et al., 2018). Strain hardening behavior was seen in our study, showing as a more than linear increase in the stress as a function of strain. The rate of the rearrangements occurring outside the linear region is often described as a strain hardening index (SHI). Although differences in hardness_40% related to the activity of the enzymes were obtained, no significant difference in SHI were found for the studied samples (Fig S3.1 in supplementary information). Also for even larger deformation, leading to fracture events, no effects of plasmin and chymosin were observed. All cheeses showed similar fracture strain, around 61.8 ± 3.6% (Fig S3.2 in supplementary information), and fracture stress, around 300.0 ± 97.7 KPa (Fig S3.2 in supplementary information). So even though changes in the casein network due to hydrolysis led to differences in textural properties and some rheological properties, the effect on large deformation was limited.

3.3.3.2.2 Textural properties obtained from TPA test

Besides the large compression test, a TPA (double compression) test was also conduced to gain more information of textural properties. The results are shown in Fig 3.6. At pH 6.2, when plasmin was inactivated, both resilience and cohesion remained constant during storage (solid lines in Fig 3.6a&c). This may be attributed to the low activity of chymosin and the inactive plasmin. In the case that plasmin was active, resilience and cohesion decreased rapidly (dashed line in Fig 3.6a&c). Same phenomenon was observed also at pH 5.9 (Fig 3.6b&d). The decrease in resilience and cohesion was in fact more pronounced at pH 5.9 due to a synergistic effect of plasmin and chymosin. Also an increase in chymosin induced a decrease in cohesion and resilience. We thus conclude that both chymosin and plasmin showed influence on resilience and cohesion.

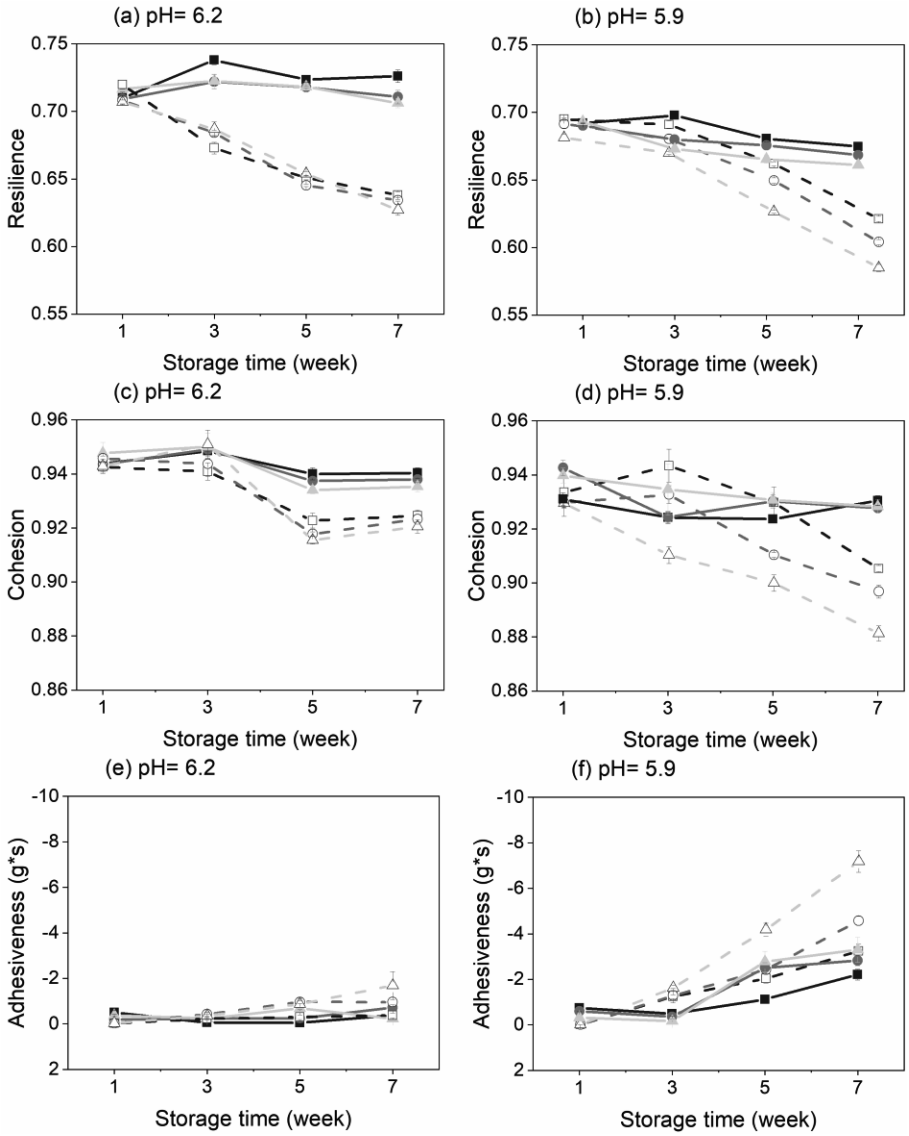

Figure 3.6. Resilience (a, b), cohesion (c, d) and adhesiveness (e, f) of model cheeses during 7 weeks of storage, determined by a TPA test. Left: pH=6.2, right: pH=5.9. Cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 µl/kg milk: ■, □; 50 µl/kg milk: ●, ○; 80 µl/kg milk: ▲, △) were added.

Concerning adhesiveness, the effect of pH was larger than that of the type of enzyme (see Fig 3.6e&f). In fact, adhesiveness significantly increased after 3 weeks of storage at lower pH (5.9). The same trend was observed by Watkinson et al. (2001), who

investigated the effect of pH (5.2-6.2) on the textural properties of a semi-hard full-fat model cheese. In their study, adhesiveness rapidly increased after 28 days of ripening at low pH, while limited change was found at higher pH. Fig 3.6f shows that both chymosin and plasmin had an impact on the adhesiveness, especially after 7 weeks, as both samples with inactive plasmin and active plasmin showed an increase in adhesiveness.

To conclude, chymosin had more effect on the Young's modulus while plasmin played a key role in the decrease of resilience and cohesion during storage. Adhesiveness was mainly correlated with the pH, even though both chymosin and plasmin were responsible for an increase in adhesiveness. Also hardness_40% was highly dependent on both chymosin and plasmin. However, parameters related to larger deformation, such as the strain hardening index, fracture strain and fracture stress, were not affected by the enzymes in our study.

3.3.4 Linking casein hydrolysis to rheological and textural properties

The results presented above show how plasmin and chymosin affected the rheological and textural properties of our model cheeses through changes in the protein network induced by casein hydrolysis. To gain a better understanding of the role of the different casein fractions, the obtained rheological and textural parameters were plotted as a function of the degree of casein hydrolysis (protein fraction soluble at pH 4.6) and the intact casein fractions, respectively. As at pH 5.9 the degradation of α_{s1}-CN was more extensive and the intact β-CN fraction decreased more gradually with ongoing proteolysis, we chose to present only the results at this pH value (Figure 3.7-3.9). An overview of all results is provided in Fig S 3.3-3.8 in the supplementary information.

A decrease in G' and Young's modulus was found with an increase of the hydrolysis of both α_{s1}-CN (Fig 3.7b&e) and β-CN (Fig 3.7c&f). This decrease was independently on whether plasmin was active or inactive. Therefore, we believe that the decrease in G' and Young's modulus was dominated by the breakdown of α_{s1}-CN. The same

phenomenon was seen at pH 6.2, although the difference were less pronounced (Fig S3.4-S3.5). The plots with pH 4.6 soluble protein, presented in Fig 3.7a&d, are similar to the plots with β-CN (Fig 3.7c&f). This confirms that the soluble protein fraction was dominated by plasmin-induced hydrolysis of β-CN.

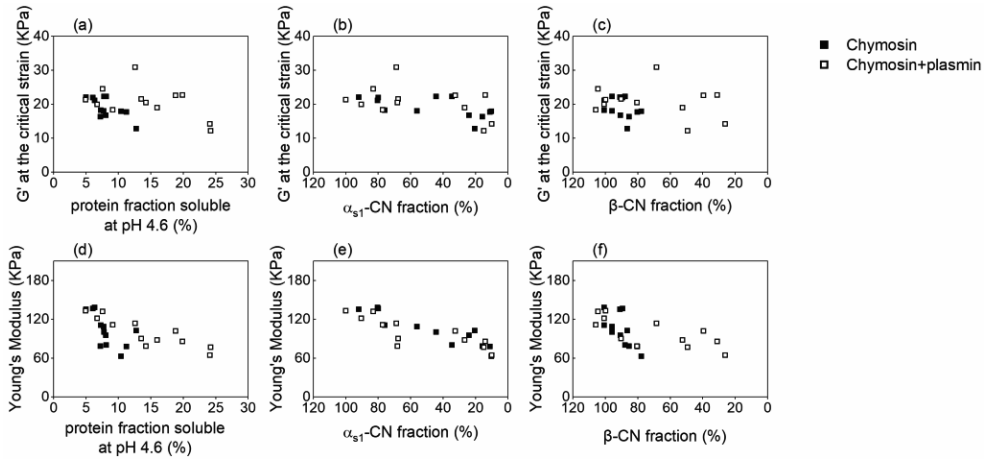

Figure 3.7. G' at the critical strain (a, b, c) and Young's modulus (d, e, f) as a function of the protein fraction soluble at pH 4.6 and the intact casein (α_{s1}- and β-CN) fraction, in model cheeses made at pH 5.9, with inactive (■, chymosin) and active plasmin (□, chymosin + plasmin).

In general, hydrolysis of intact casein decreases the structural integrity of casein micelles (Gagnaire et al., 2001) and consequently changes the skeleton network of the cheese matrix. Thus, the strength of the protein network was supposed to decrease with a reduction of intact casein. This was clearly shown for hydrolysis of α_{S1}-CN. However, the hydrolysis of β-CN showed less impact. As result of the hydrolysis of β-CN by plasmin, a rearrangement of the protein network is expected to occur as a result of hydrophobic interactions among ϒ-CNs (Lucey et al., 2003) and thus new-bonds were formed, which was expected to remain the strength of the network. This is in line with our findings in Fig 3.3, showing that the G' for cheeses with active plasmin was higher than those with inactive plasmin. On the other hand, no extra rearrangement of the protein network occurred when α_{s1}-CN was hydrolyzed, as the main hydrolyzed product α_{s1}-CN (f24–199) was a large fragment and was not easy to move within the

network. Therefore, the decrease in the strength of the protein network was more related to the intact α_{s1}-CN fraction than the β-CN fraction.

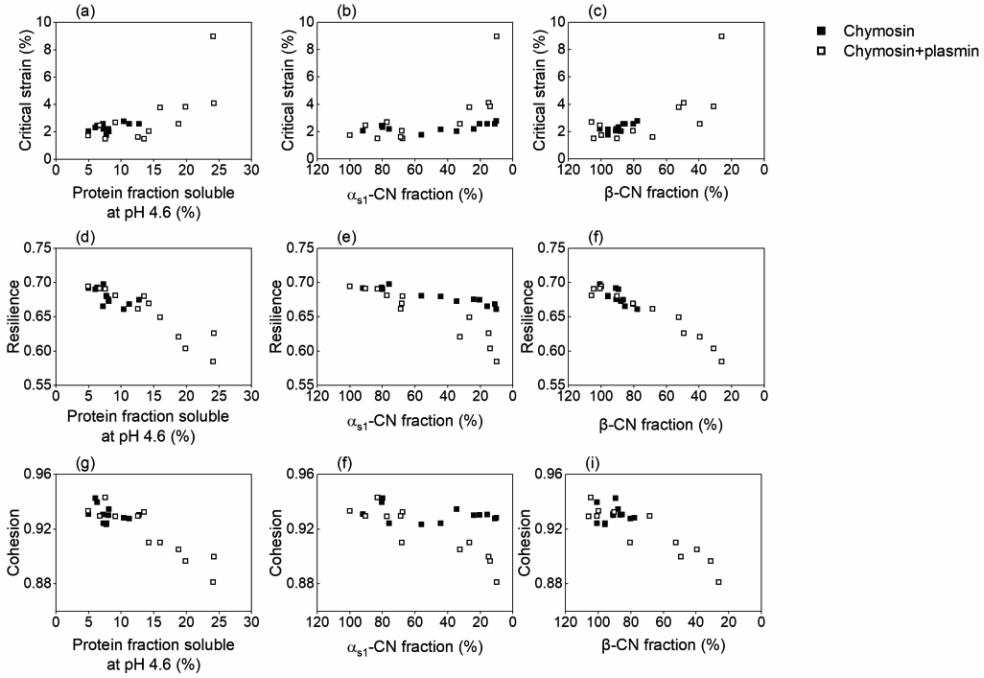

Figure 3.8. Critical strain (a, b, c), resilience (d, e, f) and cohesion (g, h, i) as a function of the protein fraction soluble at pH 4.6 and the intact casein (α_{s1}- and β-CN) fraction, in model cheeses made at pH 5.9, with inactive (■, chymosin) and active plasmin (□, chymosin + plasmin).

Our results showed that the hydrolysis of β-CN played a crucial role in the changes of three parameters, as critical strain, resilience and cohesion. In contrast to G' and the Young's modulus, these parameter had no clear correlations with intact α_{s1}-CN fraction in cheeses with only chymosin active (black squares in Fig 3.8b, e, h). However, these parameters significantly changed with the occurrence of β-CN hydrolysis by plasmin (white squares in Fig 3.8c, f, i). That the hydrolysis of intact β-CN induced an increase in critical strain can be attributed to the new (hydrophobic) interactions among hydrolyzed products. No significant changes in the critical strain was found when only intact α_{s1}-CN was hydrolyzed, as no extra rearrangement of the network occurred. In addition, the synergistic effect of chymosin and plasmin was responsible for the

increase in critical strain, since the change in critical strain was not visible at pH 6.2 (Fig S3.5a). As we mentioned above, in presence of plasmin, chymosin is able to further hydrolyze β-CN (Fig 3.2d) and more hydrolyzed fragments are released (Fig 3.1b). Consequently, the rearrangement was enhanced due to the interactions among these hydrolyzed fragments. As a result, more bonds formed and the critical strain increased rapidly.

The other two parameters resilience and cohesion are associated with the occurrence of permanent network damage, which is caused by the breakage of bonds under deformation of strain 20%. The hydrophobic interactions among the hydrolyzed ϒ-CNs after rearrangement were likely insufficient to resist the deformation of strain 20%. This can be explained by the fact that the fragments (ϒ-CNs) involved in the relevant bonds were relatively short and thus the bonds tended to easily break. Thus, resilience and cohesion showed strong correlation with the hydrolysis of β-CN (Fig 3.8f&i). When α_{s1}-CN was broken down, a rearrangement of protein network was not visible and likely no new-bonds were formed. Thus, we observed that resilience and cohesion remained constant (Fig 3.8e&h). When comparing systems in which both enzymes were active with those in which plasmin was inactive (Fig 3.8 and Fig S3.6), we found that the synergistic effect between chymosin and plasmin led to a faster decrease in resilience and cohesion. This might be explained by a higher number of broken bonds when both enzymes were active.

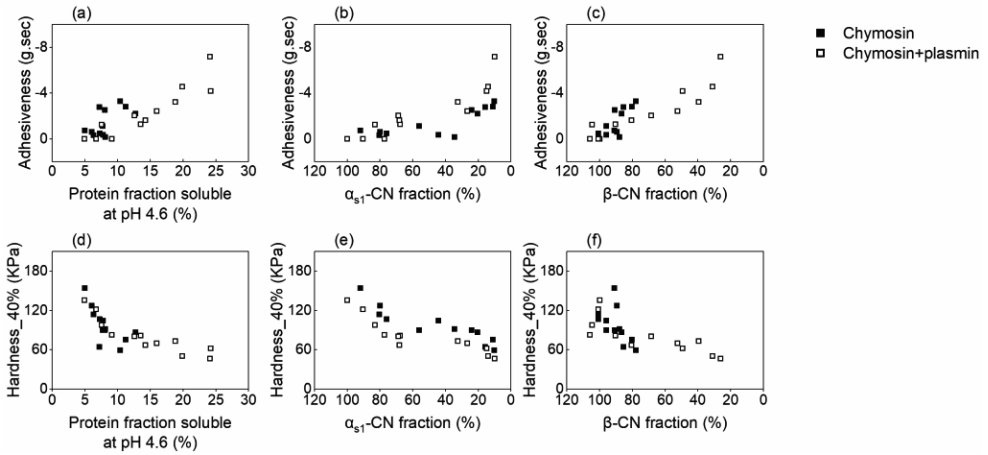

Figure 3.9. Adhesiveness (a, b, c) and Hardness_40% (d, e, f) as a function of the protein fraction soluble at pH 4.6 and the intact casein (α_{s1}- and β-CN) fraction, in model cheeses made at pH 5.9, with inactive (■, chymosin) and active plasmin (□, chymosin + plasmin).

We saw that both the hydrolysis of α_{s1}-CN fraction and β-CN fraction were associated with two parameters as adhesiveness and harness_40% (Fig 3.9), although for adhesiveness this was only visible at pH 5.9. The strong correlation between adhesiveness and soluble protein faction was in agreement with the fact that the hydrolysis products, e.g. peptides, potentially enhance the adhesiveness of casein products, which has also been shown by others (Bye, 1990). This was attributed to the high absorption energy of products with more hydroxyl groups after hydrolysis (Clerc et al., 2017). Therefore, adhesiveness increased both with hydrolysis of α_{s1}-CN and β-CN. Our study also highlights the importance of pH on the changes of adhesiveness of cheese during storage. That the synergistic effect between chymosin and plasmin leads to more release of hydrolyzed products explains the rapid increase in adhesiveness at pH 5.9, while this was not seen in samples at pH 6.2 in which plasmin was inactive (Fig S3.6e). Also for the parameter hardness_40%, the hydrolysis of both α_{s1}-CN and β-CN played a role. Hardness_40% rapidly decreased with a decreasing intact α_{s1}-CN fraction (black squares in Fig 3.9e) when plasmin was inactive. When plasmin was active, the hydrolysis of β-CN led to lower hardness_40% as well (white squares in Fig 3.9f). This

indicates that both the breakdown of α_{s1}-CN and β-CN contributes to the cheese softening during storage, while the effect from α_{s1}-CN was more dominant. The application of stress (hardness_40%) would break both the bonds between hydrolyzed products and the bonds between intact caseins. Especially the α_{s1}-CN fractions showed a great impact on hardness, as α_{s1}-CN has a high number of phosphate groups. This allows α_{s1}-CN to generate more bonds with colloidal calcium phosphate nanoclusters, which helps to form the core of casein micelle (Dalgleish and Corredig, 2012; Huppertz et al., 2017).

Overall, the change in rheological and textural properties of our samples highly depended on the hydrolysis of specific casein fractions (α_{s1}- and β-CN) by plasmin and chymosin. The hydrolysis of α_{s1}-CN dominated the decrease in G' and Young's modulus, which are parameters related to the strength of the protein network. The rearrangements of protein network led to the formation of new-bonds, and thus induced increase in critical strain. Parameters related to rearrangements of the protein network, as resilience and cohesion, were affected by interactions among hydrolyzed fragments, and decreased with the hydrolysis of β-CN by plasmin. Adhesiveness was shown to depend on the pH of the system and increased with the hydrolyzed products released both from α_{s1}-CN and β-CN. Hardness_40% of cheese was altered by the breakdown both of α_{s1}-CN and β-CN, and the intact α_{s1}-CN fraction played a greater role than β-CN. Due to a synergistic effect, the hydrolysis of β-CN was enhanced when both plasmin and chymosin were present, and more hydrolyzed fragments were released. In this case, a significant increase in critical strain and adhesiveness and a faster decrease in resilience and cohesion were observed. The findings of the present study offer new insights into the mechanisms behind textural changes resulting from proteolysis, by taking the role of both α_{s1}- and β-CN hydrolysis into account. Such knowledge may help to control the specific textural properties by modulating the activity of chymosin, plasmin or both.

3.4 CONCLUSION

In this study, we investigated the relations between specific casein fractions (α_{s1}-CN and β-CN) hydrolyzed by chymosin and plasmin and physical (rheological and textural) properties in model cheeses. Our results revealed the individual role of the hydrolysis of α_{s1}-CN and β-CN in the changes of different physical properties. The breakdown of intact α_{s1}-CN led the weakening of the protein network. The hydrolysis of β-CN and the hydrolyzed products played a critical role in the rearrangement of the protein network. This was more pronounced when more hydrolyzed products were released due to a synergistic effect when both chymosin and plasmin were active. Hardness depended on both the hydrolysis of α_{s1}-CN and β-CN, but the intact α_{s1}-CN fraction had a larger impact. That the hydrolysis of specific casein fractions (α_{s1}-CN and β-CN) showed different effects on each physical property can be explained by the different hydrolysis patterns caused by chymosin and plasmin. The findings help to better understand the texture development arising from the hydrolysis of different casein fractions, which may be used to control cheese texture and to design new products with desired textures.

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SUPPLEMENTARY INFORMATION

Table S3.1. Changes of pH during 7 weeks of storage. Experimental model cheeses were produced with 20 µL/kg, 50 µL/kg and 80 µL/kg chymosin, with(+) and without(-) aprotinin, at pH 6.2 and pH 5.9.

Cheese sample	Storage time (week)			
	1	3	5	7
pH 6.2, aprotinin (-), C20	6.22 ± 0.01^{b}	6.19 ± 0.00^{b}	6.17 ± 0.03^{b}	6.22 ± 0.01^{b}
pH 6.2, aprotinin (-), C50	6.22 ± 0.02^{b}	6.22 ± 0.01^{b}	6.24 ± 0.02^{b}	6.21 ± 0.01^{b}
pH 6.2, aprotinin (-), C80	6.20 ± 0.01^{b}	6.20 ± 0.01^{b}	6.23 ± 0.01^{b}	6.24 ± 0.01^{b}
pH 6.2, aprotinin (+), C20	6.23 ± 0.11^{b}	6.21 ± 0.00^{b}	6.21 ± 0.00^{b}	6.22 ± 0.01^{b}
pH 6.2, aprotinin (+), C50	6.17 ± 0.05^{b}	6.21 ± 0.01^{b}	6.23 ± 0.00^{b}	6.22 ± 0.02^{b}
pH 6.2, aprotinin (+), C80	6.23 ± 0.04^{b}	6.22 ± 0.04^{b}	6.21 ± 0.01^{b}	6.20 ± 0.01^{b}
pH 5.9, aprotinin (-), C20	5.95 ± 0.05^{a}	5.94 ± 0.06^{a}	5.88 ± 0.01^{a}	5.87 ± 0.01^{a}
pH 5.9, aprotinin (-), C50	5.91 ± 0.02^{a}	5.90 ± 0.01^{a}	5.93 ± 0.01^{a}	5.91 ± 0.02^{a}
pH 5.9, aprotinin (-), C80	5.90 ± 0.04^{a}	5.92 ± 0.01^{a}	5.91 ± 0.01^{a}	5.93 ± 0.01^{a}
pH 5.9, aprotinin (+), C20	5.88 ± 0.03^{a}	5.92 ± 0.04^{a}	5.88 ± 0.01^{a}	5.90 ± 0.01^{a}
pH 5.9, aprotinin (+), C50	5.91 ± 0.01^{a}	5.92 ± 0.01^{a}	5.90 ± 0.05^{a}	5.91 ± 0.01^{a}
pH 5.9, aprotinin (+), C80	5.93 ± 0.04^{a}	5.92 ± 0.03^{a}	5.93 ± 0.02^{a}	5.90 ± 0.01^{a}

$^{a-b}$ *Means within a column with the same superscript were not significantly different (α = 0.05).*

Table S3.2. Changes of dry matter (%) during 7 weeks of storage. Experimental model cheeses were produced with 20 µL/kg, 50 µL/kg and 80 µL/kg chymosin, with(+) and without(-) aprotinin, at pH 6.2 and pH 5.9.

Cheese sample	Storage time (week)			
	1	3	5	7
pH 6.2, aprotinin (-), C20	37.7 ± 0.6	38.4 ± 0.8	38.8 ± 0.6	38.6 ± 0.2
pH 6.2, aprotinin (-), C50	37.6 ± 0.5	38.3 ± 0.5	38.0 ± 0.7	38.2 ± 0.6
pH 6.2, aprotinin (-), C80	38.2 ± 1.0	37.1 ± 1.0	38.0 ± 1.4	37.3 ± 0.6
pH 6.2, aprotinin (+), C20	36.5 ± 0.6^{A}	37.9 ± 0.6^{B}	37.5 ± 0.3^{B}	37.5 ± 1.4^{B}
pH 6.2, aprotinin (+), C50	36.7 ± 0.3^{A}	38.2 ± 0.5^{B}	37.6 ± 0.5^{B}	38.6 ± 0.3^{B}
pH 6.2, aprotinin (+), C80	36.8 ± 1.3^{A}	36.7 ± 0.3^{B}	37.8 ± 0.4^{B}	38.6 ± 0.3^{B}
pH 5.9, aprotinin (-), C20	37.7 ± 0.9	38.4 ± 0.8	38.8 ± 0.2	37.4 ± 1.4
pH 5.9, aprotinin (-), C50	37.6 ± 0.7	38.3 ± 0.7	37.8 ± 0.5	38.3 ± 0.6
pH 5.9, aprotinin (-), C80	38.2 ± 0.3	37.1 ± 1.3	38.1 ± 0.2	38.5 ± 1.1
pH 5.9, aprotinin (+), C20	37.5 ± 0.6	37.9 ± 1.9	37.5 ± 1.0	38.3 ± 1.4
pH 5.9, aprotinin (+), C50	37.7 ± 0.3	38.2 ± 0.6	37.6 ± 0.7	38.2 ± 0.3
pH 5.9, aprotinin (+), C80	37.8 ± 0.6	37.7 ± 1.5	37.8 ± 0.3	37.2 ± 0.5

$^{A-B}$ *Means within a row with the same superscript were not significantly different (α = 0.05).*

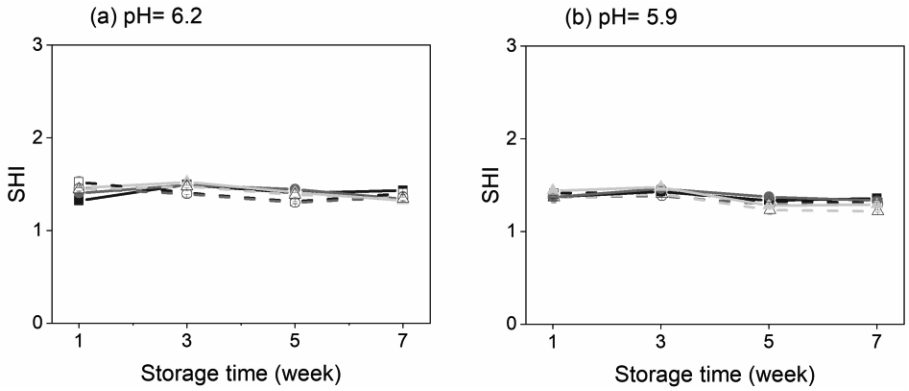


Figure S3.1. Strain hardening index of model cheeses during 7 weeks of storage, determined by a compression test. (a) pH=6.2, (b) pH=5.9. Cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 µl/kg milk: ■, □; 50 µl/kg milk: ●, ○; 80 µl/kg milk: ▲, △) were added.

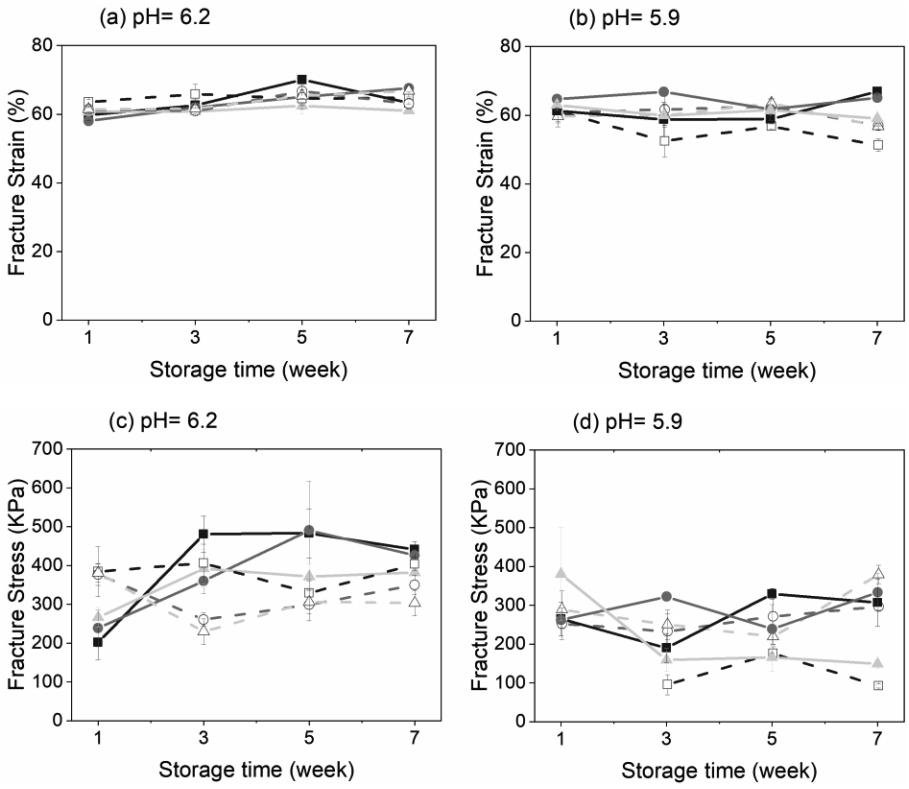


Figure S3.2. Fracture strain (a, b) and fracture stress (c, d) of model cheeses during 7 weeks of storage, determined by a compression test. Left: pH=6.2, right: pH=5.9. Cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 µl/kg milk: ■, □; 50 µl/kg milk: ●, ○; 80 µl/kg milk: ▲, △) were added.

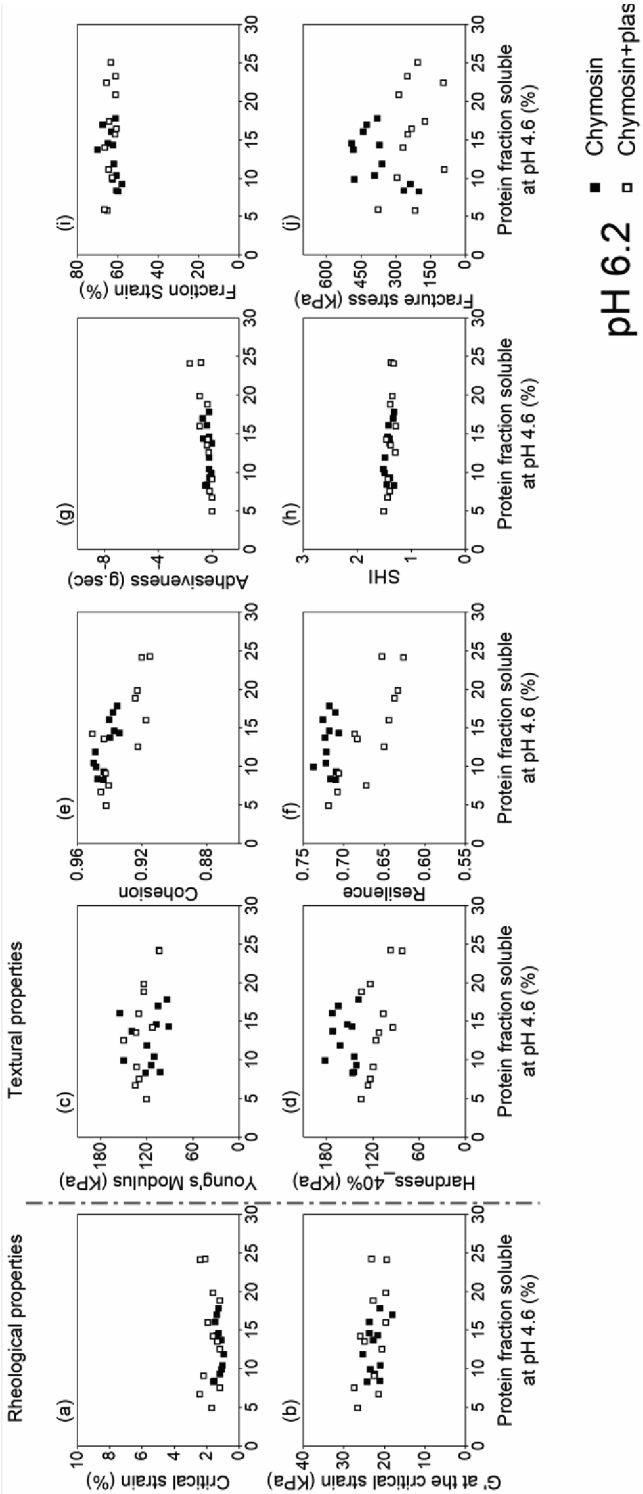


Figure S3.3. Rheological properties (a, critical strain; b, G' at the critical strain) and textural properties (c, Young's modulus; d, hardness_40%; e, cohesion; f, resilience; g, adhesiveness; h, strain hardening index; I, fracture strain; j, fracture stress) of model cheese made at pH 6.2 as a function of protein fraction soluble at pH 4.6. Data for cheese with inactive plasmin at week 1 is exclude.

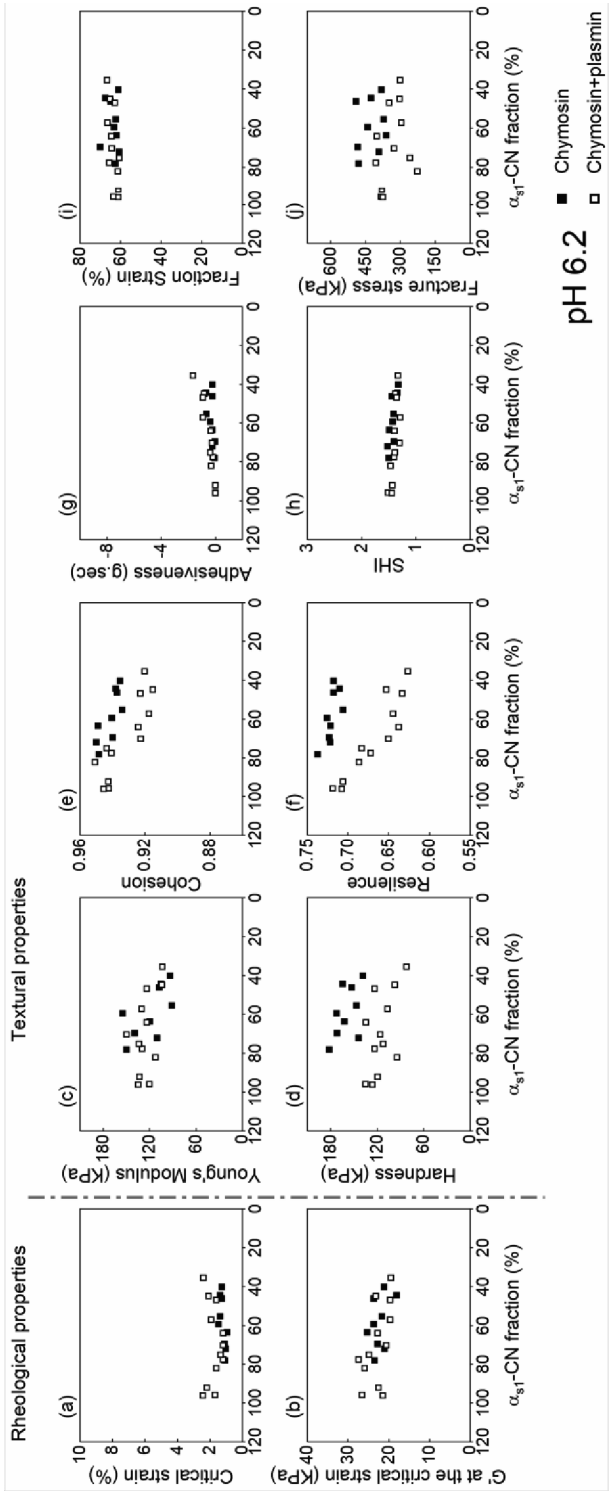


Figure S3.4. Rheological properties (a, critical strain; b, G' at the critical strain) and textural properties (c, Young's modulus; d, hardness_40%; e, cohesion; f, resilience; g, adhesiveness; h, strain hardening index; I, fracture strain; j, fracture stress) of model cheese made at pH 6.2 as a function of intact α_{s1}-CN fractions. Data for cheese with inactive plasmin at week 1 is exclude.

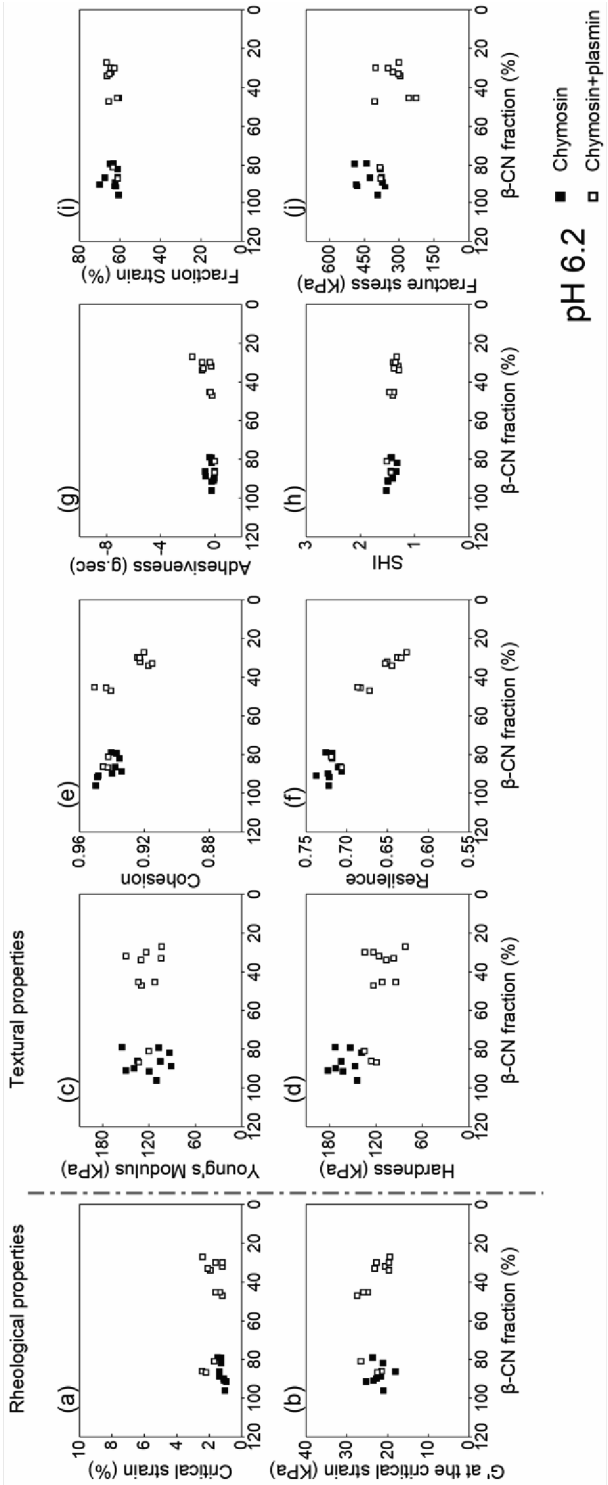


Figure S3.5. Rheological properties (a, critical strain; b, G' at the critical strain) and textural properties (c, Young's modulus; d, hardness_40%; e, cohesion; f, resilience; g, adhesiveness; h, strain hardening index; I, fracture strain; j, fracture stress) of model cheese made at pH 6.2 as a function of intact β-CN fractions. Data for cheese with inactive plasmin at week 1 is exclude.

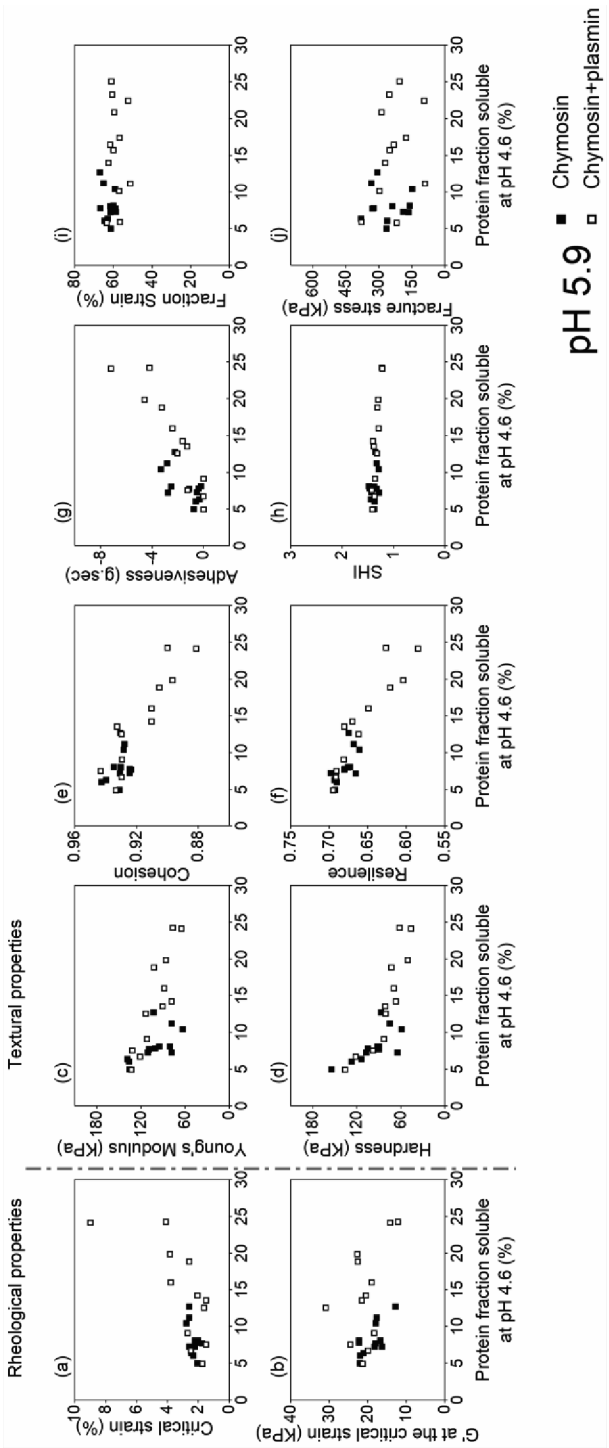


Figure S3.6. Rheological properties (a, critical strain; b, G' at the critical strain) and textural properties (c, Young's modulus; d, hardness_40%; e, cohesion; f, resilience; g, adhesiveness; h, strain hardening index; I, fracture strain; j, fracture stress) of model cheese made at pH 5.9 as a function of protein fraction soluble at pH 4.6. Data for cheese with inactive plasmin at week 1 is exclude.

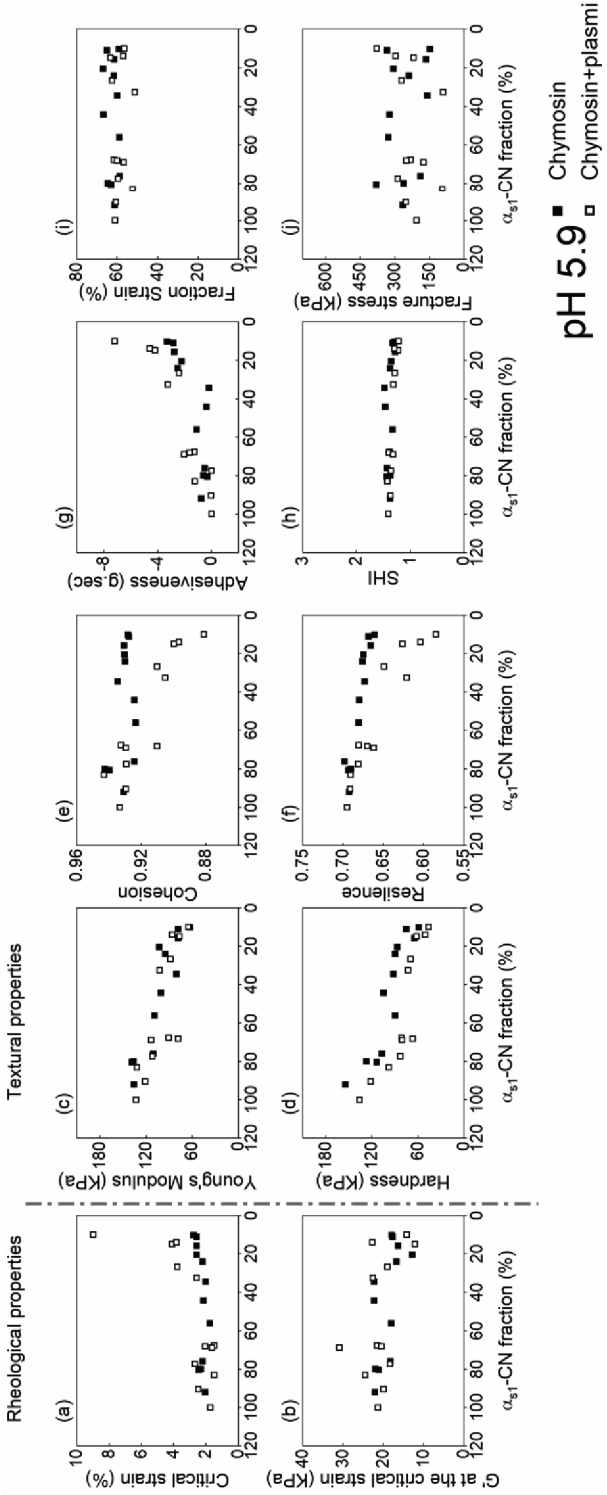


Figure S3.7. Rheological properties (a, critical strain; b, G' at the critical strain) and textural properties (c, Young's modulus; d, hardness_40%; e, cohesion; f, resilience; g, adhesiveness; h, strain hardening index; I, fracture strain; j, fracture stress) of model cheese made at pH 5.9 as a function of intact α_{s1}-CN fractions.

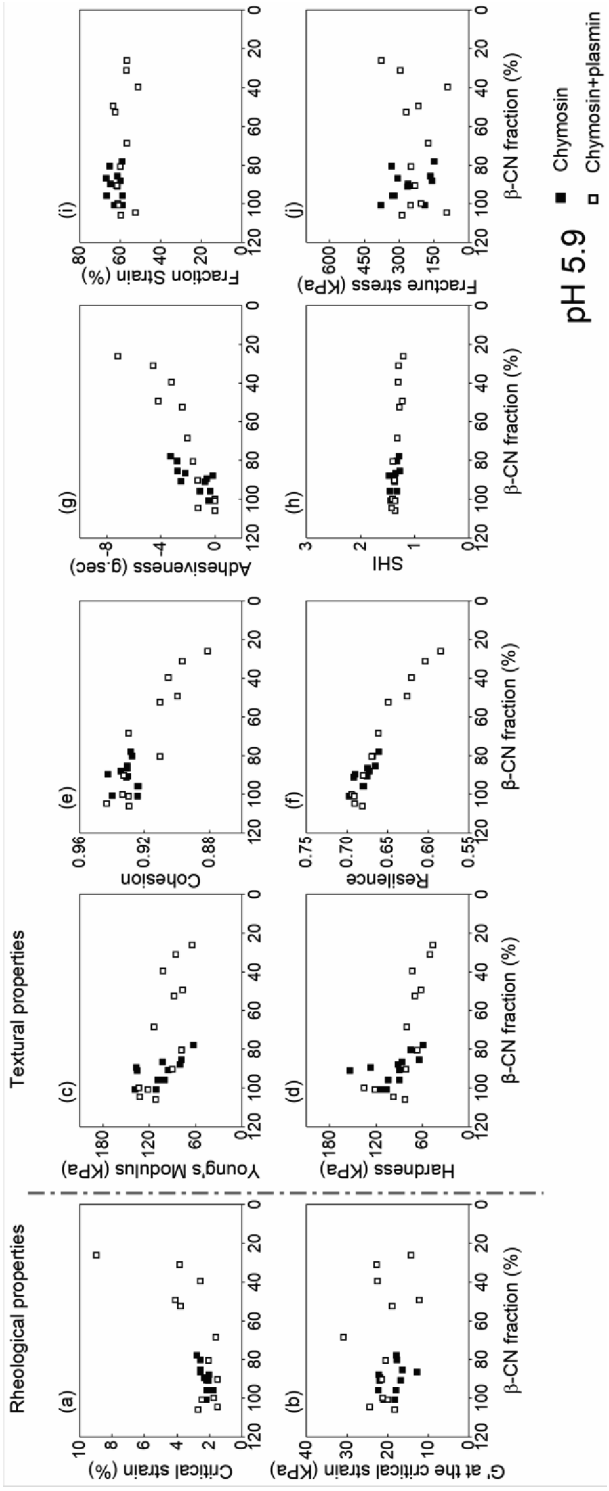
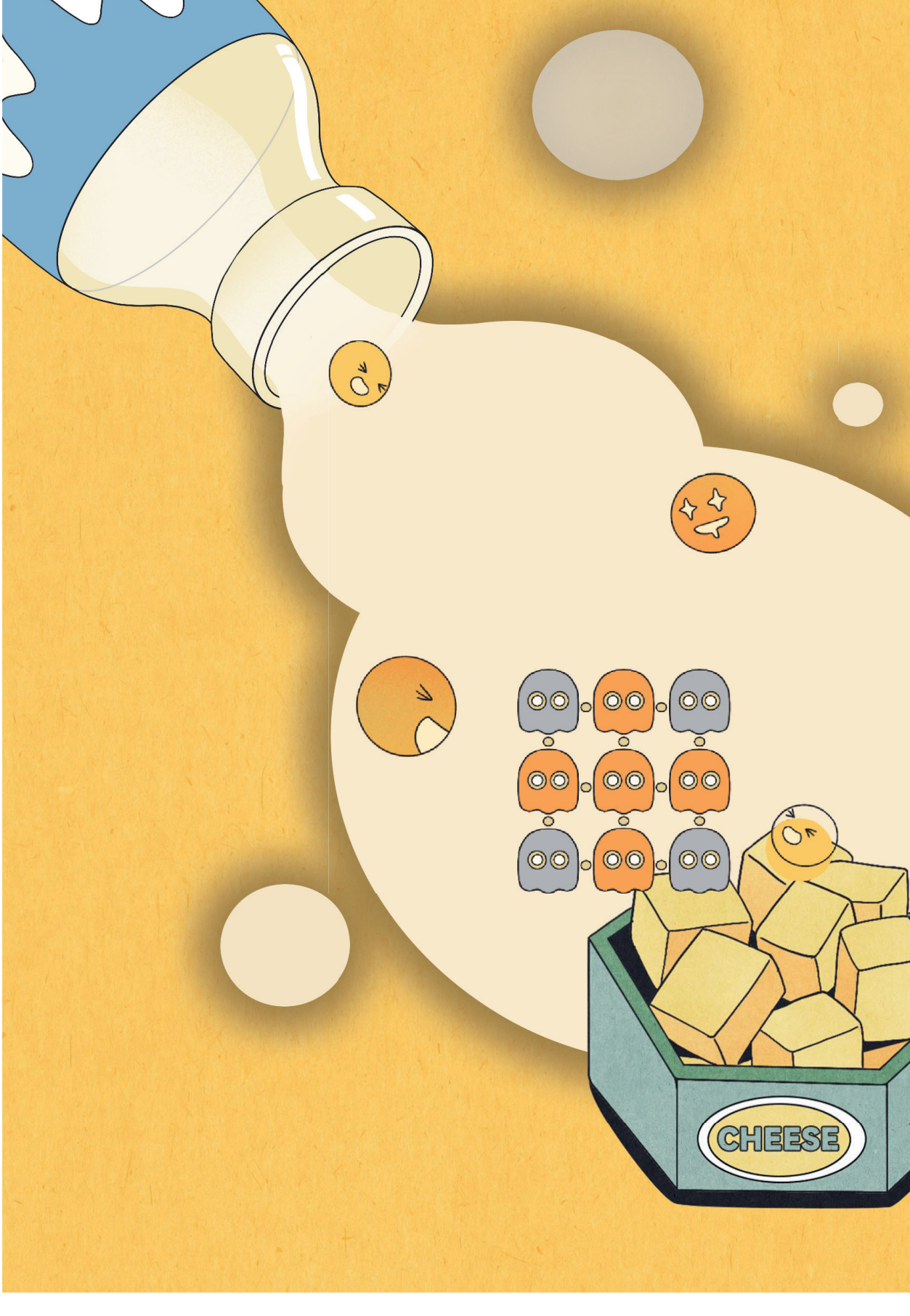


Figure S3.8. Rheological properties (a, critical strain; b, G' at the critical strain) and textural properties (c, Young's modulus; d, hardness_40%; e, cohesion; f, resilience; g, adhesiveness; h, strain hardening index; I, fracture strain; j, fracture stress) of model cheese made at pH 5.9 as a function of intact β-CN fractions


CHEESE

Chapter 4

Effect of chymosin/pepsin ratio on casein hydrolysis and physical properties of model cheeses

To be submitted as:

Huifang Cai, Etske Bijl, Huabin Luo, Elke Scholten, Guido Sala. Effect of chymosin/pepsin ratio on casein hydrolysis and physical properties of model cheeses.

ABSTRACT

Coagulants are of major importance for the cheese industry because they affect milk coagulation properties, curd composition, casein hydrolysis and the consequent development of cheese texture. Chymosin and pepsin are two major components in coagulants. Limited knowledge is available on the effect of the chymosin/pepsin ratio on the mentioned factors. This study aimed (1) to better understand the effect of chymosin/pepsin ratio on coagulation properties of milk curd, casein hydrolysis, rheological and textural properties of model cheese during storage, and (2) to link casein hydrolysis to the rheological and textural properties.

Our results showed that even when milk clotting activity was standardized, coagulants with different chymosin/pepsin ratios had significant effect on curd firming rate and firmness, which could be attributed to the different specificity of chymosin and pepsin for hydrolyzing κ-CN. During 4 weeks of storage, the chymosin/pepsin ratio influenced the hydrolysis of α_{s1}-CN, as well as the properties of the protein network and the cheese texture. For coagulant with higher proportion of pepsin, less intact α_{s1}-CN and higher degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) were found due to its high activity of hydrolyzing α_{s1}-CN. Correspondingly, the cheese had a weaker and less brittle network and was softer, less elastic and more sticky. Both the degree of casein hydrolysis and the intact α_{s1}-CN fractions showed correlations with different rheological and textural properties. Parameters related to the strength of the network (G' and Young's modulus) were mainly determined by the intact α_{s1}-CN fraction. However, parameters related to protein rearrangement, as resilience, highly depended on the hydrolyzed products. Hardness_40% (stress of strain 40%) of cheese was determined by both intact α_{s1}-CN fraction and the degree of casein hydrolysis. Overall, the findings in the study can be used as a strategy to control and design cheese with desired texture. For example, coagulant with varying chymosin/pepsin ratio or from a specific source can be used to modulate the casein hydrolysis.

4.1 INTRODUCTION

The traditional coagulant for cheese production is calf rennet and is obtained from the abomasum of recently born calves (Soodam et al., 2015; Liburdi et al., 2018; Andrén, 2021). In general, calf rennet contains both chymosin and pepsin at varying ratios, which highly depends on the feeding regime and the age of the cattle (Scott, 1986; Andrén, 2021). According to O'Connor (1993), rennet from a young milk-fed calf contains approximately 88-94% chymosin and 6-12% pepsin, while rennet from an adult bovine contains about 6-10% chymosin and 90-94% pepsin. Currently, commercial rennet used for milk clotting is extracted from mixtures of ground abomasum tissues, including calf and adult bovine (Andrén, 2021), and it is reported to contain 50 - 95 % chymosin and 5% - 50% pepsin (Winwood, 2007; Jacob et al., 2011).

In the 1950's, cheese consumption increased while the availability of calf rennet decreased, which was partly related to restrictive ethical concerns on the extraction of rennet from young calves (Garg and Johri, 1994; Sousa et al., 2001; Winwood, 2007). Consequently, the interest in calf rennet substitutes increased, and coagulants from plant and microbial sources have increasingly been investigated as potential alternatives. Today, the most used alternative coagulants are recombinant chymosins (Andrén, 2021), which are produced by fermentation of genetically modified microorganisms and comprise 70-80% of the global coagulants market according to the estimation of Johnson and Lucey (2006). Different coagulants with varying chymosin/pepsin ratios are now available on the market, and they are standardized to obtain desired milk clotting activities for various cheeses.

During curd formation, both chymosin and pepsin can hydrolyze κ-casein (κ-CN) at the bond of Phe_{105}-Met_{106}, which reduces both the net negative charge and steric repulsion of the casein micelle (Walstra, 1990; Lucey, 2002), thus leading to aggregation and eventually formation of curd (Dalgleish and Corredig, 2012). Chymosin is known to have high specificity to hydrolyze κ-CN and shows a high milk clotting activity (MCA),

while pepsin has a low MCA (Fox et al., 2017a). Based on kinetic studies using oligopeptides, the preferential cleavage of the Phe_{105}-Met_{106} bond by chymosin is believed to be a consequence of conformational changes in a region located at the entrance to the active site of the enzyme, which are not found in pepsin (Safro and Andreeva, 1990; Uniacke-Lowe and Fox, 2017). Except hydrolyzing κ-CN, chymosin and pepsin also breakdown other caseins (e.g. α- and β-CN). Pepsin is reported to have a broader proteolytic specificity towards α_{s1}- and β-CN than chymosin (Uniacke-Lowe and Fox, 2017). Thus, even though coagulants with different chymosin/pepsin ratios can have the same MCA, the properties of milk coagulated with such enzymes (such as curd firmness) may still differ as the casein hydrolysis may be different. Due to the differences in casein hydrolysis between chymosin and pepsin, the texture development of cheese in products made with different coagulants may also vary. Recent studies have focused on the effect of coagulant concentration (Madadlou et al., 2005; Santoso et al., 2020) and coagulant sources (Leite Júnior et al., 2017; García-Gómez et al., 2020) on milk gelation, casein hydrolysis, texture and sensory properties of cheese. However, knowledge on the effect of the chymosin/pepsin ratio on milk coagulation properties, curd composition, casein hydrolysis and the physical properties including cheese texture is still lacking.

The objective of this study was to understand the effect of chymosin/pepsin ratio on the link among the mentioned properties of a model cheese. Mixtures of recombinant chymosin and porcine pepsin with different ratios between the two enzymes (100/0, 80/20, 50/50, 20/80 and 0/100) were used. A precision fermentation-produced chymosin, was used, as it has been proposed as an ideal alternative to bovine chymosin due to its high MCA /proteolytic activity ratio (Kappeler et al., 2006; Alinovi et al., 2018). Porcine pepsin was used due to its similarity to bovine pepsin with respect to hydrolyzing milk caseins (Fox, 1969; Chu and Nakagawa, 1982). A bovine rennet, containing approximately 80% chymosin and 20% pepsin, was also used as comparison. During curd formation, the relative amount of released casein macropeptide (CMP),

casein degradation and rheological properties of the curd were monitored. After manufacturing, model cheeses were stored for 4 weeks at 16˚C to accelerate casein hydrolysis. During this period, dry matter, degree of casein hydrolysis (protein fraction soluble at pH 4.6), pattern of casein hydrolysis (expressed as intact casein fractions), rheological properties (critical strain, storage modulus, creep parameters) and textural properties (Young's modulus, hardness_40%, strain hardening index, resilience and adhesiveness) of the model cheeses were studied.

4.2 MATERIALS AND METHODS

4.2.1 Materials

All model cheeses were made using the same batch of pasteurized skimmed milk purchased from a local supermarket ('magere melk', Jumbo brand, the Netherlands), which, according to the supplier, contained 3.6% protein and less than 0.1% fat. Experiments on milk coagulation (section 4.2.2&4.2.3) were carried out using another batch of milk produced in the same month, with the same composition. Recombinant chymosin (CHY-MAX® M 1000, 1000 IMCU/ml; Chr. Hansen, Denmark) and pepsin from porcine gastric mucosa (P7000, 599 units/mg; Sigma-Aldrich, Germany) were used. Calf rennet (Ceska® Kalase 150, 150 IMCU/ml; CSK Food Enrichment, the Netherlands), which contains approximately 80% chymosin and 20% pepsin according to the supplier, was also used as comparison. Aprotinin from bovine lung (A1135, 3-8 TIU/mg; Sigma Aldrich, Germany) was used to inhibit plasmin activity during storage. Protein standards (β-CN and α_S-CN) and other chemicals were obtained from Sigma Aldrich.

4.2.2 Calculation of the content of added coagulant

4.2.2.1 Preparation of coagulants

Recombinant chymosin and calf rennet were diluted to 10% (v/v) in distilled water and these stock solutions were used for all experiments. A pepsin solution (1 mg/ml) was

prepared by dissolving the pepsin powder in a 0.01 M HCl aqueous solution. All coagulant solutions were stored in the fridge (4 °C) and were used within 3 days.

4.2.2.2 Determination of the content of coagulants

To exclude the potential effect of curd differences on cheese properties, coagulant with the same MCA were formulated for cheese preparation, as the curd properties highly depend on the MCA and influence syneresis during cheese making, which will consequently affect the texture of final products. As the activity of pepsin was not expressed in units similar to those of chymosin, we first used a rheological method (explained in section 4.2.3.2) to determine its MCA and to relate it to that of chymosin. Rennet clotting time (RCT), defined as the time at which the G' of milk gel reached 10 Pa, was used to indicate the MCA (McMahon and Brown, 1983; Carlson et al., 1985). Later, the content of required coagulants for cheese preparation was standardized based on a certain MCA (50 MCU/kg milk).

Four chymosin activities (10, 20, 50 and 80 MCU/kg milk) and six pepsin contents (1, 3, 4, 5, 6 and 7 mg/kg milk) were selected to make calibration curves. The calibration curves were obtained by plotting the RCT as a function of the reciprocal of MCA (or enzyme content) according to Holter–Foltmann equation (Foltmann, 1959). The required content (mg/kg milk) of pepsin to reach the same MCA (10, 25, 40, 50 MCU /kg milk) level as for chymosin was estimated based on the calibration curve of pepsin. The content of each enzyme in the studied mixtures was then calculated based on different chymosin/pepsin ratios and the same total MCA level of chymosin, i.e. 50 MCU/kg milk, which is an activity recommended for cheese making according to the chymosin supplier. The proportion of each enzyme (chymosin and pepsin) and the content in the used coagulants are presented in Table 4.1.

Table 4.1. Proportions and contents of chymosin and pepsin in coagulants with same MCA level (50 MCU/kg milk).

Sample	Chymosin (100 MCU/ml)		Pepsin (1 mg/ml)	
	Proportion (%)	Content, ml/kg milk	Proportion (%)	Content, ml/kg milk
100C/0P	100	0.50	0	0.00
80C/20P	80	0.40	20	0.77
50C/50P	50	0.20	50	1.90
20C/80P	20	0.10	80	2.71
0C/100P	0	0.00	100	3.30

To compare the results obtained with the chymosin-pepsin mixtures prepared by us to those of a calf rennet, a coagulant obtained from CSK was also used. To determine the amount of calf rennet required for the experiments, a calibration curve of diluted rennet (10%) was obtained by plotting the RCT as a function of calf rennet content (range 0.05-0.5 ml/kg milk). A content of 3.29 (ml/kg milk) of the diluted calf rennet (10%) was used to reach the required MCA level of 50 MCU /kg milk.

4.2.3 Analysis of casein hydrolysis and rheological properties during milk coagulation

4.2.3.1 Sample preparation

To investigate the effect of chymosin/pepsin ratio on the hydrolysis of κ-casein, the kinetics of casein macropeptide (CMP) release was studied. Samples were prepared based on the method of Giroux et al. (2015). Duplicate milk samples were heated to 33 °C, after which 0.6% (w/w) GDL was added to reduce the pH to 5.9 in 30 min. Then, coagulants with different chymosin/pepsin ratios were added. At set time intervals (3, 5, 10, 15, 30 min), the enzymatic reaction was terminated by addition of a 20% trichloroacetic acid (TCA) solution, to reach a final TCA concentration of 5%. Thereafter, the samples were centrifuged (Centrifuge 5424 R, Eppendorf, Germany) at 5000 g for 15 min at room temperature and the supernatant was collected. The supernatants

were then filtered through 0.2 µm RC filters and the relative CMP amount was determined. At a 30 min time interval, also the pellets of the curds were collected and analyzed for the degradation of α_{s1}-casein and β-casein fractions.

For the analysis of rheological properties during milk coagulation, after adding the coagulants, the milk samples (4.7 ml) were immediately transferred to the measuring cup. Low viscous paraffin oil was used to cover the samples to prevent evaporation. Then an oscillatory experiments were performed.

4.2.3.2 Determination of CMP release and casein hydrolysis during milk coagulation

The relative CMP amount in the filtered supernatants was determined using reversed-phase high-performance liquid chromatography (RP-HPLC, Thermo ScienceTM UltiMate 3000; Waltham, USA) according to a method described by de Vries et al. (2015). An Aeris 3.6 µm Widepore XB-C18 column (250×4.6 mm, Phenomenex, the Netherlands) was used. Furthermore, the degradation of α_{s1}-casein and β-casein of the collected pellets at a 30 min time interval were determined using same method, with sample preparation according to Chapter 2. Casein standards (α_S-CN and β-CN) were used as references to determine the elution times of the different major casein fractions. We focused on the results of α_{s1}-CN and β-CN as they are the major components of casein network.

The relative amount of released CMP (%) at different sampling moments was calculated based on its peak area in the chromatogram obtained from RP-HPLC analysis. The peak area of CMP of the sample made with 100C/0P at 30 min was set as 100% so that a relative comparison could be made among samples. The results of CMP at 30 min was also used to calculate the degradation of κ-casein (%), which was set as 100% for the sample made with 100C/0P. For the results obtained from the pellets collected at a time of 30 min after adding coagulant, the degradation of α_{s1}-CN and β-CN (%) was also calculated based on their peak areas. We assume that the degradation of α_{s1}-CN and β-CN (%) of the sample made with 100C/0P were negligible,

and thus they were set as 0%. All reported values for the different casein fractions are thus relative, and are not based on absolute values.

4.2.3.3 Investigation of the rheological properties during milk coagulation

Changes in the G' during curd formation were monitored with a MCR501 Rheometer (Anton Paar, Graz, Austria) using a measuring cup (CC17/Ti-3677) and a concentric cylinder geometry (CC17/Ti-3955). Oscillatory experiments were performed by applying a constant frequency of 1 Hz and a strain of 1%. Measurements were taken every 30 s for a period of 40 min at a temperature set at 33 °C, controlled by a water bath. As the coagulation already started before the rheological properties were measured, we included this additional waiting time to obtain the real coagulation time. We extracted rennet clotting time (RCT), maximum curd-firmness rate (MCFR), time at MCFR, maximum storage modulus (G'_{max}) and time at G'_{max} from the obtained data. RCT (min) was defined as the time when the G' reached 10 Pa. MCFR (Pa/min) was defined as the maximum slope of the gelation curve where the G' increases with time. G'_{max} was defined as the G' at its reached plateau values. All measurements were carried out in triplicate and average values were reported.

4.2.4 Cheese preparation

Model cheese samples were produced with coagulants containing different chymosin/pepsin ratios and calf rennet. The procedure used to prepare the model cheeses was based on the general cheese production process described in Chapter 3, with modification of the cutting time. The cutting time was set as 25 min after the addition of coagulant. Model cheeses were manufactured in duplicate cheese making trials. The weight of model cheeses from each trial was recorded to calculate the cheese yield.

4.2.5 Determination of dry matter

The dry matter of cheese whey and model cheese was determined in triplicate according to the method described by Lynch et al. (1997) and Patrignani et al. (2019).

1.0-1.5 g cheese whey or 0.3-0.4 g model cheese was dried overnight at 105 °C using a drying oven (Venticell 111, MMM Medcenter; Germany). The dry matter content was calculated based on the mass difference before and after drying.

4.2.6 Casein hydrolysis during cheese storage

4.2.6.1 Degree of casein hydrolysis

The degree of casein hydrolysis was determined as described in Chapter 2. The degree of casein hydrolysis was expressed as protein fraction soluble at pH 4.6.

4.2.6.2 Hydrolysis of intact casein fraction by RP-HPLC

The pattern of casein hydrolysis during storage was investigated using RP-HPLC in triplicate, to determine the changes of intact casein fractions in the model cheeses (de Vries et al., 2015). Sample preparation was the same as described in Chapter 2. The intact casein fractions were calculated based on their peak area. The intact specific casein fraction (%) at the different sampling moments (1, 2, 3, 4 week) was expressed as the peak area of the specific caseins (α_s-CN and β-CN) divided by the specific casein peak area of cheese made with pure chymosin at week 1.

4.2.7 Rheological and textural properties of model cheese during storage

4.2.7.1 Rheological properties

The rheological properties of cheese were investigated in triplicate using a MCR501 Rheometer (Anton Paar, Graz, Austria) at a temperature of 20 °C. A parallel-plate geometry with a smooth stainless steel plate (diameter of 25 mm, code: PP25/P2/SS) was used. To avoid slipping of samples, a serrated plate was used as lower plate. Vacuum sealed cheese was taken out from the incubator (16 °C) and was equilibrated at room temperature for 1 h. Then the cheese was cut in a cylindrical shape (diameter of 25 mm, height of 5 mm) using a stainless-steel punch of 25 mm diameter and a device with parallel stainless-steel wires of 5 mm gap. The cheese cylinders were put on the lower plate and the upper geometry was lowered until the desired normal force was reached. The normal force was set as 0.25 N for small amplitude oscillatory shear

(SAOS) analysis and 1 N for creep analysis, based on common settings used in literature (Bähler et al., 2015; Faber et al., 2017; Jõudu et al., 2017; Zad Bagher Seighalani et al., 2020). After loading the cheese sample between the two plates, the exposed surface area of cheese was covered with low viscous paraffin oil to minimize drying out during measurements. A waiting time of 1 min was set in order to relax any normal stress induced during sample loading and also to attain test temperature (20 °C) equilibrium. Then, SAOS analysis and creep measurements were performed on separate samples.

To determine the linear viscoelastic region, small amplitude oscillatory shear (SAOS) analysis was carried out at a frequency of 1 Hz with a logarithmic increase of the strain amplitude from 0.01 to 100 %. From the SAOS data, we extracted the critical strain and the G' at this strain. Critical strain was defined as the strain at which the G' changed by more than 5% from the previous value.

The time-dependent rheological behavior of cheese was evaluated using a creep test, to evaluate possible permanent failure of the protein network. Creep measurements were carried out within the linear viscoelastic region at a frequency of 1 Hz. An instantaneous stress (τ_0=300 Pa) was applied to the sample and maintained for a period of 165 s. The resultant strain (ϒ) was measured as a function of time. The results were expressed in terms of creep compliance $J(t)= \Upsilon(t)/\tau_0$, as a function of time. The creep behavior was characterized using a four-component Burger model consisting of a Maxwell and Kelvin-Voigt model in series (Burgers, 1939; Andrés et al., 2008; Karaman et al., 2016). The creep behavior is described as:

$$J(t) = J_m + J_k\left(1 - e^{-\frac{t}{\lambda_k}}\right) + \frac{t}{\eta_m} \quad (1)$$

where J(t) is the creep compliance at time t, J_m is the instantaneous elastic compliance (Pa^{-1}) of the Maxwell spring, J_k is the retarded compliance (Pa^{-1}) that represents the retarded elastic region associated with the Kelvin–Voigt element, λ_k is the retardation

time (s) related to the Kelvin–Voigt element and η_m is the Newtonian viscosity (Pa*s) associated with the Maxwell dashpot (Olivares et al., 2009b; Karaman et al., 2016).

4.2.7.2 Textural properties

One hour after taking the samples from the incubator (16 °C), the cheese was cut in a cylindrical shape (diameter and height of 1 cm). Texture profile analyses (TPA) and large deformation tests were carried out in triplicate, using a Stable Micro Systems TA-TX plus (Stable Micro Systems TA-TX plus, United Kingdom) equipped with a 50 mm diameter cylindrical probe (perspex) and a 5 kg load cell. A TPA test was performed at a rate of 2 mm/s with a strain of 20%. From the obtained TPA curves, resilience (upstroke peak area of first compression/downstroke peak area of first compression) and adhesiveness (the negative peak area between two compressions) were extracted. For large deformation tests, samples were compressed until a strain of 85 % at a rate of 2mm/s. The Young's modulus was extracted from the linear region (1-3% strain) of stress-strain curves. Even though the hardness is usually represented by the fracture stress, for some samples we could not determine this hardness as their strain-stress curves did not show a clear fracture. Instead, we used as a parameter hardness_40%, which was determined as the stress at a strain of 40%. This has also been used by others as a measure of cheese hardness (Ak and Sundaram, 1997; Alvarez et al., 2000). The strain hardening index (SHI) was calculated according to the empirical equation suggested by Kokelaar et al. (1996) and van Vliet (2008) as:

$$\sigma = a \cdot \varepsilon^{b} \quad (2)$$

where σ is the stress, a is the strength coefficient (Pa), ε is the deformation strain (-), and b is the strain hardening index (-). Eqn 2 was fitted (R2~0.98–0.99) to stress-strain data over the strain range before fracture, which was from 0.02 to 0.6.

4.2.8 Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey Post Hoc test was used to evaluate significant differences of dry matter and mechanical properties (both rheological and

textural parameters) between the six batches of model cheese in each week, using IBM SPSS Statistics 25 (IBM Corporation, NY, USA). The significance level was set at 0.05.

4.3 RESULTS AND DISCUSSION

4.3.1 Casein hydrolysis and milk coagulation properties during curd formation

4.3.1.1 Casein macropeptide (CMP) release

Six coagulants with varying chymosin/pepsin ratios but the same milk-clotting activity level (50 MCU/kg milk) were added to milk to produce model cheese samples. To assess the effect of chymosin/pepsin ratio on the hydrolysis of κ-CN during coagulation, the kinetics of CMP release was investigated.

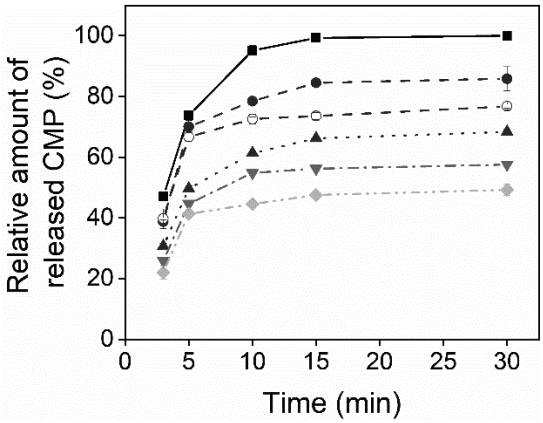


Figure 4.1. Relative amount of released casein macropeptide (CMP) as a function of time in milk gelled with coagulants with different chymosin/pepsin ratio: 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆), and calf rennet (○). All results were calculated based on the peak area. The peak area of the sample made with 100% chymosin at 30 min was taken as 100%.

As shown in Fig 4.1, the chymosin/pepsin ratio of the coagulant had a significant impact on CMP release rate and final amount of released CMP. The higher chymosin/pepsin ratio, the faster and the more CMP was released, due to the higher chymosin specificity in hydrolyzing the Phe_{105}-Met_{106} bond of κ-casein compared to

pepsin (Drøhse and Foltmann, 1989; Uniacke-Lowe and Fox, 2017). It is also interesting to observe that calf rennet, which contained approximately 80% chymosin and 20% pepsin, produced less CMP than the coagulant prepared by us with the same chymosin to pepsin ratio. This is in line with the fact that recombinant chymosin has a higher specificity in hydrolyzing κ-CN than bovine chymosin (Jacob et al., 2011; Biswas and Metzger, 2016).

4.3.1.2 Milk coagulation properties

To assess the effect of chymosin/pepsin ratio on gelation behavior, rheological experiments were performed. The results are shown in Table 4.2. No significant difference in RCT was found among coagulants with different chymosin/pepsin ratios, confirming that these coagulants had the same MCA although they contained different chymosin/pepsin ratios. Also the RCT of calf rennet was similar. However, the chymosin/pepsin ratio significantly altered MCFR, G'_{max} and time at G'_{max}; for lower chymosin/pepsin ratio, a lower MCFR, a longer time to reach G'_{max} , and a lower G'_{max} of milk curd were found. The calf rennet showed properties similar to those of the coagulant with the same composition (80C/20P).

Table 4.2. Rennet coagulation time (RCT), maximum curd firming rate (MCFR), time at maximum curd firming rate (time at MCFR), maximum storage modulus (G'_{max}), time at maximum storage modulus (time at G'_{max}) of milk curd made with coagulants with different chymosin/pepsin ratio and with calf rennet.

Sample	RCT (min)		MCFR (Pa/min)		Time at MCFR (min)		G'_{max} (Pa)		Time at G'_{max} (min)	
100C/0P	6.4±0.2	a	21.6±0.6	c	6.1±0.7	a	178.1±3.5	c	20.9±1.1	a
80C/20P	6.5±0.1	a	19.1±0.1	b	6.8±0.3	a	173.8±3.0	bc	21.6±0.0	ab
50C/50P	6.8±0.0	a	16.5±0.1	a	7.3±0.4	a	169.7±1.7	abc	23.6±1.3	bc
20C/80P	6.4±0.2	a	16.5±1.2	a	6.8±0.3	a	165.9±0.3	ab	20.9±2.7	abc
0C/100P	6.6±0.6	a	15.5±1.0	a	7.3±1.8	a	162.5±3.6	a	24.6±0.7	bc
Calf rennet	6.4±0.1	a	18.4±0.4	b	7.1±0.7	a	167.4±3.0	abc	23.6±0.4	bc

$^{a\text{-}c}$ Means within a column with the same superscript were not significantly different ($\alpha = 0.05$).

As shown in Table 4.2, the chymosin/pepsin ratio had significant impact on gelation properties (including MCFR and G'_{max}) and curd made with coagulants with lower

chymosin/pepsin ratio was less firm. These differences in protein network properties can be explained by the degradation of the different caseins. We therefore plotted the maximum milk curd firming rate (MCFR) against released CMP, as shown in Fig 4.2. There was a strong linear relation between maximum milk curd firming rate (MCFR) and the relative amount of released CMP (R^2 = 0.8990, P < 0.01). The higher the CMP release, the less κ-CN remained at the outer layer on the casein micelles. As chymosin releases more CMP, a higher chymosin/pepsin ratio thus led to faster coagulation and consequent shorter MFCR.

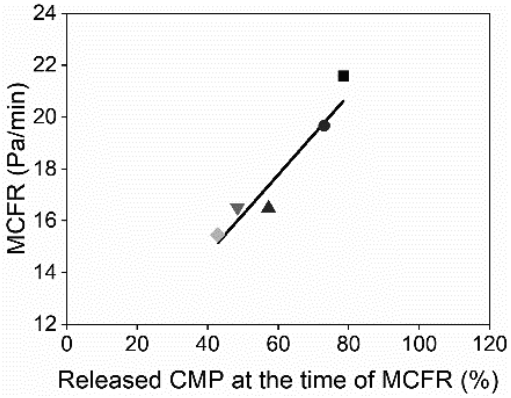


Figure 4.2. Maximum milk curd firming rate (MCFR) as a function of relative amount of released CMP at the same time. Coagulants with different chymosin/pepsin ratio were used: 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆). The line represent the best fit through the data points.

Curd firmness, as measured by G'_{max} in this study, has been suggested to be highly dependent on the composition and the network structure of casein micelles (Pellegrini et al., 1997; Mara et al., 1998; Low et al., 2006). Therefore, we plotted G'_{max} as a function of the relative degradation of the different casein fractions, given in Figure 4.3. A strong correlation between curd firmness and relative degradation of κ-CN was found as well (R^2 = 0.9150, P < 0.01). Higher degradation of κ-CN means that more CMP was released. Therefore, more interactions among the casein micelles occurred and a stronger casein network was formed. Figure 4.3 shows that the chymosin/pepsin ratio

affected the breakdown of α_{s1}-CN and β-CN during curd formation: the lower chymosin/pepsin ratio, the more α_{s1}-CN and β-CN were hydrolyzed. This may result in differences in curd firmness as well. As a matter of fact, trends were observed when plotting curd firmness versus degradation of these casein fractions, α_{s1}-CN (R^2 = 0.6321, P = 0.059) and β-CN (R^2 = 0.4522, P = 0.053). However, the relations between these parameters were not statistically significant. We could conclude that the phenomena occurring during coagulation were mainly related to κ-CN degradation. As chymosin leads to more κ-CN degradation, higher ratio of chymosin/pepsin leads to firmer curds.

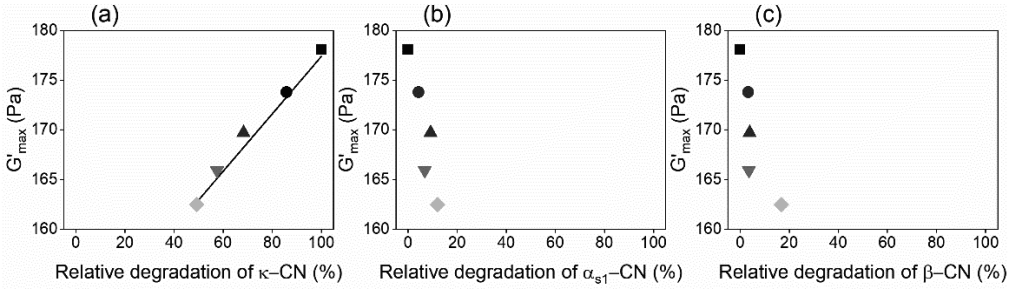


Figure 4.3. G'_{max} as a function of relative degradation of (a) κ-casein; (b) α_{s1}-casein; (c) β-casein, 30 min after adding coagulant. Coagulants with chymosin/pepsin ratio of 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆). were used. The line(a) represents the best fit through the data points.

4.3.2 Yield of cheese and dry matter in the cheese whey

As reported above, α_{s1}-CN and β-CN were degraded during curd formation when coagulants containing pepsin were used, although this degradation was not strongly related to the properties of the curd. However, the measured differences may affect the cheese yield due to the loss of casein in the whey. Thus, dry matter of the cheese whey and cheese yield were determined, and the results are shown in Table 4.3. The chymosin/pepsin ratio had no influence on the dry matter of the whey and the total cheese yield, even though pepsin has the ability to degrade caseins at different locations. Pepsin is known to have a preference for cleavage of bonds between non-polar amino acids with a large hydrophobic side, such as Phe_{23}-Phe_{24} in α_{s1}-CN and

Leu_{192}-Tyr_{193} in β-CN (Perna et al., 2020), which results in the formation of two large fragments, α_{s1}-CN (f24–199) and β-CN (f1-192), and one small hydrophobic peptide, β-CN (f193-209) (Upadhyay et al., 2004; Piraino et al., 2007). These large peptides tend to remain in the cheese curd. The accompanying small fragment α_{s1}-CN (f1-23) is water-soluble, and can thus probably be released into the whey (Piraino et al., 2007; Albenzio et al., 2015). Since only 4.2-15.4% α_{s1}-CN was degraded during sample preparation, the soluble fraction was assumed to be minimal. So, the reason why the dry matter content of the whey was not significantly affected by the chymosin/pepsin ratio in the coagulant was that the majority of the caseins and its hydrolyzed products remained in the cheese curd. Accordingly, the yields of cheeses made with coagulants with different chymosin/pepsin ratio and by calf rennet were similar.

Table 4.3. Dry matter of cheese whey and yield of model cheese, made with coagulants containing different chymosin/pepsin ratio and with calf rennet.

Sample	Dry matter of Whey (%)	Yield (%, g cheese/ 100 g milk)
100C/0P	7.5 ± 0.07	10.91 ± 0.196
80C/20P	7.5 ± 0.08	10.73 ± 0.155
50C/50P	7.5 ± 0.04	10.55 ± 0.397
20C/80P	7.5 ± 0.08	10.29 ± 0.025
0C/100P	7.5 ± 0.07	10.61 ± 0.001
Calf rennet	7.5 ± 0.07	10.82 ± 0.103

4.3.3 Dry matter and casein hydrolysis of cheese during storage

4.3.3.1 Dry matter changes during storage

During a storage time of 4 weeks, the dry matter of the cheeses remained constant at 34.1 ± 0.7%, as shown in Table S4.1 of the supplementary information. No significant difference in dry matter was found among cheese samples prepared with different coagulants during the entire 4 weeks. Different chymosin/pepsin ratios and type of coagulant had thus no impact on the dry matter of cheese.

4.3.3.2 Casein hydrolysis during storage

4.3.3.2.1 Degree of casein hydrolysis

To understand the effect of different coagulants on casein degradation, the degree of casein hydrolysis was determined over time (Fig 4.4). Although the coagulants were used upon standardization of the MCA, clear differences in casein hydrolysis were observed during 4 weeks of storage. For coagulants with higher proportion of pepsin, the degree of casein hydrolysis was higher. We also noticed that cheeses made with the three coagulants (C50/P50, C20/P80 and C0/P100) had the same trend for the degree of casein hydrolysis during storage, indicating that the effect of the enzyme ratio reached a plateau. At week 4, the protein fraction soluble at pH 4.6 ranged from 21.7% (C0/P100) to 10.8% (C100/P0). The higher degree of casein hydrolysis for lower chymosin/pepsin levels (more pepsin) can be explained by the broad proteolytic specificity of pepsin on α_{s1}-CN during storage (Upadhyay et al., 2004; Uniacke-Lowe and Fox, 2017). The calf rennet containing 80% chymosin and 20% pepsin always showed a higher degree of casein hydrolysis compared to our mixture containing 80% recombined chymosin. Bovine chymosin is more efficient in hydrolyzing α_{s1}-CN than the recombinant chymosin, which has been reported in different types of cheese, such as white brined cheese (Gumus and Hayaloglu, 2019), Cheddar cheese (Bansal et al., 2009; Soodam et al., 2015), Mozzarella cheese (Moynihan et al., 2014) and other hard/semi-hard model cheese (García-Gómez et al., 2020). Even though both bovine and recombinant chymosin show a preference for cleaving the Phe_{23}-Phe_{24} bond on α_{s1}-CN and the Leu_{101}-Lys_{102} bond on the primary hydrolysis products (α_{s1}-CN (f24-199)), bovine chymosin is also active towards bond Trp_{164}-Tyr_{165}, while recombinant chymosin is not (Møller et al., 2012). The hydrolysis of Trp_{164}-Tyr_{165} in cheese made with calf rennet resulted therefore in more hydrolysis and accumulation of smaller peptides.

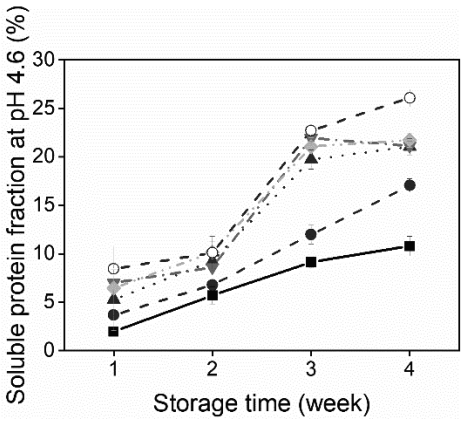


Figure 4.4. Degree of casein hydrolysis, expressed as protein fraction soluble at pH 4.6, of model cheeses during 4 weeks of storage. Coagulants with chymosin/pepsin ratio of 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆), and calf rennet (○) were used.

4.3.3.2.2 Pattern of casein hydrolysis

To understand how the pattern of proteolysis was affected by different coagulants, we investigated the changes of intact casein fractions in cheese during 4 weeks of storage (Fig 4.5). It was found that α_{s2}-CN was not degraded (data not shown). Therefore, only α_{s1}-CN and β-CN are discussed here below, as they were mostly responsible for changes in the protein network. From week 1 to week 4, α_{s1}-CN was extensively hydrolyzed, while β-CN was broken down to a limited extent (less than 20%). This can be explained by two phenomena. On one hand, chymosin and pepsin have a preference for hydrolyzing α_{s1}-CN, while they hydrolyze β-CN to a lesser extent (Ardö et al., 2017; Uniacke-Lowe and Fox, 2017). On the other hand, plasmin-induced hydrolysis of β-CN was excluded in the study, as aprotinin was added as plasmin inhibitor (Jimi et al., 1995; Y.Ardö, 2007). Limited hydrolysis of β-CN was thus obtained, and most changes were due to the degradation of α_{s1}-CN.

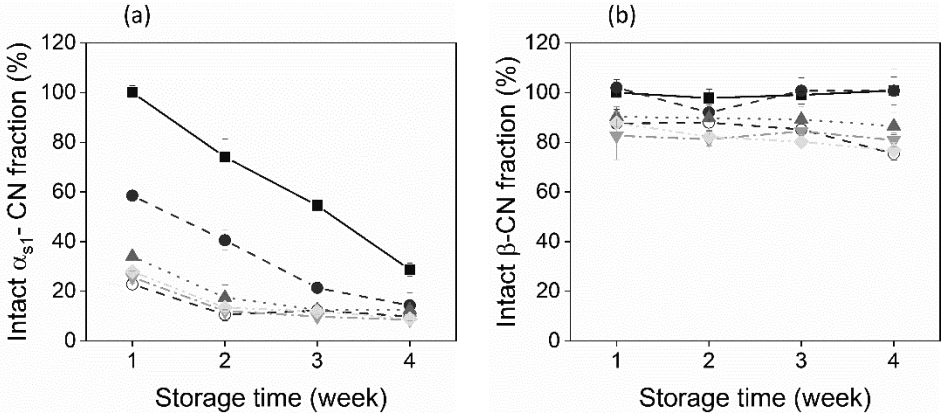


Figure 4.5. Intact (a) α_{s1}-CN and (b) β-CN as a result of casein hydrolysis in cheeses during 4 weeks of storage, determined by RP-HPLC. Coagulants with chymosin/pepsin ratio of 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆), and calf rennet (○) were used. All the casein fractions were calculated based on the peak area, the peak area of the sample made with 100% chymosin at week 1 taken as 100%.

Even though the MCA was standardized, the chymosin/ pepsin ratio had a significant effect on the pattern of casein hydrolysis during 4 weeks of storage. Fig 4.6 shows that cheese made with coagulants with lower chymosin/pepsin ratio had lower amount of intact α_{s1}-CN. This is in line with the results of protein fraction soluble at pH 4.6 (Fig 4.4). The high proteolytic activity of pepsin directly induced a high degradation of α_{s1}-CN. In case of more pepsin (ratio of 50/50, 20/80 and 0/100), it was also observed that the degradation of α_{s1}-CN became similar among samples, as the effect of pepsin reached a maximum. As we expected, cheese made with calf rennet (80% bovine chymosin) showed higher degradation of α_{s1}-CN when compared to cheese made with our mixture containing 80% recombinant chymosin. So, although the CMP release in the curd was similar (Figure 4.1), the source of the chymosin had a large effect of the casein hydrolysis during storage.

4.3.4 Rheological and textural properties of cheese during storage

To investigate how the chymosin/pepsin ratio affected the changes in the structure and cheese texture, the rheological and textural properties of the cheeses were determined during 4 weeks of storage. Rheological properties can mostly be related to the structural properties of the network (Fox et al., 2017b), while textural properties have shown a better correlation to sensory and oral processing behavior (Joyner Melito et al., 2018).

4.3.4.1 Rheological properties of cheese during storage

4.3.4.1.1 Critical strain and G'

To gain insight into the properties of the protein network, we measured the critical strain and its corresponding G'. Cheese with a higher value of critical strain is more resistant to deformation, which is related to the number, strength and type of intra- and intermolecular interactions among components (Roefs et al., 1990; Tunick and Van Hekken, 2002). G' at the critical strain is a measure of the firmness of cheese (Rogers et al., 2010).

As shown in Table 4.4, the critical strain increased from week 1 to week 4 and with decreasing chymosin/ pepsin ratio, indicating that the network became less brittle. This increase might be attributed to the rearrangements within the protein network as a result of proteolysis. With ongoing casein hydrolysis, some of the hydrolyzed fragments and peptides remained in the network due to hydrophobic and electrostatic interactions. When a deformation was applied, these hydrolyzed products started moving and induced the formation of new bonds. These new-formed bonds aided in resisting the deformation. However, these new-formed bonds were relatively weak and had less effect on the firmness of the protein network, represented as G'. G' decreased during 4 weeks storage for all model cheeses, and decreased more for lower chymosin/pepsin ratios, which means that the protein network of the samples was weakened and cheese became less stiff

Table 4.4. Critical strain and G' at critical strain of cheeses made with coagulants containing different chymosin/pepsin ratio and with calf rennet during 4 weeks of storage.

	Critical strain (%)							
Sample	Week 1		Week 2		Week 3		Week 4	
100C/0P	1.62 ± 0.18	a	2.20 ± 0.24	a	2.80 ± 0.44	a	5.62 ± 1.19	a
80C/20P	2.03 ± 0.00	a	2.53 ± 0.43	ab	4.13 ± 1.23	ab	6.11 ± 1.58	a
50C/50P	1.93 ± 0.17	a	3.26 ± 0.41	ab	3.92 ± 1.21	ab	7.13 ± 0.82	ab
20C/80P	3.26 ± 0.41	b	3.52 ± 0.39	bc	5.12 ± 1.63	abc	7.08 ± 0.00	ab
0C/100P	2.93 ± 0.27	b	3.42 ± 0.32	bc	6.11 ± 0.95	bc	8.74 ± 1.12	bc
Calf rennet	4.11 ± 0.37	c	4.43 ± 0.00	c	8.28 ± 0.00	c	9.21 ± 1.35	c
	G' at critical strain (KPa)							
Sample	Week 1		Week 2		Week 3		Week 4	
100C/0P	14.7 ± 3.1	c	12.0 ± 2.7	c	11.5 ± 0.8	c	6.6 ± 0.8	c
80C/20P	9.5 ± 1.3	b	9.5 ± 0.3	bc	8.5 ± 1.8	b	6.3 ± 1.4	bc
50C/50P	9.9 ± 3.0	b	8.8 ± 1.9	b	7.1 ± 0.6	ab	6.1 ± 0.6	bc
20C/80P	9.6 ± 1.6	b	8.4 ± 0.7	b	8.0 ± 2.7	b	5.6 ± 0.8	bc
0C/100P	9.1 ± 0.7	ab	8.3 ± 1.9	b	4.8 ± 1.1	a	5.0 ± 0.4	ab
Calf rennet	6.1 ± 1.5	a	4.5 ± 1.3	a	4.8 ± 0.3	a	3.9 ± 0.4	a

[a-c] Means within a column with the same superscript were not significantly different (α = 0.05).

Cheese made with calf rennet showed higher critical strain and lower G' when comparing cheeses with similar casein hydrolysis (C20/P80 and C0/100, Fig 4.6). The variations of the degree of casein hydrolysis were the main factor dominating these changes (Fig 4.5). Even though these cheeses had the same level of intact α_{s1}-CN level after 4 weeks of storage, more rearrangement of protein network occurred for cheese with calf rennet due to the high degree of casein hydrolysis. Thus, more new-bond were formed and led a higher critical strain. Also, a high degree of casein hydrolysis means that more large insoluble fragments were further hydrolyzed. This was responsible for the lowest firmness of the protein network for cheese made with calf rennet.

4.3.4.1.2 Parameters from creep measurement

The previous results already showed that the protein network was affected by casein hydrolysis, and that rearrangements in the network occurred. This will also lead to differences in the time-dependent viscoelastic behavior of the cheeses. To get more

insights into the differences in these properties, we performed a creep test, using the Burger model to obtain different rheological parameters. The results for all cheeses during 4 weeks of storage are shown in Table 4.5. The R^2 values of the fitting of J=f(t) based on the Burger model (Eqn 1) were always higher than 0.94, indicating that the Burger model was a good model to describe the stress relaxation characteristics of our cheese samples. Such model also provided good correlations in studies on different types of cheeses, such as Cheddar, Kashar, Gouda and Mozzarella cheese (Kuo et al., 2000; San Martín-González et al., 2007; Biswas et al., 2008).

J_m, the instantaneous elastic compliance, gives information on the elastic behavior of a material (Lynch and Mulvihill, 1994; MA et al., 1996; 1997). Table 4.5 shows that J_m of all cheeses increased as storage weeks increased, and it slightly increased when the proportion of pepsin increased up to 50% and remained constant with a further increase. A higher J_m means the network is relatively free to rearrange under the applied stress (Ojijo et al., 2004). The increase in J_m is related to the decrease in G' (weakening protein network), as a weaker network is easier to move and to rearrange. The cheese made with calf rennet had the highest J_m after 4 weeks of storage, corresponding to the further proteolysis of large fragments by bovine chymosin, even though it had levels of intact casein fractions similar to those of two other cheeses (C20/P80 and C0/100, Fig 4.5).

During creep measurement, the application of an instantaneous shear stress causes breakage and reformation of gel network bonds, but can also induce flow of the viscoelastic material, which generates creep behavior (Chattong et al., 2007; Kontou et al., 2019). The rate of breakage and reformation of these bonds vary and contribute differently to the temporal retarded compliance (J_k). A higher J_k represents a network with more rearrangements, reflecting a softer and less rigid nature of the cheese matrix. In our study, J_k increased from week 1 to week 4 (Table 4.5). Higher values of J_k were obtained for cheese with a higher proportion of pepsin. Calf rennet showed highest J_k compared with other coagulants. The results confirmed that cheeses made

with a higher proportion of pepsin had a weaker network, with more rearrangements, and were softer and less rigid. The rearrangement of the protein network has often been related to the small hydrolyzed fragments produced by proteolysis (Panyam and Kilara, 1996; Watkinson et al., 2001; Lucey et al., 2003). The broad cleavage sites on α_{s1}-CN by pepsin and bovine chymosin was probably responsible of the higher J_k of cheese made with more pepsin and with calf rennet, as more small hydrolyzed products were released and enhanced the rearrangement (Fig 4.4).

Newtonian viscosity (η_m) provides information on the properties of the viscous behavior of the material (Olivares et al., 2009a). A higher η_m suggests a network with greater overall resistance to flow. Overall, η_m slightly decreased with increasing storage time and decreasing chymosin/pepsin ratios, which is in line with the decrease in G' (weakening of the protein network) (Table 4.5). The continuous degradation of caseins was the main cause of the lower η_m. Cheese made with calf rennet showed the lowest values of η_m, confirming that this sample had the weakest protein network. Retardation time (λ_k) is known as the time it takes to retard the viscoelastic part of a material and to start deformation after the retardation (Olivares et al., 2009b). Surprisingly, neither the storage time nor the chymosin/ pepsin ratio showed an effect on λ_k. In fact, λ_k is related to both the elastic and the viscous behavior of a network. It was evident that with more casein hydrolysis, i.e. longer storage time and more pepsin, J_m and J_k increased, and η_m decreased, which suggests a decrease in both elastic and viscous behavior. The less elastic cheese should have a lower λ_k (i.e. shorter time), while the softer (less viscous behavior) cheese tends to show higher λ_k (longer time). Upon proteolysis during storage, the elastic and viscous behavior changed simultaneously, which can explain the lack of effect of the studied parameters on the retardation time. Thus, we can infer that the differences in casein hydrolysis did not affect the retardation time (λ_k). Sharma et al. (2017) already reported that λ_k was not significantly influenced when both the elastic and the viscous behavior increased for Mozzarella cheese in a creep measurement.

Table 4.5. Rheological parameters, J_m, J_k, λ_k and η_m, of cheeses made with coagulants containing different chymosin/pepsin ratio and with calf rennet, during 4 weeks of storage.

	J_m (10^{-5} Pa^{-1})							
Sample	Week 1		Week 2		Week 3		Week 4	
100C/0P	3.76 ± 1.60	a	5.82 ± 0.95	a	6.90 ± 0.91	a	6.74 ± 0.54	a
80C/20P	5.49 ± 0.64	b	6.07 ± 0.97	ab	8.95 ± 2.11	ab	8.79 ± 1.13	b
50C/50P	6.19 ± 0.84	bc	6.95 ± 0.44	bc	10.16 ± 2.89	b	11.42 ± 0.42	c
20C/80P	7.40 ± 0.48	cd	7.54 ± 0.12	cd	11.48 ± 0.33	b	10.79 ± 0.77	c
0C/100P	7.65 ± 0.30	cd	7.76 ± 0.23	cd	10.92 ± 0.74	b	11.67 ± 1.11	c
Calf rennet	8.33 ± 0.16	d	8.11 ± 0.10	d	11.72 ± 0.78	b	14.45 ± 0.49	d
	J_k (10^{-5} Pa^{-1})							
Sample	Week 1		Week 2		Week 3		Week 4	
100C/0P	7.6 ± 0.5	a	8.1 ± 1.5	a	9.3 ± 1.8	a	9.7 ± 0.7	a
80C/20P	9.4 ± 1.0	ab	8.5 ± 1.5	a	14.7 ± 5.5	b	12.9 ± 2.0	ab
50C/50P	9.4 ± 1.1	ab	9.3 ± 1.5	ab	13.3 ± 3.3	ab	14.5 ± 4.7	bc
20C/80P	11.9 ± 1.0	b	10.2 ± 1.4	ab	12.7 ± 3.1	ab	20.3 ± 1.0	d
0C/100P	14.7 ± 2.4	b	11.3 ± 1.2	b	17.8 ± 0.3	bc	18.2 ± 1.1	cd
Calf rennet	16.2 ± 3.1	c	11.2 ± 1.8	b	20.9 ± 1.7	c	23.8 ± 2.5	e
	λ_k (s)							
Sample	Week 1		Week 2		Week 3		Week 4	
100C/0P	4.35± 0.31		4.08 ± 0.01		3.83 ± 0.17		3.88 ± 0.11	
80C/20P	4.19 ± 0.07		4.14 ± 0.08		4.37 ± 0.01		3.75 ± 0.21	
50C/50P	4.26 ± 0.30		4.03 ± 0.34		3.76 ± 0.46		3.84 ± 0. 16	
20C/80P	4.23 ± 0.36		3.96 ± 0.24		3.96 ± 0.21		4.41 ± 0.52	
0C/100P	3.90 ± 0.25		4.32 ± 0.10		4.53 ± 0.35		4.05 ± 0.47	
Calf rennet	4.08 ± 0.06		4.19 ± 0.09		4.54 ± 0.12		3.52 ± 0.00	
	η_m (Pa·s)							
Sample	Week 1		Week 2		Week 3		Week 4	
100C/0P	115.5 ± 8.0	c	119.9 ± 22.6	b	71.7 ± 12.8	bc	84.3 ± 2.7	c
80C/20P	97.4 ± 11.3	b	107.7 ± 24.9	ab	73.9 ± 25.0	bc	74.5 ± 19.2	b
50C/50P	94.6 ± 6.9	b	111.4 ± 17.3	ab	76.1 ± 18.9	bc	78.8 ± 31.7	bc
20C/80P	85.8 ± 8.7	b	99.0 ± 9.5	ab	81.3 ± 16.8	c	58.3 ± 1.9	ab
0C/100P	81.7 ± 7.2	b	81.5 ± 11.0	a	51.2 ± 1.1	ab	54.8 ± 4.8	ab
Calf rennet	60.0 ± 8.2	a	82.0 ± 12.7	a	44.3 ± 3.4	a	40.7 ± 8.3	a

$^{a-d}$ Means within a column with the same superscript were not significantly different ($\alpha = 0.05$).

To conclude, the chymosin/pepsin ratio and the source of coagulant had an impact on the protein network during cheese storage. Cheese made with lower chymosin/pepsin ratio (more pepsin) and calf rennet had a weaker network, which was reflected in

lower values for G'. For these cheeses, also more rearrangement of protein network occurred under deformation, leading to higher values of critical strain, J_m , and J_k.

4.3.4.2 Textural properties of cheese

To investigate whether the changes in the protein network also affected the textural properties of cheese, large deformation tests in compression and TPA were carried out. The results are shown in Table 4.6 and Table 4.7.

Table 4.6. Young's modulus, hardness_40% and strain hardening index (SHI) of cheeses made with coagulants containing different chymosin/pepsin ratios and with calf rennet during 4 weeks of storage.

	Young's Modulus (KPa)							
Chymosin/pepsin	Week 1		Week 2		Week 3		Week 4	
100C/0P	1082.7 ± 49.9	a	753.3 ± 69.3	a	501.5 ± 37.3	a	424.1 ± 40.7	a
80C/20P	741.5 ± 93.3	b	621.2 ± 117.5	ab	439.2 ± 31.5	ab	397.6 ± 67.6	ab
50C/50P	693.5 ± 149.0	bc	568.0 ± 39.6	bc	406.2 ± 25.2	bc	339.5 ± 69.0	bc
20C/80P	545.2 ± 86.4	cd	432.0 ± 27.8	cd	372.0 ± 46.9	bc	365.6 ± 34.5	abc
0C/100P	497.0 ± 50.9	d	307.0 ± 21.6	d	337.2 ± 89.7	cd	320.5 ± 25.2	c
Calf rennet	403.1 ± 63.9	d	324.8 ± 71.1	d	253.5 ± 48.8	d	240.8 ± 38.8	d
	Hardness_40% (KPa)							
Chymosin/pepsin	Week 1		Week 2		Week 3		Week 4	
100C/0P	111.8 ± 12.9	a	114.1 ± 11.5	d	83.2 ± 2.8	d	70.8 ± 3.5	d
80C/20P	111.2 ± 9.3	a	79.4 ± 3.4	bc	70.3 ± 5.5	c	65.0 ± 2.4	cd
50C/50P	104.8 ± 11.1	a	84.3 ± 3.6	c	61.8 ± 4.1	bc	59.3 ± 2.9	bcd
20C/80P	83.9 ± 6.3	a	85.1 ± 8.6	c	56.0 ± 3.9	b	51.2 ± 3.8	ab
0C/100P	85.7 ± 5.3	a	54.8 ± 4.8	ab	53.8 ± 4.7	b	55.0 ± 3.3	bc
Calf rennet	85.5 ± 4.5	a	48.6 ± 3.5	a	40.0 ± 2.0	a	41.7 ± 3.8	a
	SHI (-)							
Chymosin/pepsin	Week 1		Week 2		Week 3		Week 4	
100C/0P	1.94 ± 0.13	a	1.95 ± 0.02	a	1.93 ± 0.13	b	2.13 ± 0.06	b
80C/20P	2.12 ± 0.14	a	1.99 ± 0.06	a	2.08 ± 0.10	ab	2.31 ± 0.03	a
50C/50P	2.12 ± 0.11	a	2.00 ± 0.09	a	2.23 ± 0.01	a	2.35 ± 0.03	a
20C/80P	2.02 ± 0.16	a	2.03 ± 0.13	a	2.17 ± 0.09	a	2.38 ± 0.04	a
0C/100P	2.00 ± 0.23	a	2.08 ± 0.09	a	2.23 ± 0.05	a	2.35 ± 0.03	a
Calf rennet	2.19 ± 0.06	a	2.08 ± 0.09	a	2.20 ± 0.05	a	2.38 ± 0.02	a

[a-c] Means within a column with the same superscript were not significantly different (α = 0.05).

The Young's modulus decreased during 4 weeks of storage and in samples with lower chymosin/pepsin ratio (Table 4.6), which is coherent with the occurrence of proteolysis and the weakening of the protein network. These results are in line with the result of

G' at the critical strain in SAOS analysis (Table 4.4). Samples made with calf rennet showed relatively lower values of the Young's modulus. Hardness_40% showed a trend similar as that of the Young's modulus. Cheese became softer with longer storage time and with lower chymosin/pepsin ratio.

The strain hardening index (SHI) provides information on the rate of rearrangements among the elements under large deformation (Sharma et al., 2018). Table 4.6 shows that no significant differences in SHI among cheeses were observed. Even though the coagulant showed effect on the rearrangement among elements upon small deformation (creep measurement), this effect was not seen under large deformations.

Table 4.7. Resilience and adhesiveness of cheeses made with coagulants with coagulants containing different chymosin/pepsin ratios and with calf rennet during 4 weeks of storage.

	Resilience (-)							
Chymosin/pepsin	Week 1		Week 2		Week 3		Week 4	
100C/0P	0.668±0.007	a	0.669±0.006	a	0.667±0.009	a	0.635±0.002	a
80C/20P	0.656±0.009	a	0.657±0.012	a	0.659±0.007	ab	0.629±0.003	ab
50C/50P	0.659±0.013	a	0.667±0.006	a	0.652±0.009	b	0.628±0.005	ab
20C/80P	0.665±0.007	a	0.654±0.004	a	0.646±0.007	bc	0.626±0.009	ab
0C/100P	0.663±0.006	a	0.635±0.013	b	0.639±0.010	c	0.616±0.010	b
Calf rennet	0.660±0.011	a	0.624±0.014	b	0.635±0.005	c	0.601±0.003	c
	Adhesiveness (g·s)							
Chymosin/pepsin	Week 1		Week 2		Week 3		Week 4	
100C/0P	-1.117±0.727	ab	-1.202±0.250	a	-4.598±0.495	a	-4.814±0.973	a
80C/20P	-1.656±0.968	ab	-1.456±0.500	a	-5.261±0.708	ab	-4.850±0.810	a
50C/50P	-1.769±0.649	b	-4.946±1.805	b	-5.307±0.143	ab	-5.929±0.886	ab
20C/80P	-0.644±0.288	a	-4.780±0.117	b	-6.753±0.400	b	-5.821±0.745	ab
0C/100P	-0.766±0.530	ab	-4.662±0.519	b	-7.215±2.020	b	-7.524±0.431	b
Calf rennet	-0.638±0.445	a	-4.388±0.655	b	-5.289±0.789	ab	-5.412±1.342	ab

[a-c] Means within a column with the same superscript were not significantly different (α = 0.05).

TPA tests were performed to mimic the first two compressions occurring during consumption and to provide more information about possible relations between casein hydrolysis and material properties. We chose to shown resilience and adhesiveness, as other parameters such as cohesion and springiness obtained from the TPA test showed no significant differences among cheese samples. Table 4.7 shows

that the resilience of all cheeses slightly decreased with increasing storage time and with decreasing chymosin/pepsin ratio. From week 2 onwards, cheese made with calf rennet had significantly lower resilience than that of cheeses made with other coagulants.

An increase in adhesiveness was found with increasing storage time and decreasing chymosin/pepsin ratio. This was mainly related to a more extensive casein hydrolysis and produced peptides. This was also reported by Bye (1990), who showed that the adhesiveness of casein products was enhanced by peptides. The increase in adhesiveness after casein hydrolysis has been reported to be caused by the high absorption energy of products with more hydroxyl groups (Clerc et al., 2017). Although calf rennet had extensive proteolytic activity, it did not lead to high adhesiveness. From literature, we know that pepsin further breaks down α_{S1}-CN with cleavage site on the C-terminal region (Michaelidou et al., 1998). Bovine chymosin in calf rennet has higher affinity for α_{S1}-$CN_{164}/_{165}$ than for recombinant chymosin in the other coagulants, resulting in more extensive hydrolysis and exposure of N-terminal products (Møller et al., 2012). It is therefore likely that peptides released from the C-terminal region of α_{S1}-CN dominated the increase of adhesiveness, leading to a sticky cheese, while peptides containing N-terminal residues had no influence.

Overall, our results revealed the potential of altering specific cheese texture (Young's modulus, hardness_40% and adhesiveness) during storage by modulating the chymosin/pepsin ratio in the coagulant, while maintaining other textural properties (resilience and SHI).

4.3.5 Linking casein hydrolysis to the rheological and textural properties

Our results showed that even with a standardized MCA, different coagulants had a significant effect on casein hydrolysis and accompanying changes in both rheological and textural properties. To study possible correlations between casein hydrolysis and different rheological and textural properties in more detail, the values of the obtained

parameters were plotted as a function of degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and intact casein fractions. In our study, only limited β-CN was degraded and no hydrolysis of α_{s2}-CN occurred. Thus, we focused our discussion on the α_{s1}-CN fraction. For the rheological properties, as the parameters obtained from SAOS test and creep measurement provide similar information on structural properties of the network, we here chose to present the results of critical strain and G' only. For textural parameters, Young's modulus, resilience, adhesiveness and hardness_40% were selected, since coagulants showed significant effect on these parameters. The results for selected parameters are shown in Fig 4.6-4.8. The Person correlation coefficients (R) are shown in Table 4.8. An overview of all results is provided in Figure S4.1 and Table S4.2 in the supplementary information.

As shown in Fig 4.6b&d, G' and Young's modulus gradually decreased as the intact α_{s1}-CN fraction decreased. As α_{s1}-CN is the main structural component forming the skeleton network in the cheese matrix, the breakdown of intact α_{s1}-CN directly leads to the loss of strong interactions and thus the weaker network (lower G' and Young's modulus) was shown. In term of the degree of casein hydrolysis, the decrease in G' and Young's modulus was obvious when initial proteolysis (< 12% protein fraction soluble at pH 4.6) occurred. However, they remain constant with further increase in degree of casein hydrolysis (Fig 4.6a&c). As we have already mentioned above, the arrangement of protein network was enhanced with more hydrolyzed fragments at a high degree of casein hydrolysis, and thus new-bonds formed. The new-formed bonds might help to resist the weakening of protein network that caused by the loss of interactions among intact caseins. Thus, the strength of the network (G' and Young's modulus) showed less change. The result in Table 4.8 showed that the G' and Young's modulus had higher correlation coefficients with intact α_{s1}-CN fraction than it of the soluble protein. This also confirms that intact α_{s1}-CN fraction is important in determining the strength of protein network (G' and Young's modulus).

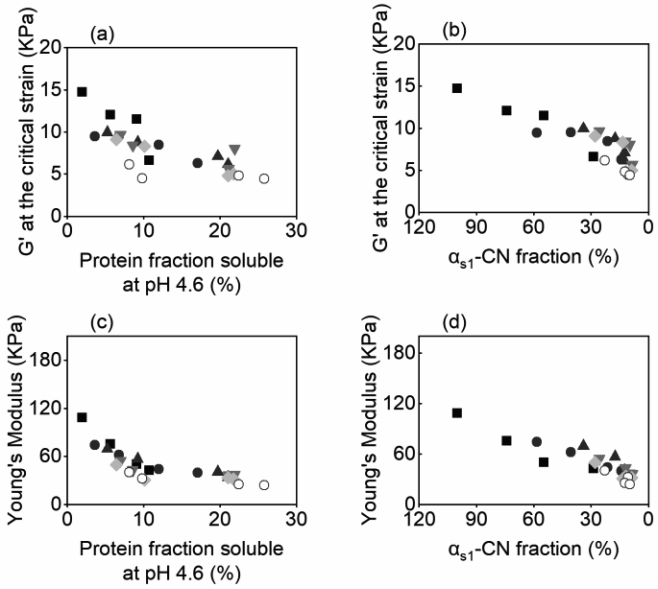


Fig 4.6. G' at the critical strain (a, b) and Young's modulus (c, d) of six model cheeses as a function of degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and intact α_{s1}-CN fraction. Coagulants with chymosin/pepsin ratio as 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆), and calf rennet (○) were used. All the casein fractions were calculated based on the peak area, the peak area of sample made with 100% chymosin at week 1 being 100%.

We also found that the degree of casein hydrolysis played important role in the changes of critical strain, resilience and adhesiveness. With increasing degree of casein hydrolysis, critical strain and adhesiveness gradually increased and resilience slightly decreased (Fig 4.7a, c&e). However, these parameters stayed almost constant until 60% of α_{s1}-CN was hydrolyzed, and changed significantly only upon further hydrolysis of this fraction (Fig 4.7b, d&f). Correspondingly, it was observed that these parameters had higher correlation coefficients with the degree of casein hydrolysis than it with intact α_{s1}-CN fractions (Table 4.8). As we already mentioned above, the formation of new bonds among hydrolyzed fragments was the main factor that induced the increase in critical strain. This is confirmed by the strong correlation between critical strain and the degree of casein hydrolysis (Table 4.8). Although new bonds were formed, they tend to be broken at small deformation. This can be explained by the fact that the

fragments formed the bonds are relatively short. As a result, the bonds are easily broken and it induced permanent changes in the protein network in early stages of compression, and thus a decrease in resilience occurred. This explains why also resilience was highly associated with the degree of casein hydrolysis (Fig 4.7c). For the reasons mentioned in section 4.3.4.4.2, it was not surprising to find a strong correlation between adhesiveness and pH 4.6 soluble protein (Fig 4.7e). Overall, we conclude that the degree of casein hydrolysis was more dominant in the changes of the parameters related to the rearrangements of the protein network that the intact casein fraction. A higher correlation with the degree of hydrolysis was also found for the creep parameters (J_m and J_k) and SHI in Table S4.2.

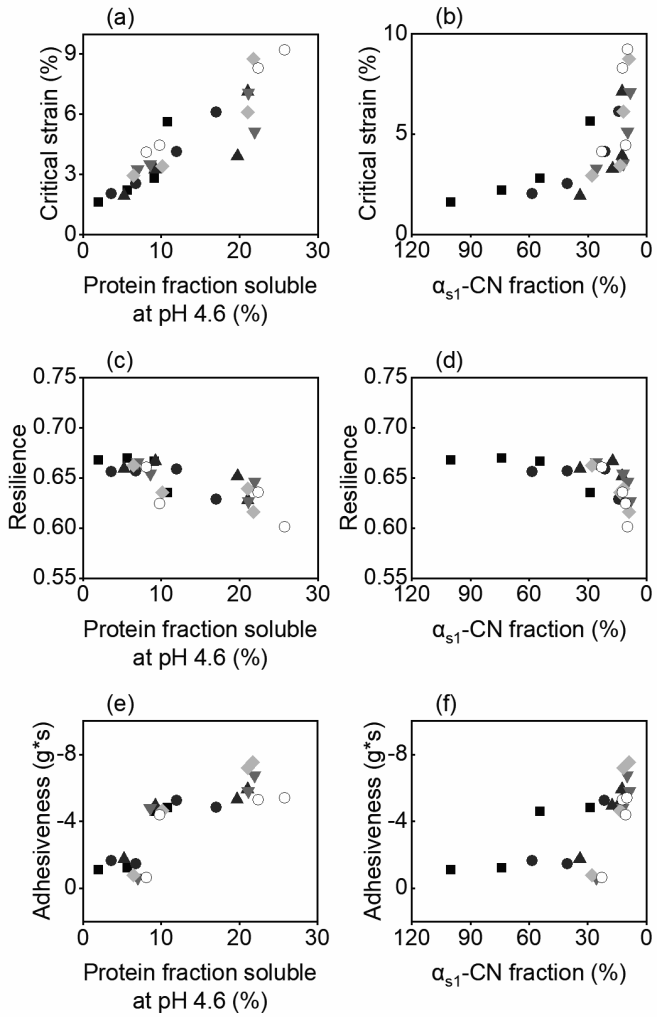


Fig 4.7. Critical strain (a, b), resilience (c, d) and adhesiveness (e, f) of six model cheeses as a function of the degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and the intact α_{s1}-CN fraction. Coagulants with chymosin/pepsin ratio as 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆), and calf rennet (○) were used. All the casein fractions were calculated based on the peak area, the peak area of sample made with 100% chymosin at week 1 being 100%.

Although the above mentioned parameters have a higher correlation with the degree of casein hydrolysis, hardness_40% was found to have a strong relation to both the degree of casein hydrolysis and intact α_{s1}-CN fractions (Fig 4.8 and Table 4.8). At a deformation of 40%, most bonds within the cheese matrix would be broken, including

the interactions among intact caseins and the bonds resulting from rearrangements of protein network. In this case, both the intact α_{s1}-CN fraction and the degree of casein hydrolysis were responsible for the changes in hardness_40%.

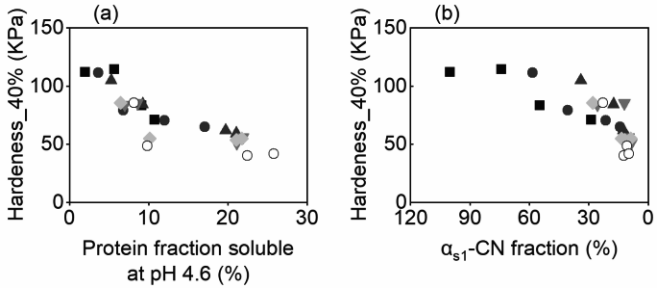


Fig 4.8. Hardness_40% of six model cheeses as a function of the degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and the intact α_{s1}-CN fraction. Coagulants with chymosin/pepsin ratio as 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆), and calf rennet (○) were used. All the casein fractions were calculated based on the peak area, the peak area of sample made with 100% chymosin at week 1 being 100%.

TABLE 4.8. Pearson's correlation coefficients (R) between mechanical parameters of model cheese and intact casein fractions.

	pH 4.6 soluble protein	α_{s1}-CN fraction	Dominated factor
G'	-0.76**	0.85**	Intact α_{s1}-CN fraction
Young's Modulus	-0.76**	0.91**	
Critical Strain	0.90**	-0.65**	Degree of casein hydrolysis
Resilience	-0.76**	0.61**	
Adhesiveness	-0.82**	0.64**	
Hardness_40%	-0.85**	0.81**	Intact α_{s1}-CN fraction and degree of casein hydrolysis

Overall, the effect of the coagulants on different rheological and textural properties can be explained based on both the degree of casein hydrolysis (expressed as protein fraction soluble at pH 4.6) and the intact α_{s1}-CN fraction. The use of the coagulant with more pepsin and the calf rennet led a less brittle network within the cheese matrix, as more hydrophobic products (fragments and peptides) were released and more

rearrangement of the protein network occurred. The cheese was less stiff, less elastic and softer due to the weaker protein network as a result.

The findings obtained in this study present new insight into the development of cheese texture, by taking the composition of coagulant into account. The knowledge provide a potential for controlling cheese texture and designing cheese with desired texture by modulating the levels of intact α_{s1}-CN casein fractions and the degree of casein hydrolysis. Using coagulant with different chymosin/pepsin ratio, for example from different sources, can be considered a convenient strategy to alter the structure and probably the subsequent texture.

4.4 CONCLUSION

In this study, we investigated how the chymosin/pepsin ratio in coagulants affects the coagulation properties during milk curdling and the rheological and textural properties of model cheese during storage. Next to mixtures of the mentioned enzymes, a calf rennet (CSK rennet) was also used as comparison. Our results show that the chymosin/pepsin ratio in coagulant has an impact on gelation properties (e.g. curd firming rate and curd firmness) and casein macropeptide (CMP) release. During 4 weeks of storage, higher degradation of α_{s1}-CN, higher degree of casein hydrolysis and a weaker network were found for cheese made with a higher proportion of pepsin, due to its high activity of hydrolyzing α_{s1}-CN. The breakdown of intact α_{s1}-CN significantly led to the weakening of protein network (decreased G' and Young's modulus). The higher degree of casein hydrolysis corresponded to higher critical strain and adhesiveness, and lower resilience. This is attributes to the enhanced rearrangement of protein network due to the high amount of hydrolyzed products. The cheese softening was related to both the interactions among intact α_{s1}-CN and the interactions among hydrolyzed products. The findings of this study provide the potential of using coagulant with different chymosin/pepsin ratio to modulate the

hydrolysis of α_{s1}-CN and the network. This likely further helps us to control cheese texture and to design cheese with desired texture.

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SUPPLEMENTARY INFORMATION

Table S4.1 Dry matter of cheeses made with coagulants with different chymosin/pepsin ratio and with rennet from CSK during 4 weeks of storage.

Chymosin/pepsin	Week 1	Week 2	Week 3	Week 4
100/0	35.8 ± 0.7%	35.8 ± 0.6%	35.0 ± 0.7%	34.4 ± 0.3%
80/20	35.6 ± 1.2%	34.7 ± 0.8%	35.4 ± 0.2%	34.8 ± 0.8%
50/50	35.6 ± 1.2%	35.8 ± 1.5%	35.4 ± 0.3%	34.7 ± 0.9%
20/80	35.1 ± 1.2%	34.3 ± 0.6%	33.7 ± 0.8%	32.7 ± 1.2%
0/100	34.3 ± 1.9%	33.3 ± 1.3%	34.5 ± 2.0%	33.7 ± 0.4%
Calf rennet	33.9 ± 0.6%	35.4 ± 1.4%	33.1 ± 1.0%	34.5 ± 1.7%

TABLE S4.2. Pearson's correlation coefficients (R) between mechanical parameters of model cheese and intact casein fractions.

	pH 4.6 soluble protein	α_{s1}-CN fraction	Dominated factor
G'	-0.76**	0.85**	Intact α_{s1}-CN fraction
Young's Modulus	-0.76**	0.91**	
Critical Strain	0.90**	-0.65**	Degree of casein hydrolysis
J_m	0.96**	-0.75**	
J_k	0.80**	-0.60**	
Resilience	-0.76**	0.61**	
SHI	0.84**	-0.63**	
Adhesiveness	-0.82**	0.64**	
η_m	-0.77**	0.63**	Intact α_{s1}-CN fraction and degree of casein hydrolysis
Hardness_40%	-0.85**	0.81**	

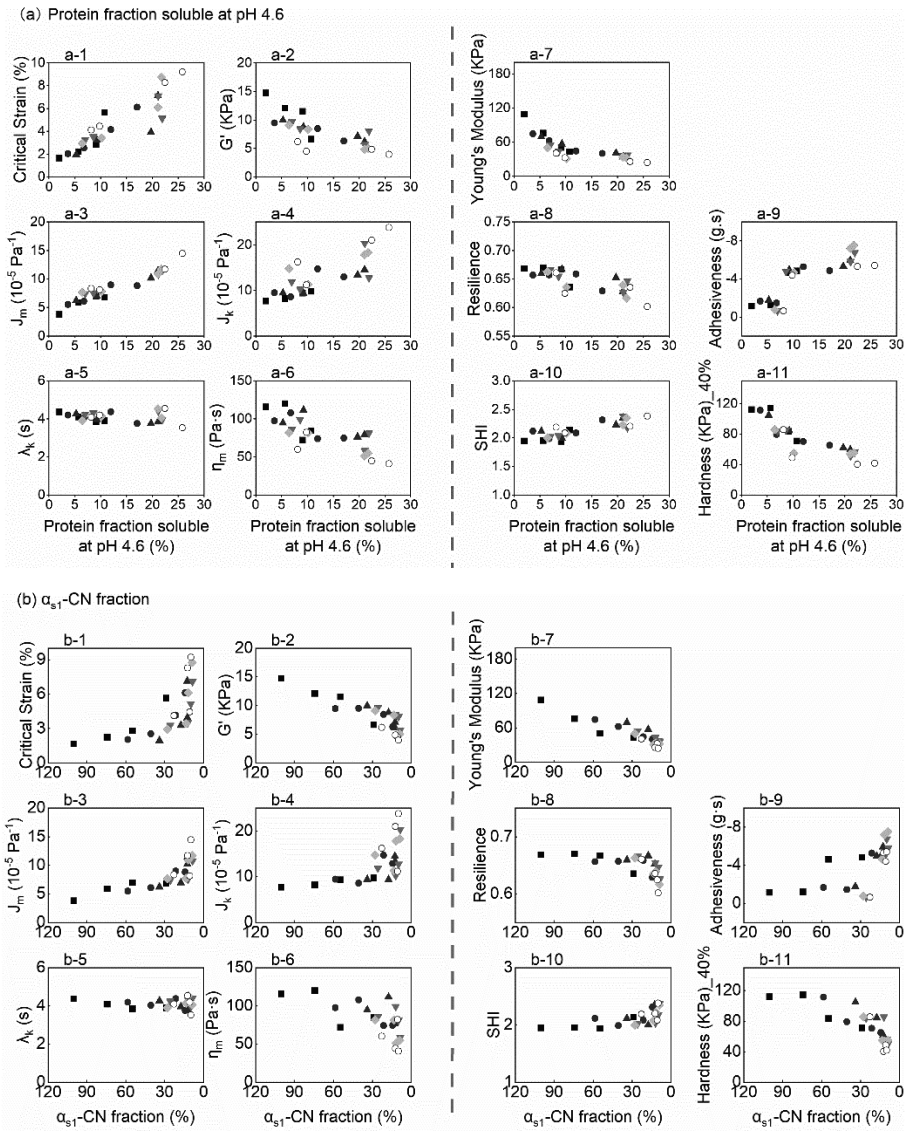

Fig S4.1. Rheological properties (a1-6 & b1-6; critical strain, G' at the critical strain, J_m, J_k, λ_k and ηm) and textural properties (a7-11 & b7-11; Young's modulus, resilience, adhesiveness, strain hardening index and hardness_40%) of six model cheeses as a function of the degree of casein hydrolysis (a) and the intact α_{s1}-CN fraction (b). Coagulants with chymosin/pepsin ratio as 100/0(■), 80/20(●), 50/50(▲), 20/80(▼) and 0/100(◆), and calf rennet (○) were used. All the casein fractions were calculated based on the peak area, the peak area of sample made with 100% chymosin at week 1 being 100%.


CHEESE

Chapter 5

Role of bolus properties in understanding complex texture attributes of cheese

To be submitted as:

Huifang Cai, Etske Bijl, Elke Scholten, Guido Sala. Role of bolus properties in understanding complex texture attributes of cheese.

ABSTRACT

Simple texture attributes of cheese, such as hardness and brittleness, can often be directly linked to its composition and mechanical properties. However, for more complex texture attributes, the role of oral processing and the characteristics of the formed bolus also have to be taken into account. In the present study, we investigated the relation between cheese compositional and physical properties, bolus formation and texture perception to better understand complex sensory attributes of this food product. Five commercial cheeses with two fat content levels and varying ripening time were selected, and the texture of these samples was characterized by compression measurements and Texture Profile Analysis. In addition, the bolus properties were characterized by several parameters, including compositional and mechanical properties, particle size distribution and lubrication properties.

Our results confirmed that simple texture attributes such as hardness, brittleness and elasticity are mostly related to the mechanical properties of the cheeses (i.e. fracture strain and resilience). However, these properties were insufficient to explain more complex attributes like smoothness, creaminess and fattiness. In this case, role of bolus properties should be taken into account. The bolus formation was largely influenced by the fat content and mechanical properties (e.g. hardness and brittleness) of cheese products. As fat melted during mastication, a higher fat content contributed to the formation of a softer, more cohesive and better lubricating bolus, and the cheeses were perceived as being smoother and creamier. For low fat cheeses, the cohesion and lubrication properties of bolus had a great influence on the perception of smoothness and creaminess. When the cheese contained harder particles, the bolus provided less lubrication (higher μ), and the cheese was perceived as less smooth and creamy. These findings offer new insights into the mechanisms behind the perception of complex texture attributes for cheese. These insights may help to engineer the sensory properties of cheese by allowing to identify innovations in cheese manufacture leading to a control of bolus formation.

5.1 INTRODUCTION

Texture perception is one of the major factors determining the overall quality of food and the preference of consumers (Guinard and Mazzucchelli, 1996). It is the result of multimodal sensory sensations and is usually described by many texture attributes. It is known that these attributes are perceived at different stages during oral processing (Pascua et al., 2013; Foegeding et al., 2015; Devezeaux de Lavergne et al., 2017). During oral processing, food is reduced in size while saliva is incorporated to form a bolus suitable for swallowing (Koç et al., 2013). These modifications are responsible for changes in the texture of foods and therefore contribute to the complexity of texture perception (Devezeaux de Lavergne et al., 2017).

Several studies have already shown that some texture attributes of solid foods can be explained by phenomena occurring during the first bite, which can be regarded as the first stage of oral processing. In general, attributes as hardness and firmness have been linked to mechanical properties, such as fracture stress, fracture strain, Young's modulus and work to fracture (Foegeding et al., 2003; Everard et al., 2006), which can be directly linked to the composition of the product. For example, it has been widely reported that a higher dry matter results in a stiffer and harder cheese, which also leads to higher perceived hardness and firmness (Xiong et al., 2002; Adhikari et al., 2003). However, the mechanical properties are not sufficient to predict more complex texture attributes such as smoothness, creaminess and fattiness, and low correlations are often found between these aspects (Saint-Eve et al., 2015; Ningtyas et al., 2019). The main reason is that these attributes are mainly perceived at later stages of oral processing, during the chew down and swallowing phase (Foegeding et al., 2015; Devezeaux de Lavergne et al., 2017). During the oral processing of cheese, next to the general phenomena already mentioned above, fat is released from the cheese matrix as result of melting (Chen, 2009; Foegeding et al., 2011), and this changes the physical properties of the food. Attributes that are perceived during later stages of consumption are therefore not directly related to the characteristics of the food, and

the properties of the obtained bolus become more relevant. Up to now, few studies have related bolus properties to texture attributes. For liquid products (e.g. water, yogurt, tomato juice, ketchup and mustard), Seo et al. (2007) indicated that the texture attribute slipperiness was related to the rheological properties of the bolus during swallowing. For emulsion-filled gels, Devezeaux de Lavergne et al. (2015b) found that these systems were perceived as either creamy or grainy at the end of oral processing, depending on the specific breakdown behavior. The attribute creaminess was associated with a high bolus flowability, while graininess could be explained by a high number of particles within the bolus. For commercial cooked ham, Rizo et al. (2019) reported that fibrousness was linked to the number of particles in the bolus, which was used to represent the degree of fragmentation during mastication.

Although the link between bolus properties and sensory perception has been investigated for different foods, there is little knowledge about the effect of oral processing on the sensory perception of cheese. Only few studies have taken oral processing and bolus properties into account. For example, Melito et al. (2013) investigated oral processing characteristics of three different types of commercial cheeses (Cheddar, Mozzarella and American cheese). They showed that first chewing cycle and jaw's closing velocity during mastication were positively correlated with the nonlinear viscoelastic properties of cheese, but they did not link this to sensory attributes. The few studies that did take into account sensory attributes were more focused on flavor attributes instead of texture attributes. For example, Saint-Eve et al. (2015) investigated the effect of composition and physical properties of commercial cheeses on dynamic sensory perception in terms of saltiness, sourness and overall aroma. They showed that these attributes were linked to the rheological properties of the bolus, confirming that oral processing is indeed important for sensory perception. The role of cheese bolus formation on aroma release was also studied by Feron et al. (2014). Neither of these studies included texture attributes, such as smoothness,

coating, creaminess and stickiness, even though these attributes are important for product liking (Foegeding and Drake, 2007; Yates and Drake, 2007).

The objective of the present study was to investigate the relation between compositional and mechanical properties of cheese, bolus formation and texture perception. Five commercial cheeses with two levels of fat content and different ripening times were used. Two levels of fat content (full and low) were selected to obtain cheese with vary compositional properties, as protein content and dry matter are also altered if fat content varies. A comprehensive understanding on texture perception of cheese can be used to develop strategies to steer sensory perception by controlling the properties of cheese.

5.2 MATERIALS AND METHODS

5.2.1 Samples

Five commercial Gouda cheeses (around 500 g each) from the same brand (AH) were purchased from a local supermarket (Albert Heijn, the Netherlands). A description of all samples is given in Table 5.1. The cheeses were stored at 4 °C and were used within three weeks. For all the experiments, the outer crust was discarded (see Fig S5.1) to obtain a homogeneous sample.

Table 5.1. Description of five commercial Gouda Cheese

Product name	Description
jong belegen 48+	7 weeks of ripening, Full fat
belegen 48+	16 weeks of ripening, Full fat
oud 48+	48 weeks of ripening, Full fat
jong belegen 30+	7 weeks of ripening, Low fat
belegen 30+	16 weeks of ripening, Low fat

5.2.2 Cheese characterization

5.2.2.1 Composition

The dry matter content of the cheeses was determined by oven drying at 105 °C (Lynch et al., 1997). The protein content was determined by the DUMAS method (Flash EA

1112 series Dumas, Interscience, the Netherlands). A nitrogen conversion factor of 6.38 was used. The fat content was analyzed using a Schmid-Bondzynski-Ratzlaff gravimetric method (IDF, 1986).

5.2.2.2 Mechanical properties

The cheeses were cut into specimens with cylindrical shape with a diameter and height of 1 cm. The mechanical properties of the cheeses were measured using a Texture Analyzer (TA-TX plus, Stable Micro Systems Ltd., UK) equipped with a 50 mm diameter cylindrical probe (stainless steel) and a 5 kg load cell. Young's modulus, hardness_25% (stress at strain of 25%), fracture strain and fracture stress were extracted from the large deformation test, as described in more detail in Chapter 2. The measurements were performed with a compression speed of 2 mm/s until a strain of 85%. A texture profile analysis (TPA) was performed at a speed of 2 mm/s and with a strain of 20% for both compressional cycles. From the obtained TPA curves, resilience, adhesiveness and cohesion were extracted. More information on the calculations of these parameters can be found in Chapter 2.

5.2.3 Sensory evaluation

Sensory evaluation of the cheese products was performed using a Rate-all-that-apply (RATA) method a with 9-box scale according to the description of Oppermann et al. (2017) and Meyners et al. (2016). Seventy-seven individuals (48 females / 29 males, age between 18 and 31 years) were recruited. Inclusion criteria were good general health, BMI (18.5–25 kg/m^2), no dairy intolerance and good dental health. Before joining the study, all participants signed an informed consent form. After completion of the study participants received a financial compensation.

For the sensory characterization, the cheeses were cut into cubes (15×15×15 mm; 9.8 ± 0.3 g) at the same day of the evaluation. The cubes containing "eyes" were discarded. Cheese cubes were placed into lidded 70-ml cups (Natureko Biodisposables B.V., the Netherlands) with random 3-digit codes, were stored at 4 °C and were brought to room

temperature 15 min before sensory evaluation. The samples were served in randomized order. Participants were instructed to consume the cheese cubes as they would normally do and to evaluate nine sensory attributes (hardness, brittleness, elasticity, smoothness, stickiness, creaminess, fattiness, overall flavor and saltiness). Subjects were asked to choose "0" if the attributes were not perceived. The intensity of the perceived attributes was rated from "1" (low) to "9" (high). The order of the sensory attributes was randomized within two blocks of categories ("Texture" and "Basic flavor and taste") for each participant. A list of attribute definitions was provided (Table S5.1 in the supplementary information). Participants were instructed to rinse their mouth with water and eat a cracker (LU Mini Crackers Naturel, Jumbo, the Netherlands) before the evaluation of a new sample.

5.2.4 Characterization of bolus properties

Twenty participants (10 males / 10 females) were randomly selected from the sensory evaluation panel to investigate the properties of the bolus obtained in a separate session. Participants were asked to chew each of the 5 cheese cubes as they would normally do and expectorate the bolus into lidded 70-ml cups before swallowing. The mastication time of each cheese cube was recorded. Two boluses of each sample were collected. For each bolus, 2 g were used to determine the dry matter content and saliva incorporation. The remainder of one replicate was used to determine the mechanical properties of the bolus. The rest of the other replicate was used to analyze the tribological properties and the particle size of the bolus. All measurements were performed immediately after expectoration. The remainder of the two boluses were combined again and stored at -20 °C for further analysis of the fat content.

5.2.4.1 Mechanical properties of bolus

The mechanical properties of boluses were determined immediately after expectoration, using a two cycle puncture test, according to the method described by van Eck et al. (2019). A Texture Analyzer equipped with a 4 mm diameter cylindrical probe (stainless steel) and a 500 g load cell was used. The bolus samples were prepared

according to an approach described by Aguayo-Mendoza et al. (2021). Around 1.5 g bolus was transferred to the center of a plastic plate (diameter=100 mm) and was gently shaped into a cylinder (around 25 mm in diameter and 8 mm in height). The probe punctured the bolus with a speed of 2 mm/s up to a strain of 75% of the initial bolus height. The probe was then retrieved at the same speed and a resting time of 5 s was applied before the second puncture.

Bolus hardness, adhesiveness and cohesion were extracted from the obtained force-time curves as described by van Eck et al. (2019). Bolus hardness was defined as the maximum force during the first puncture. Adhesiveness was calculated as the negative area under the force-time curve during the first cycle. Cohesion was defined as the ratio of the positive force area of the second puncture to that of the first puncture. Each bolus (total 100) was measured in triplicates and the average value and standard error are reported.

5.2.4.2 Dry matter content and saliva incorporation

The determination of dry matter content was carried out within 30 min after expectoration to avoid moisture evaporation from the samples. Oven drying at 105 °C was used as described in literature (Lynch et al., 1997). Saliva incorporation per gram dry bolus was calculated by subtracting the moisture content of the cheese on a dry weight basis from the moisture content of the bolus on dry weight basis (van Eck et al., 2019). Subsequently, the saliva content per gram bolus was calculated based on the dry matter of the bolus. Each bolus (total 200) was measured in duplicate and the average value and standard error are reported.

5.2.4.3 Fat content

The analysis of fat content of cheese bolus was also determined according to the Schmid-Bondzynski-Ratzlaff gravimetric method (IDF, 1986). Forty boluses (8 persons × 5 bolus) were selected for fat content determination. The average value and standard error are reported. The amount of fat released in mouth and the percentage

of released fat were calculated based on the fat content and weight of the cheese and cheese bolus as:

Released fat in mouth =

(fat content in cheese × weight of cheese) - (fat content in bolus × weight of bolus) (1)

$$\text{Percentage of released fat (\%)} = \frac{\text{Released fat in mouth}}{\text{Fat content in cheese} \times \text{weight of cheese}} \quad (2)$$

5.2.4.4 Size distribution of particles in bolus

To determine the size distribution of the particles present in the collected boluses, 1 g of cheese bolus was transferred in a 50 ml tube. Lukewarm water (20 ml, around 37 °C) was added. The particles were separated by shaking the tube (lidded) for 1 min and then the mixture was gently poured into a plastic petri dish (120×120×17 mm, Greiner Bio, the Netherlands). The particles left on the tube surface and lid were gently rinsed with 20 ml lukewarm water and poured to the petri dish as well. Petri dishes were scanned to obtain images in greyscale with a resolution of 1200 dpi using a scanner (CanonScan 9000F markII). The images were analyzed using ImageJ (Version 1.51). The total number of particles and three diameter parameters, D_{10}, D_{50} and D_{90}, corresponding to the 10, 50, and 90% points in the cumulative curves of the particle size distribution, were quantified. Each bolus (total 100) was measured in triplicates and the average value and standard error are reported.

5.2.4.5 Tribological properties of bolus

The friction coefficient, μ, of the boluses was determined using a tribometer (TriboLab, Bruker, Karlsruhe, Germany) equipped with a 20 N load sensor (DFM-2G, Bruker, Billerica USA), according to the method described by van Eck et al. (2020), and especially designed in house to measure solid-like foods. A roughened PDMS substrate (55×45×5mm) was made to mimic the in-mouth surface, according to a procedure described by Fuhrmann et al. (2020). The upper probe was designed as a PDMS cylinder (diameter of 27 mm and height of 20mm) with a rough bottom surface. The rough surfaces of the upper probe and the substrate were created in moulds that were coated with sandpaper (code P180, corresponding to an average particle diameter of

75 µm, according to ISO6344-3 (1988)) to mimic the rough nature of the tongue and the palate. Bolus (1 g) was placed on the substrate and gently leveled with a spoon. The applied normal force exerted by the probe was set as 0.25 N. The friction force was measured at velocities ranging from 4.7 mm/s to 47.5 mm/s. The PDMS probe and PDMS substrate were cleaned with detergent, ethanol and water prior to each measurement. The friction coefficient (µ) at each velocity was calculated using an advanced oscillating algorithm analysis (UMT viewer software, Bruker, USA). As µ was not velocity-dependent, we report µ as the average value obtained over the entire speed range from 4.7 mm/s to 47.5 mm/s. Each bolus (total 100) was measured in duplicate and the average value and standard error are reported.

5.2.5 Statistical data analyses

For cheese characterization, one-way analysis of variance (ANOVA) with a Tukey Post Hoc test was performed to investigate the significant difference of obtained results among five cheeses. For bolus characterization and sensory intensities, a two-way analysis of variance (ANOVA) (product, subjects and interaction) was applied to determine the significant differences between different products or subjects. Pearson's correlation coefficients were calculated to investigate the correlations between properties of cheese/bolus and sensory attributes (only texture category) using $p < 0.05$ as the level of significance. All described analyses were performed using IBM SPSS Statistics 25 (IBM Corporation, NY, USA). The correlations between properties of cheese/bolus and sensory attributes were also summarized using a Principal Component Analysis (PCA) on subject averaged data (RStudio, version 1.0.143).

5.3 RESULTS AND DISCUSSION

5.3.1 Cheese characterization

As we expected, the composition and mechanical properties differed significantly with different levels of fat content and varying ripening time (Table 5.2).

Table 5.2. Composition and mechanical properties of the 5 studied cheeses. Different letters indicate significant difference between products at p < 0.05. Mean values ± standard error of the mean are given.

	Full fat			Low fat	
Ripening time (weeks)	7	16	48	7	16
Composition					
Dry matter (wt%)	58.3±0.2^{b}	62.9±0.1^{c}	65.5±0.3^{d}	50.8±0.2^{a}	57.8±0.1^{b}
Fat content (wt%)	30.3±1.2^{b}	30.01±1.1^{b}	32.46±1.2^{c}	17.28±0.7^{a}	18.86±0.7^{a}
Protein content (wt%)	24.3±0.2^{a}	27.7±1.0^{b}	27.1±0.2^{b}	29.0±0.5^{b}	32.1±1.4^{c}
FDM[1] (%)	52.1	47.7	49.5	34.0	32.6
PDM[2] (%)	41.8	44.0	41.3	57.1	55.5
P/F ratio[3]	0.80	0.92	0.83	1.68	1.70
Mechanical properties					
Young's modulus (KPa)	110.3±5.2^{a}	341.7±24.3^{b}	435.1±43.1^{b}	121.4±11.5^{a}	769.0±86.9^{c}
Fracture strain (%)	61.1±0.3^{d}	47.2±0.5^{c}	29.0±0.6^{a}	58.5±0.5^{d}	39.5±0.5^{b}
Fracture stress (KPa)	130.5±2.7^{a}	219.1±10.0^{b}	135.6±2.8^{a}	181.2±10.5^{c}	303.7±14.0^{d}
Hardness_25% (KPa)	32.1±0.7^{a}	116.2±5.2^{b}	129.0±3.4^{b}	44.1±2.2^{a}	217.5±9.1^{c}
Resilience	0.48±0.002^{b}	0.47±0.003^{b}	0.38±0.003^{a}	0.58±0.003^{c}	0.47±0.004^{b}
Cohesion	0.86±0.003^{c}	0.83±0.002^{b}	0.76±0.003^{a}	0.92±0.006^{d}	0.83±0.001^{b}
Adhesiveness (g·s)	-5.7±0.6^{a}	-6.6±1.3^{a}	-6.2±1.4^{a}	-0.9±0.2^{b}	-4.4±0.4^{ab}

[1-3] Fat content in dry matter (FDM), protein content in dry matter (PDM) and the ratio of protein to fat (P/F ratio).

Compared to low fat cheeses, full fat cheeses had higher fat content and dry matter, and lower protein content. With increasing ripening time, the dry matter content increased due to moisture loss for both the full fat and low fat cheeses. Correspondingly, protein and fat content slightly increased. Consequently, Young's modulus, hardness and fracture stress increased with ripening time. In contrast, fracture strain, resilience and cohesion decreased. The decrease in resilience and

cohesion was not directly related to the dry matter content, but more to the properties of the protein network as a result of proteolysis during ripening, as also reported in Chapter 2-3 and other researches (Romeih et al., 2002; Sahan et al., 2008; Jung et al., 2013).

Low fat cheeses showed significant higher Young's modulus, hardness and fracture stress than full fat cheeses, even though the latter type had a higher dry matter content. This can be explained by the high protein/fat ratio in low fat cheeses. The higher protein content in low fat cheeses leads to a more dense protein network, and, therefore, these cheeses had a firmer texture.

Ripening time, i.e. dry matter content, did not seem to directly influence the adhesiveness of the cheese, which was strongly affected by the fat content. Full fat cheeses were more adhesive than low fat cheeses, which was also reported in other studies (Bryant et al., 1995; Gwartney et al., 2002; Rogers et al., 2009). Fat globules may be present at the surface of the cheese, and fat, due to its "sticky" nature, can adhere to the probe of the Texture Analyzer, thus resulting in higher adhesiveness. This was also found in a study on cheese analogues with varying oil content (Shabani et al., 2016).

5.3.2 Sensory perception

To investigate the effect of composition and mechanical properties of cheeses on their sensory profile, a sensory evaluation was performed including 9 sensory attributes: 7 texture attributes and 2 flavor and taste attributes. The scores of the perceived intensities of each attribute are shown in Table 5.3.

Table 5.3. Intensity scores of the sensory attributes evaluated with the Rate-All-That-Apply (RATA) method (N=77). Mean values ± standard error are given. Different letters indicate significant difference between products at p < 0.05.

	Full fat			Low fat	
Ripening weeks	7	16	48	7	16
Texture					
Hardness	2.3 ± 0.2^{a}	3.5 ± 0.2^{b}	6.7 ± 0.2^{d}	4.2 ± 0.2^{c}	6.1 ± 0.2^{d}
Brittleness	2.1 ± 0.2^{a}	2.6 ± 0.2^{ab}	4.6 ± 0.3^{c}	3.4 ± 0.3^{b}	4.4 ± 0.2^{c}
Elasticity	5.0 ± 0.3^{bc}	4.7 ± 0.2^{bc}	3.3 ± 0.2^{a}	5.4 ± 0.2^{c}	4.2 ± 0.2^{ab}
Stickiness	4.5 ± 0.2^{b}	4.6 ± 0.2^{b}	4.6 ± 0.2^{b}	3.3 ± 0.2^{a}	3.9 ± 0.2^{a}
Smoothness	6.3 ± 0.2^{c}	5.6 ± 0.2^{c}	3.5 ± 0.2^{a}	4.0 ± 0.2^{b}	3.5 ± 0.2^{a}
Creaminess	6.2 ± 0.2^{b}	5.9 ± 0.2^{b}	3.9 ± 0.2^{a}	3.9 ± 0.2^{a}	3.8 ± 0.2^{a}
Fattiness	5.3 ± 0.2^{b}	5.6 ± 0.2^{b}	4.4 ± 0.2^{a}	3.9 ± 0.2^{a}	4.2 ± 0.2^{a}
Basic flavor and taste					
Overall flavor	5.5 ± 0.2^{ab}	5.6 ± 0.2^{bc}	6.5 ± 0.2^{d}	4.7 ± 0.2^{a}	6.3 ± 0.2^{cd}
Saltiness	4.2 ± 0.2^{a}	5.4 ± 0.2^{b}	6.7 ± 0.2^{c}	4.1 ± 0.2^{a}	6.4 ± 0.2^{c}

As expected, the full fat cheeses with longer ripening time were perceived harder, more brittle and less elastic, which was consistent with the trend observed for dry matter. This was also seen for low fat cheese when ripening time increased from 7 to 16 weeks. Ripening time also showed an influence on the attributes smoothness, creaminess and fattiness: the intensity scores for these three attributes significantly decreased during 6 to 48 weeks of ripening for full fat cheese. For low fat cheeses, a higher score for smoothness was found for longer ripening time (16 weeks). When we look at the difference between cheeses with different fat levels, it was found that low fat cheeses were perceived as harder and more brittle compared to the full fat cheeses. The effect of fat content on elasticity was less clear. Low fat cheeses had also lower scores for the attributes smoothness, creaminess and fattiness than full fat cheeses. For the attribute stickiness, the fat content had a large influence as well, while the ripening time had no effect. Full fat cheeses were perceived more sticky than low fat cheeses, which is in agreement with findings from other studies (Bryant et al., 1995; Gwartney et al., 2002; Liu et al., 2008; Rogers et al., 2009).

It is not surprising to find that cheese with longer ripening time had higher overall flavor intensity, as the development of cheese aroma and flavor highly depends on the biochemical and microbiological events mediated by different enzymes and cultures during ripening (Visser, 1993; Khattab et al., 2019). No significant difference in overall flavor intensity was found between cheeses with different fat levels. Saltiness increased during ripening, which could be attributed to the increased salt content due to moisture evaporation. The results of the sensory evaluation show that some texture attributes were more affected by ripening time and others by fat content.

5.3.3 Correlation between cheese characteristics and texture attributes

To gain more insights into the link between texture attributes and cheese characteristics, Pearson's correlation coefficients (R) between the perceived texture attributes and both the composition and the mechanical properties of the cheeses were determined. The results are shown in Table 5.4. In addition, to visualize the relationship between these parameters, we also included a Principal Components Analysis (PCA) plot (Fig 5.1).

Stickiness was positively related to dry matter ($R = 0.90$, $P < 0.05$). Also the fat content played an important role in the perception of this attribute, as a strong correlation ($R = 0.94$, $P < 0.01$) was found with stickiness, and fat content and stickiness are located close to each other in the PCA plot in Fig 5.1b. As a high level of fat is linked to a low protein in dry matter content and protein/fat ratio, a strong correlation was seen between stickiness and parameters of dry matter content and protein/fat ratio as well. Except stickiness, the results from Table 5.4 demonstrate that almost no direct relations were found between cheese composition and texture attributes. This was expected; it is difficult to link attributes to one component only since the properties of the cheese matrix are complex, arising from different interactions among various compounds.

Table 5.4. Pearson's correlation coefficients (R) between sensory attributes and composition and physical properties of the studied cheeses.

	Sensory attributes						
	Hardness	Brittleness	Elasticity	Stickiness	Smoothness	Creaminess	Fattiness
Composition of cheese							
Dry matter	0.30	0.17	-0.78	***0.90****	0.07	0.23	0.51
Fat content	-0.08	-0.19	-0.48	***0.92****	0.40	0.50	0.65
Protein content	0.61	0.66	-0.17	-0.57	-0.74	-0.71	-0.60
FDM[1] (%)	0.23	0.34	0.37	***0.93****	-0.54	-0.63	-0.72
PDM[2] (%)	-0.25	-0.36	-0.31	***-0.87****	0.53	0.61	0.68
P/F ratio[3]	0.19	0.31	0.40	***-0.95****	-0.52	-0.61	-0.74
Mechanical properties of cheese							
Young's modulus	0.73	0.71	-0.66	0.06	-0.60	-0.49	-0.24
Fracture strain	***-0.88****	***-0.82****	***0.96****	-0.33	0.65	0.51	0.20
Fracture stress	0.31	0.34	-0.05	-0.32	-0.37	-0.32	-0.19
Hardness_25%	*0.72*	0.70	-0.66	0.07	-0.58	-0.47	-0.20
Resilience	-0.48	-0.38	***0.91****	-0.79	0.12	-0.01	-0.26
Cohesion	-0.61	-0.50	***0.96****	-0.71	0.27	0.12	-0.17
Adhesiveness	0.03	0.15	0.55	***-0.98****	-0.40	-0.54	-0.76

*P<0.05, **P<0.01.

[1-3] Fat content in dry matter (FDM), protein content in dry matter (PDM) and the ratio of protein to fat (P/F ratio).

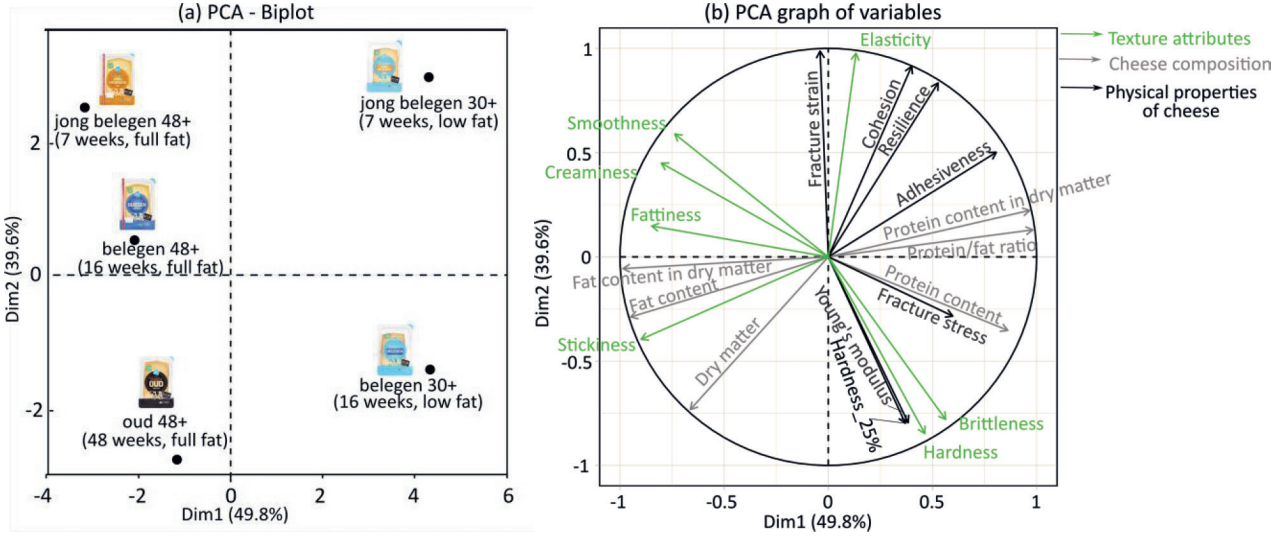

Figure 5.1. Principal Component Analysis (PCA) of the characteristics and texture attributes of the five studied cheeses, which had different fat levels (full fat and low fat cheese) and ripening time (7, 16 and 48 weeks). The individual samples map is shown on the left (a), and the correlation circle displaying the relationship between the variables describing composition (grey), mechanical properties (black) and texture attributes (green) of dimensions 1 and 2 is shown on the right (b). The level of confidence was 0.95.

It seems that the mechanical properties of the cheese were more relevant to explain some of the texture attributes. Table 5.4 shows strong negative correlations between fracture strain and the two attributes hardness and brittleness, i.e. attributes perceived at the first bite. Fig 5.1 demonstrates that other mechanical properties of cheese, such as Young's modulus and hardness of cheese, were close to these two texture attributes (brittleness and hardness). Although the relation is clear, the correlations between these parameters were not significant, as evidenced by low R values ($P > 0.05$) in Table 5.4. Even though fracture stress has been shown to be related to the texture attribute hardness in brittle food materials, such as fruits, vegetables and biscuits (Vincent, 2004; Barrangou et al., 2006; Kim et al., 2012), we do not see such a correlation in the present study (Table 5.4). It should be noted that for the full fat cheeses in our study, the perceived hardness increased with increased ripening time. However, the fracture stress initially increased after 16 weeks ripening, but decreased again after 48 weeks ripening (Table 5.2). The initial increase can be attributed to the increase in dry matter, and the subsequent decrease at later ripening time was probably caused by extensive proteolysis after 48 weeks. This may explain why the fracture stress showed no direct link to the perceived hardness in our study. Such a low correlation between perceived hardness and fracture stress was also found by Guichard et al. (2021), who summarized the results from 6 different research projects related to sensory perception of cheese.

The fracture strain was highly correlated to elasticity, a texture attribute that is perceived at the early mastication stage (Devezeaux de Lavergne et al., 2017). A low fracture strain is related to an easy break-up of the sample, and, therefore, the perceived elasticity is low. Such a correlation between fracture strain and sensory elasticity was also reported by Jaros et al. (1997) in Emmental cheeses. Besides fracture strain, also resilience and cohesion were closely related to perceived elasticity (Table 5.4). These parameters are also well known to be associated with sensory elasticity, and high correlations have also been seen for white cheeses (Baysal and

Ozcan, 2020) and emulsion-filled agar/gelatin gels (Devezeaux de Lavergne et al., 2015b). A strong correlation was found also between stickiness and adhesiveness, which is not surprising, as both parameters are used to describe how foods stick to the probe/teeth and palate (Fiszman and DamÁSio, 2000; Karagul-Yuceer et al., 2007).

For the attributes smoothness, creaminess and fattiness, no correlations were found with the mechanical properties of cheese. The PCA plots also show that these three attributes were located far away from most of the cheese properties. These results demonstrate that cheese properties are not sufficient to explain such complex sensory attributes. This has already been acknowledged in literature, as these attributes are believed to be more related to the structural changes in the food during oral processing and are perceived mainly at the end of the mastication process (Devezeaux de Lavergne et al., 2015b). For example, fattiness is considered to be related to in-mouth fat release during oral processing, which often requires structural break down of the food, and migration of the fat from the food (Koç et al., 2013).

5.3.4 Bolus formation and bolus properties

The previous results showed that complex attributes, as smoothness, creaminess and fattiness, cannot be explained on the basis of the mechanical properties, and that changes in cheese properties after oral processing need to be taken into account. As the mentioned attributes are perceived during the later stages of mastication, we also investigated the properties of the cheese boluses and how these properties relate to the compositional and mechanical characteristics of the studied samples. Finally, we evaluated possible correlations between bolus properties and these complex texture attributes.

5.3.4.1 Chewing time and saliva incorporation

To understand bolus formation during oral processing, we studied how total chewing time and saliva incorporation depended on the properties of the different cheese samples. The results are shown in Table 5.5.

Table 5.5. Chewing time and saliva incorporation (N=20) during oral processing of five cheese samples. Different letters indicate significant difference between products at p < 0.05. Mean values ± standard error are given.

	Full fat			Low fat	
Ripening weeks	7	16	48	7	16
Total chewing time (s)	21.4±2.1^{a}	25.9±2.0^{ab}	27.9±1.9^{b}	28.4±2.3^{b}	28.2±2.1^{b}
Saliva incorporation (mg/g of dry food)	397±31^{a}	554±35^{b}	455±32^{ab}	522±41^{b}	681±39^{c}

The composition and mechanical properties of cheeses influenced the total chewing time and incorporated saliva. Table 5.5 shows that for both full and low fat cheeses, chewing time increased with increased ripening time (i.e. increased dry matter and hardness). This is in line with the fact that for dry and hard foods a longer time is required to achieve a bolus consistency suitable for swallowing (Hutchings and Lillford, 1988; Chen et al., 2013; Panouillé et al., 2014). We found that saliva incorporation increased with longer chewing time (7 and 16 weeks of ripening), both for full and low fat cheeses. However, this trend was not maintained for longer ripening time, as saliva incorporation decreased for the sample ripened for 48 weeks, even though chewing time increased. The difference in saliva incorporation was most likely due to the water absorption capability of the different cheese matrices, as also discussed before for breads and potatoes (van Eck et al., 2020). In their research, breads were able to absorb more moisture during oral processing than boiled potatoes, and thus high saliva incorporation was reported for bread. Even though the sample had more time to incorporate saliva, the cheese matrix was apparently too hard to absorb the saliva into the cheese bolus. This was reflected in the lower saliva incorporation of the hard cheese with a ripening time of 48 weeks. This was also seen in the study of Lorieau et al. (2018), who examined the influence of the texture of model dairy products on bolus properties; a slightly lower amount of saliva was incorporated for harder model cheeses than for softer model cheeses while they had the same composition and same level of chewing time.

Not only the ripening time, but also the fat content influenced chewing time. Shorter chewing time and less saliva corporation were observed for full fat cheeses compared to the low fat cheeses. Saliva is incorporated to soften the bolus and also to lubricate the bolus for safe swallowing (Bongaerts et al., 2007; Ben Tobin et al., 2020). The fat present in the cheeses had a lubricating function as well, which can facilitate the process of swallowing. Samples with higher fat content thus required less saliva incorporation. The fact that more saliva was needed for the low fat cheeses was also clear from the results of the moisture content of their boluses. The two low fat cheese boluses had levels of moisture (58.4 ± 0.5%) higher than the three full fat cheese boluses (51.6 ± 0.4%). Drago et al. (2011) investigated the saliva incorporation in model dairy products and also found that more saliva was incorporated into the bolus of non-fat samples compared to samples containing fat. In addition, the lower fracture strain for samples with the same ripening time may also lead to a faster break up, and therefore a shorter chewing time.

That the chewing time was influenced by dry matter, hardness and fat content of food was also found in other studies on crackers (van Eck et al., 2020), bread (Panouillé et al., 2014), and model gels (Devezeaux de Lavergne et al., 2015b; Almotairy et al., 2021). However, in our study no obvious correlations were found between chewing time and different compositional factors and mechanical properties (Table S5.2). Only saliva incorporation had a strong correlation with protein content and fracture stress, which could be related to the change in both dry matter content and protein/fat ratio.

5.3.4.2 Bolus properties at moment of swallowing

The bolus properties at the moment of swallowing are shown in Table 5.6. As the full fat cheeses already had a higher dry matter content and less saliva was incorporated (Table 5.5), boluses of high fat cheese had a higher dry matter than low fat cheese samples. Boluses of full fat cheese of course contained a higher amount of fat. When considering the properties of a bolus, not only saliva, dry matter and fat content in the bolus, but also the release of fat from the bolus should be taken into account (Loret et

al., 2011; Guo et al., 2013). We estimated the amount of released fat in mouth based on the fat content of the bolus and the fat content of the cheese products. The full fat cheeses showed a higher degree of fat release, in agreement with a higher fat content in the cheese itself. Surprisingly, for these three full fat cheeses, the highest fat content (48 weeks) (Table 5.2) corresponded to the lowest fat release (table 5.6). The limited fat release is most likely related to the high hardness of the sample (Table 5.2), which limited saliva incorporation (Table 5.5), but also made it more difficult to expel fat from the matrix (Table 5.6).

Table 5.6. Composition of bolus, fat released in mouth and physical properties of the boluses obtained for the five studied cheeses. Different letters indicate significant difference between products at $p < 0.05$. Mean values ± standard error of the mean are given.

	Full fat			Low fat	
Ripening weeks	7	16	48	7	16
Composition of bolus (N=20)					
Dry matter (wt%)	47.1±0.7^{b}	47.2±0.5^{b}	51.0±0.6^{a}	41.1±0.8^{c}	42.0±0.5^{c}
Fat content (wt%)[1]	24.9±0.9^{bc}	22.6±1.2^{b}	26.8±0.7^{c}	15.0±0.9^{a}	15.4±0.8^{a}
Fat released in mouth (N=8)					
Released fat in mouth (g)	1.7±0.4^{b}	1.9±0.2^{b}	1.6±0.2^{b}	0.6±0.1^{a}	0.7±0.1^{a}
Percentage of released fat (%)	21.6±2.5^{b}	23.7±4.4^{b}	21.0±4.0^{b}	16.9±1.2^{a}	19.3±1.6^{a}
Size distribution of particles in bolus (N=20)					
Number of particles	158±18^{a}	172±22^{a}	237±21^{ab}	220±20^{a}	284±11^{b}
D_{10} (mm)	1.60±0.06^{a}	1.57±0.02^{a}	1.48±0.02^{a}	1.53±0.02^{a}	1.51±0.02^{a}
D_{50} (mm)	2.53±0.33^{a}	2.25±0.03^{a}	2.09±0.03^{a}	2.16±0.04^{a}	2.13±0.03^{a}
D_{90} (mm)	5.69±1.45^{a}	4.40±0.15^{a}	4.34±0.15^{a}	4.33±0.15^{a}	4.12±0.11^{a}
Mechanical properties of bolus (N=20)					
Bolus hardness (N)	1.6±0.1^{a}	1.6±0.1^{a}	1.8±0.1^{ab}	2.0±0.1^{b}	1.9±0.1^{b}
Adhesiveness (g·s)	-28.8±1.9^{b}	-29.1±2.2^{b}	-45.0±2.9^{a}	-16.4±1.5^{c}	-19.0±1.5^{c}
Cohesion (-)	0.34±0.01^{c}	0.26±0.01^{b}	0.25±0.01^{ab}	0.24±0.01^{ab}	0.21±0.01^{a}

[1]Fat content is given as an average value from 40 cheese boluses from 8 persons (N=8).

Table 5.6 shows that the number of particles in the bolus was higher for low fat cheeses than those of full fat cheeses with corresponding ripening time (7 and 16 weeks). Boluses for cheese with longer ripening time had more particles. These differences in

number of particles may be related to two aspects: the structural break down of the samples and the chewing time. A lower fracture strain (more brittle) for low fat cheeses (Table 5.2) resulted in a faster break-up, and thus in more fragmentation during mastication. These cheeses also had a longer chewing time (Table 5.5), which resulted in more mastication to reduce the bolus particle size. Cheeses with longer ripening time or lower fat content consequently produced more pieces in the obtained bolus. A larger number of particles should be reflected in smaller particles for the same sample volume, which was indeed shown in our result (Table 5.6). For low fat cheeses, the particles in the bolus were smaller. However, the differences were not statistically significant. All cheese boluses had a similar level of particle sizes (D_{10}, D_{50} and D_{90}), even though the total number of particles was different. Overall, we see limited effects on the particles within the obtained boluses.

Regarding the mechanical properties of the cheese boluses, hardness was lower for full fat cheeses, even though they contained higher dry matter (Table 5.6). An explanation could be that the fat melted in the mouth at body temperature, which contributed to the softening of the bolus. The effect of fat melting on the softening effect of the bolus was more pronounced in the full fat cheeses, resulting in a lower hardness (1.6-1.8) compared to the low fat cheeses (1.9-2.0). In term of adhesiveness, we do not see a clear relation with saliva incorporation, even though a high saliva incorporation has been shown to correspond to a more adhesive bolus for other food products (van Eck et al., 2019; Pematilleke et al., 2021). Adhesiveness was higher for full fat cheeses (ranging from -28.8 to -45) than for low fat cheeses (ranging from -16.4 to -19.0), even though full fat cheeses had less saliva incorporation. This indicates that the fat content played a more important role than saliva incorporation. Saliva incorporation and bolus formation also had an effect on cohesion, which represents how the particles stick to each other to resist bolus flow (Panouillé et al., 2014; Young et al., 2016). Even though high saliva incorporation is often linked to high bolus cohesion in solid samples (Mosca and Chen, 2017), this was not shown in our results,

indicating that saliva was not the only parameter to influence cohesion. The bolus of low fat cheese, with more saliva incorporation, showed lower cohesion (Table 5.6). This can be explained by two reasons. On one hand, the fat present in the bolus of high fat cheeses was able to strongly glue the particles to each other, which resulted in higher cohesion for the full fat cheeses. On the other hand, for the bolus of low fat cheese, the slightly higher number of particles might be responsible for the lower cohesion. A higher number of particles is related to a higher surface area, and would therefore require more saliva incorporation to glue all the particles together. Even though more saliva was incorporated, the amount of saliva might have been insufficient to cover enough surface area to form a high cohesive bolus. In fact, a strong negative correlation was observed between cohesion and the number of particles ($R = -0.83$, $P < 0.05$), confirming this hypothesis. The influence of the number of particles on cohesion was also observed in sausage boluses by Aguayo-Mendoza et al. (2020). Thus, we conclude that both fat content and the number of particles influenced bolus cohesion.

The results discussed above show that the cheese fat content had a large influence on bolus properties, such as hardness, adhesiveness and cohesion. This was most likely related also to the lubrication properties of fat that make the bolus easier to swallow and has the ability to glue particles together into a cohesive bolus. To gain more insights into these aspects, we also measured the friction coefficient (μ, inverse of lubrication) of the different bolus samples (Table 5.7) between two rough surfaces.

Table 5.7. Friction coefficient (μ) of the boluses obtained for the five studied cheeses (N=20). Different letters indicate significant difference between products at $p < 0.05$. Mean values ± standard error of the mean are given.

	Full fat			Low fat	
Ripening weeks	7	16	48	7	16
μ [1]	0.50±0.02[a]	0.52±0.02[a]	0.59±0.02[a]	0.52±0.02[a]	0.75±0.05[b]

[1]*Friction coefficient (μ) is given as an average value of μ measured at 8 different speeds, ranging from 4.7 mm/s to 47.5 mm/s.*

The μ for full fat cheeses was similar, with values of 0.50, 0.52 and 0.59, independently of the ripening time. This may be attributed to the similar high levels of fat content in the bolus. Even though the fat content differed slightly, the similar lubrication properties of the full fat cheeses may be explained by a saturation effect of fat. Such a saturation effect of fat was also reported by Chojnicka et al. (2009) in emulsion-filled gels. The friction coefficient, μ, of gelatin gels containing Tween-stabilized emulsion droplets decreased to 0.15 with 5 wt% oil addition, but it remained the same with a further increase of the oil content. Thus, above a certain critical fat content, a further increase in fat content does not contribute to more lubrication (lower μ). In the case of the low fat cheese, the cheese with 7 weeks ripening showed a friction coefficient, μ, of 0.52, similar to the values of the full fat cheeses. Even though it had a lower fat content, it could be that this fat content was already in the range of the saturation concentration. However, the bolus of low fat cheese with 16 weeks ripening had a significantly higher μ. As they had the same fat content, this difference cannot be attributed to fat content only. Next to fat content, other characteristics of the bolus thus also influence μ. The higher μ for the low fat cheese with longer ripening time (16 weeks) may be related to the fact that the bolus contained more and harder particles. It is known that hard particles can increase friction, especially when they are irregular in shape (Liu et al., 2016). As the bolus of the full fat sample with even harder particles (48 weeks) did not show higher values for μ, we can conclude that in this case, fat influenced μ more than the mechanical properties of the particles itself.

In conclusion, these results show that composition and the physical properties of cheese influence the bolus formation and bolus properties. Cheese fat content and hardness determine to a large extent how much saliva is incorporated and how long the sample needs to be chewed to obtain a bolus suitable for swallowing. The difference in composition and chewing time influences then bolus hardness, number of particles, and the lubrication properties of the bolus.

5.3.6 Variability between participants

According to literature, sensory perception can be influenced by many differences in eating behavior among participants (e.g. fast eaters vs. slow eaters) (Shah et al., 2014; McCrickerd et al., 2017; Goh et al., 2021). This is supposed to provide food boluses with different properties and may also influence the perception of cheese. The different eating behavior is reflected in differences in oral processing time, also known as eating/chewing time. In the study, the participants (n=20) could be divided into three groups according to the chewing time; (1) a short chewing time group (6 persons, 17.3 ± 3.19 sec/g), (2) a mid chewing time group (9 persons, 26.0 ± 7.69 sec/g), and (3) a longer chewing time group (5 persons, 37.88 ± 5.48 sec/g). This was linked to differences in saliva incorporation: higher eating rates were related to low saliva incorporation, whereas low eating rates led to more saliva incorporation. Differences in chewing time was found to have a significant influence on bolus hardness and bolus adhesiveness, which is summarized in Fig 5.2.

In the case of full fat cheese, longer chewing time gave a softer bolus, corresponding to a more extended breakdown of the cheese and a higher degree of melted fat during mastication. However, this effect was not evident in low fat cheese. For these cheeses, less fat could be released, and the effect of chewing time on cheese breakdown did not have a significant impact. With longer chewing time, the bolus also became more sticky, corresponding to a higher adhesiveness. Although we did not see differences in bolus hardness for the low fat cheeses, we did see differences in bolus adhesiveness. This can be attributed to both the higher fat release and saliva incorporation for a higher chewing time. Devezeaux de Lavergne et al. (2015a) reported similar results for a study on sausages. Participants with longer chewing time provided a softer and more adhesive bolus with more fat release and saliva incorporation. The difference in bolus properties may induce differences in sensory perception among groups. It would be worth investigating inter-oral responses of healthy individuals during chewing concerning the bolus properties and the sensory perception in the future. This would

be beneficial for the design of cheese based on target groups of consumers. Nevertheless, the present study focused on understanding the relation between bolus properties and complex sensory attributes. Although participants showed differences in chewing time and bolus properties, we analyzed any correlation between bolus properties and sensory perception for all participants together.

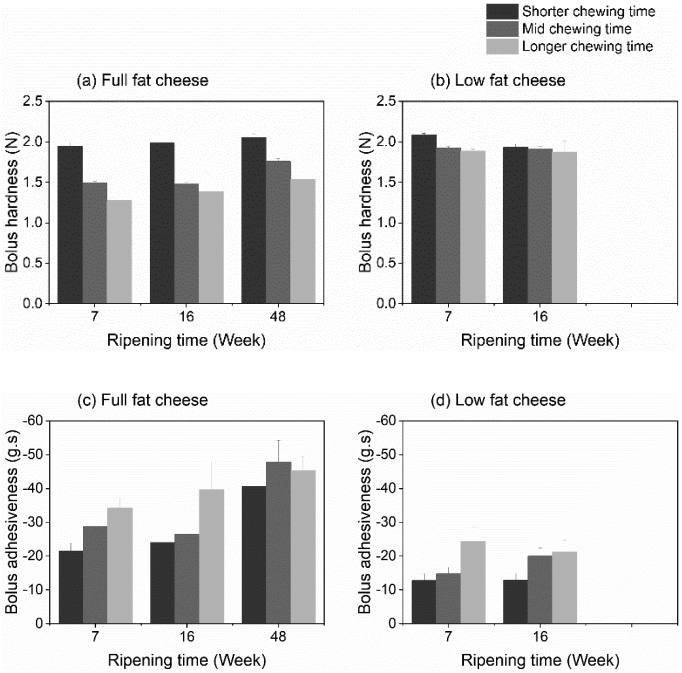

Figure 5.2. Hardness(a, b) and adhesiveness (c, d) of bolus obtained from participants with short (black), mid (dark gray) and long (gray) chewing time; a&c: full fat cheese, b&d: low fat cheese.

5.3.5 Correlation between oral processing, bolus properties and sensory perception

To understand the role of cheese oral processing in sensory perception, the correlations between bolus properties and the different texture attributes were investigated. The results are shown in Table 5.8 and in the Principal Components Analysis (PCA) plot of Fig 5.3. The texture attributes related to the first bite and early mastication stage, such as hardness, brittleness, and elasticity showed no significant correlation with bolus properties, with the exception of a negative relationship

between brittleness and bolus cohesion ($R = -0.81$, $P < 0.05$). This was expected, since these texture attributes are mostly related to the cheese properties themselves (section 5.3.3). On the other hand, for the complex attributes smoothness, creaminess and fattiness, the correlations with the cheese properties were low. We expected these attributes to have a higher correlation with the bolus properties arising from oral processing. Table 5.8 shows indeed that smoothness, creaminess, fattiness and stickiness had strong correlations with different bolus properties such as bolus hardness, particle distribution and cohesion.

Table 5.8. Pearson's correlation coefficients (R) between sensory attributes and oral processing parameters and bolus properties. Calculations were carried out using the average value of each parameters.

		Sensory attributes						
		Hardness	Brittleness	Elasticity	Stickiness	Smoothness	Creaminess	Fattiness
Bolus formation and fat release	Total chewing time	0.80	***0.83****	-0.36	-0.53	***-0.90****	***-0.88****	-0.72
	Saliva incorporation	0.44	0.47	-0.11	-0.39	-0.51	-0.47	-0.32
	Released fat in mouth	-0.40	-0.51	-0.19	0.69	***0.93****	0.78	***0.89****
	Percentage of released fat	-0.30	-0.41	-0.26	0.62	***0.93****	0.74	***0.92****
Bolus properties	**Composition of bolus**							
	Dry matter	0.07	-0.04	-0.60	***0.88****	0.25	0.35	0.51
	Fat content[1]	-0.12	-0.23	-0.44	***0.89****	0.43	0.51	0.60
	Size distribution of partials in bolus							
	Number of particles	***0.88****	***0.92****	-0.52	-0.45	***-0.93****	***-0.90****	-0.78
	D_{10}	***-0.97****	***-0.98****	0.69	0.29	***0.96****	***0.93****	0.76
	D_{50}	***-0.87****	***-0.88****	0.52	0.37	***0.91****	***0.86****	0.65
	D_{90}	-0.75	-0.76	0.38	0.38	0.80	0.74	0.52
	Mechanical properties of bolus							
	Bolus hardness	0.58	0.66	-0.01	***-0.84****	***-0.83****	***-0.91****	***-0.96****
	Adhesiveness	-0.28	-0.18	0.73	-0.78	-0.03	-0.13	-0.32
	Cohesion	-0.79	***-0.81****	0.40	0.42	***0.85****	0.79	0.59
	Friction coefficients							
	μ[2]	0.72	0.74	-0.54	-0.18	-0.68	-0.62	-0.46

[1]Fat content is given as an average value from 40 cheese bolus from 8 person (N=8).

[2]Friction coefficient (μ) is given as an average value of μ measured at 8 different speeds, ranging from 4.7 mm/s to 47.5 mm/s.

**P<0.05, **P<0.01.*

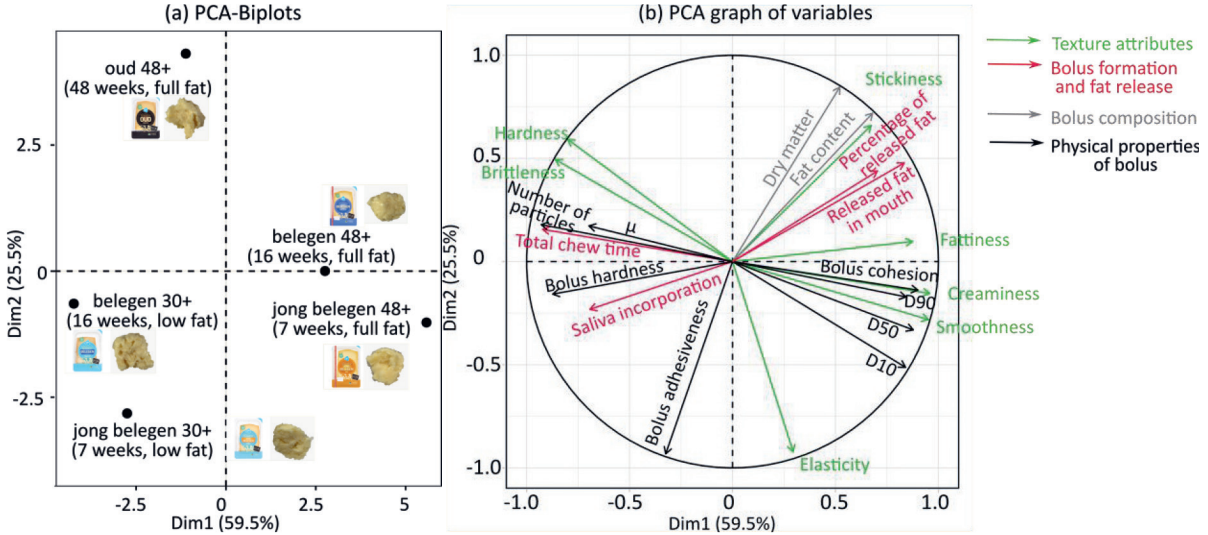

Figure 5.3. Principal Component Analysis (PCA) of texture attributes, eating behaviors and bolus properties of the five studied cheeses. The individuals map (samples) is shown on the left (a), and the correlation circle displaying the relationship between variables describing texture attributes (green), oral processing (red), composition (gray), and mechanical properties (black) of dimensions 1 and 2 is shown on the right (b). The level of confidence was 0.95.

Bolus hardness showed a strong negative correlation ($P < 0.05$) with stickiness, smoothness, creaminess and fattiness (Table 5.8), with Pearson's correlation coefficients of -0.84, -0.83, -0.91 and -0.96. As discussed in the previous section, bolus hardness was largely influenced by oral processing, due to the saliva incorporation and fat melting. For smoothness and creaminess, also a high correlation was found with number of particles and particle size distribution. A high number of particles was linked to lower smoothness and lower creaminess, as strong negative correlations were found ($R = -0.93$ and -0.90, respectively). Our results suggest that smoothness and creaminess are more associated with the formation of small particles in the cheese bolus, as higher positive correlations were found for D10, and lower correlations with D50 and D90 (Table 5.8). However, this appears to be coincidental, as we found no significant differences in the particle sizes of all samples. Other aspects related to the number or size of the particles, such as cohesion and lubrication properties of the bolus were probably more relevant to explain smoothness and creaminess.

We found a strong positive correlation between bolus cohesion and smoothness ($R = 0.85$, $P < 0.05$). As is mentioned in section 5.3.4.2, a less cohesive bolus was formed when the fat level of cheese was lower and more particles were present. Likely, both the lower fat content and the higher number of particles led to lower bolus cohesion and, consequently, a lower smoothness was perceived. Negative correlations between friction coefficient (μ) and smoothness, creaminess and fattiness were obtained (Table 5.8), although these correlations were not significant ($P > 0.05$). However, the friction coefficients of the full fat cheeses were almost the same (Table 5.7), whereas perceived smoothness and creaminess were different. This may explain that these correlations were not significant, and these results also suggest that other physical properties, such as bolus hardness and cohesion, played a large role for these texture attributes. Fat, therefore, did not directly influence sensory perception through the friction coefficient of the bolus, but more by the lubrication effects of the fat within the bolus.

Table 5.8 also shows that the attributes smoothness, creaminess and fattiness had related to a higher fat content in the cheese, but also shows that released fat provides a more smooth, creamy and fatty feeling due a physical fat coating left in the mouth. The fact that chewing time shows strong negative correlations with smoothness, creaminess and fattiness can also be explained by the fat content. High fat samples had shorter chewing time as less saliva incorporation was needed. Therefore, shorter chewing time was related to higher level of smoothness, creaminess and fattiness.

Although the complex texture attributes smoothness, creaminess and fattiness were not all correlated to the same bolus characteristics, they are located close to each other in the PCA plot (Fig 5.3b). The close relation between these three attributes was also found in many other sensory studies on cheese (Bayarri et al., 2012; Ningtyas et al., 2019; Aguayo-Mendoza et al., 2021), and is actually not surprising, as in dairy products all three parameters are linked to the presence and the concentration of fat (Frøst and Janhøj, 2007). For acidified milk drinks, yoghurt and cream cheese, smoothness has also been suggested to be closely linked to creaminess (Kokini, 1987; Kora et al., 2003; Janhøj T et al., 2006; Johansen et al., 2008).

Overall, the results reveal the important role of the properties of cheese bolus in understanding the complex texture attributes perceived at late mastication stage, such as smoothness, creaminess and fattiness. Fat content, lubrication and cohesion of particles were key factors to explain the correlations between bolus properties and these texture attributes. As fat globules melted during mastication, cheese with higher fat content formed a softer and more cohesive bolus, which was perceived as smoother and creamier. When fat content was low, the lubrication and cohesion of particles in the bolus became more dominant in determining smoothness and creaminess. For hard and low fat cheese, the limited fat content, limited saliva incorporation and more hard particles led to less cohesion of the bolus, which resulted in a lower lubrication of the bolus itself (higher μ), and thus led to a less smooth and

creamy perception. Lubrication aspects to form a cohesive bolus is thus an important factor.

The knowledge obtained in this study offers a better understanding on complex texture attributes. It may help to design strategies to control smoothness and creaminess of cheese. The fat melting was shown to play an important role in bolus hardness and it determines the sensation of smoothness and creaminess. Further control over fat melting may thus be used as a strategy to control these sensory attributes. For example, fat melting may be altered by using different melting fractions of fat. It has been reported that milk from specific seasons contain fat with different low/high melting fractions, depending on the triacylglyceride composition (Arita-Merino et al. (2022). However, further research would be needed to verify such effect.

5.4 CONSLUSION

In this study, we investigated the relation between cheese compositional and physical properties, bolus formation and sensory perception. As expected, cheese properties were not sufficient to explain more complex attributes (smoothness, creaminess and fattiness). Our results confirmed the hypothesis that these complex attributes were more related to the bolus properties, such as fat content, cohesion and friction coefficient. As fat melted during mastication, boluses containing more fat were softer and more cohesive, leading to a smoother and creamier perception. For low fat cheeses, the cohesion and friction coefficient of the bolus became more important in determining smoothness and creaminess. When cheese was harder and had low fat content, a less cohesive bolus with more hard particles was formed due to limited fat content and saliva incorporation. This eventually resulted in a lower lubrication of the bolus itself (higher μ), and thus lower smoothness and creaminess was perceived. This study provides new insights into the mechanism behind complex cheese texture attributes . The obtained knowledge may be used to design strategies to control the sensory properties of cheese by modulating the formation of boluses.

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SUPPLEMENTARY INFORMATION

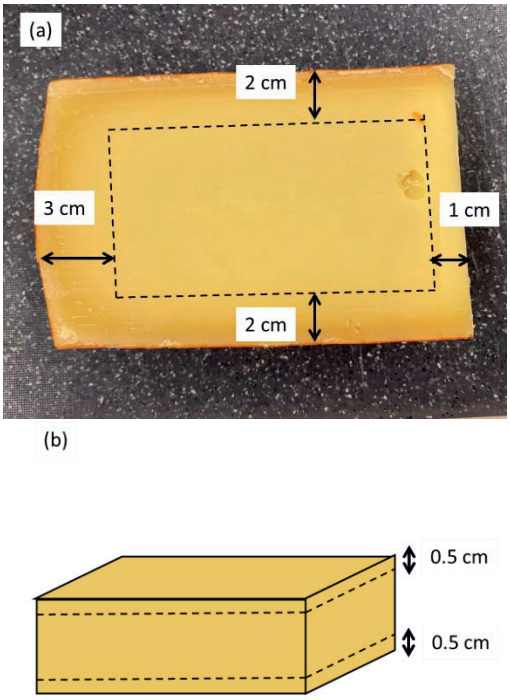

Fig. S5.1. Image of cutting the cheese samples. (a) The outer crust was discard. (b) The bottom and top part are discard.

Tables S5.1. Sensory attributes and definitions used during evaluation of cheese.

Attributes	Definition
Texture	
Hardness	The force required to bite the cheese. A sample requiring more force is harder.
Brittleness	Completely bite through cheese and evaluate the degree to which the sample fractures
Elasticity	The extent to which the cheese returns to its initial form after biting. How elastic/rubbery the sample is.
Stickiness	Overall sensation of the food sticking to the palate and around the teeth.
Smoothness	The sensation of smoothness detected when the tongue slides the sample across the palate.
Creaminess	A full, soft or velvety feeling when the food is moved through the mouth.
Fattiness	The intensity of an oily or greasy feeling in the mouth. This is related to the amount of fat that is perceived.
Basic flavor and taste	
Overall flavor	Overall intensity of the flavor
Saltiness	Salty taste, associated with salt

Table S5.2. Pearson's correlation coefficients (R) between eating behavior and the compositional and mechanical properties of cheese. Calculations was carried out by using the average value of each parameters.

	Eating behavior	
	Total chew time	Saliva incorporation
Composition		
Dry matter	-0.10	-0.17
Fat content	-0.52	-0.64
Protein content	0.79	***0.95****
Fat in dry matter	-0.69	-0.78
Protein in dry matter	0.60	0.69
Protein/fat ratio	0.63	0.73
Mechanical properties of cheese		
Young's modulus	0.51	0.78
Hardness	0.54	-0.30
Fracture strain	-0.56	0.80
Fracture stress	0.46	***0.98****
Resilience	0.05	0.18
Cohesion	-0.15	0.03
Adhesiveness	0.43	0.22

*P<0.1, **P<0.05.


CHEESE

Chapter 6

General Discussion

6.1 Introduction

Texture is an important aspect of cheese quality and is crucial for consumer appreciation. Although many studies have focused on cheese texture in relation to compositional properties, such as the protein, fat, moisture, or calcium content and pH (Green et al., 1981; Barbano et al., 1994; Bryant et al., 1995; Hennelly et al., 2005; Everard et al., 2006; Rogers et al., 2010; Ong et al., 2012; Soodam et al., 2014; Sheibani et al., 2015; Guinee, 2016), the relation between texture and the hydrolysis of specific casein (α_{s1}-CN and β-CN) fractions is not well understood yet. In addition, the sensory perception of cheese products, especially in terms of some complex texture attributes such as creaminess, smoothness and fattiness, is still not completely clear. These attributes are perceived at the late mastication stage and cheese properties alone are insufficient to explain them. This thesis aimed to better understand cheese texture from the perspective of the hydrolysis of specific casein fractions and bolus properties, focusing on both instrumental texture parameters and complex sensorial texture attributes.

In this chapter, the main findings of this thesis are summarized. Then, the relation between hydrolysis of specific casein fractions, protein network properties and cheese texture is discussed, as well as the way texture relates to other compositional properties such as the content of moisture and fat, and the pH of cheese. Subsequently, further insights into the perception of texture concerning bolus properties are provided. Meanwhile, based on the results in this thesis, some further studies on cheese texture are suggested. Lastly, the conclusion and the outlook of this field are given.

6.2 Main findings of the thesis

In Fig 6.1, a schematic representation of the main findings from the thesis is given.

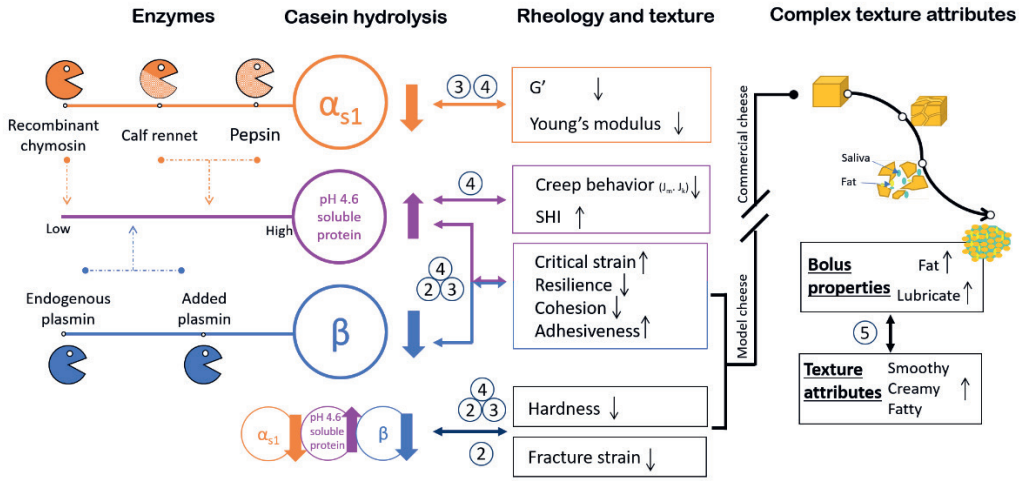

Figure 6.1. Overview of main findings of this thesis.

The results of this thesis show that the instrumental texture parameters were strongly related to the hydrolysis of specific casein fractions (α_{s1}- and β-CN) and the consequent formation of large (insoluble) hydrolyzed fragments, which highly depended on the activity and the type of the proteolytic enzymes in model cheeses (Fig 6.1). First, Young's modulus and G', two parameters measured within the linear regime of the samples (< 10% strain), decreased mainly with the breakdown of α_{s1}-CN (**Chapters 3&4**), while they were less affected by the breakdown of β-CN (**Chapter 3**) and the formation of pH 4.6 soluble protein (**Chapters 3&4**). Meanwhile, the rearrangement of the protein network as a result of newly formed hydrophobic interactions among large hydrolyzed products led to an increasing number of internal bonds, which was related to an increase in critical strain. This was more linked to the hydrolysis of β-CN (**Chapter 3**). On the other hand, the primary hydrolysis of α_{s1}-CN had less impact, as the hydrolyzed fragments were too large to induce rearrangements and generate new internal bonds. When further hydrolysis occurred and more fragments from α_{s1}-CN were released (~ 30% pH 4.6 soluble protein), these rearrangements became more significant (**Chapter 4**): critical strain thus increased and creep behavior changed as well. Hydrolysis of the caseins also led to decrease of resilience and cohesion. This decrease was found to be dominated by the hydrolysis of β-CN (**Chapters 2&3**) and

the further hydrolysis of large fragments obtained from the hydrolysis of α_{s1}-CN (**Chapter 4**). In addition, adhesiveness was more related to changes in pH , and was enhanced by the formation of small peptides (**Chapters 3&4**). For parameters obtained from large deformation (strain ≥ 40%), both hydrolysis of α_{s1}-CN and β-CN resulted in a decrease of hardness (**Chapters 2-4**).

To better understand the relation between casein hydrolysis and cheese texture, we used model cheeses in **Chapters 2-4**. Instead, commercial cheese were used to gain more insight into the sensory perception in **Chapter 5**. It was found that simple texture attributes such as hardness, brittleness and elasticity were related to the physical properties of the cheese (i.e. fracture strain and resilience), while more complex attributes, like smoothness, creaminess and fattiness were more related to the properties of the bolus. Especially the fat content and the lubrication of particles within the bolus were shown to play a critical role in the determination of smoothness and creaminess (**Chapter 5**).

6.3 Understanding texture in relation to physicochemical properties of cheese

This section gives insights into cheese texture arising from the properties of the compositional and structural properties of the cheese itself, with a focus on casein hydrolysis. First, based on the results obtained from **Chapters 3&4**, the effect of casein hydrolysis on the protein network is discussed. Then, correlations between cheese texture and the hydrolysis of specific casein fractions are examined, according to the findings in **Chapters 2-4**. Finally, other relevant factors, such as moisture content, fat content and pH, on the basis of the data obtained from **Chapters 2-5** and the works of previous researchers, are also discussed.

6.3.1 Relation between casein hydrolysis and protein network

In general, hydrolysis of intact casein (both α_{s1}-CN and β-CN) decreases the structural integrity of the casein micelles (Gagnaire et al., 2001), which is supposed to weaken the protein network. The results in **Chapters 3&4** show that hydrolysis also provided a rearrangement of the protein network. The interactions between some hydrophobic fragments released during casein hydrolysis were enhanced, and thus the number of internal bonds increased. These newly formed bonds are able to remain the strength of the casein network (**Chapter 3**). To elaborate this further, information on the protein network is given here, from the perspectives of the hydrolysis of both α_{s1}-CN and β-CN, induced by coagulant and plasmin. Fig 6.2 summarizes the effects of proteolysis on the strength of protein network.

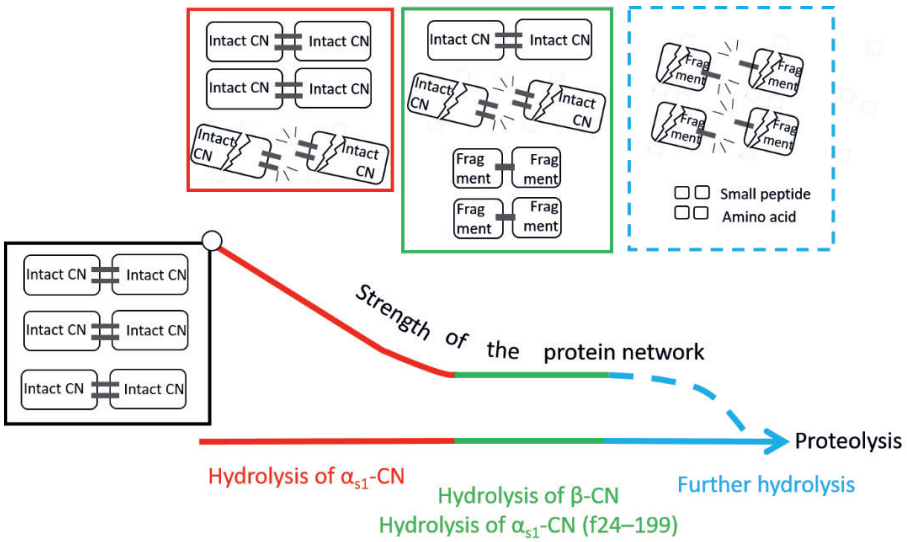

Figure 6.2. Illustration of the effect of casein hydrolysis on the interactions within the protein network of cheese.

When intact α_{s1}-CN was broken down, the strength of the protein network (G') decreased significantly (**Chapters 3&4**), due to the hydrolysis of the protein chains (marked as red in Fig 6.2). Thus, a strong correlation was found between intact α_{s1}-CN fraction and G'. A correlation between G' and the degree of casein hydrolysis was also

found for cheese made with recombinant chymosin (**Chapters 3&4**), when a low level (< 10%) of pH 4.6 soluble protein fraction was obtained. However, this relation was not shown for cheese made with calf rennet or coagulant containing a high proportion of porcine pepsin (**Chapter 4),** and a high level (~30%) of pH 4.6 soluble protein fraction was obtained. These results indicate that the strength of the protein network (G') also depends on the degree of casein hydrolysis (expressed as pH 4.6 soluble protein fraction). Although the hydrolyzed fragments were not further characterized in this thesis, knowledge on the hydrolysis of α_{s1}-CN by coagulant is well-documented (McSweeney and Fox, 1993; Piraino et al., 2007; Møller et al., 2012). During cheese ripening, chymosin initially cleaves the bond Phe_{23}-Phe_{24} in α_{s1}-CN (McSweeney and Fox, 1993; Piraino et al., 2007), yielding a small water-soluble peptide, α_{s1}-CN (f1–23), and a large water-insoluble fragment, α_{s1}-CN (f24–199). The low degree (< 10%) of casein hydrolysis induced by recombinant chymosin (**Chapters 3&4**) indicated the occurrence of initial hydrolysis, which was mainly attributed to the low amount of added recombinant chymosin and its low proteolytic activity on α_{s1}-CN (Uniacke-Lowe and Fox, 2017). The initial hydrolysis on intact α_{s1}-CN led to a decrease in G'. Subsequent hydrolysis of the larger fragment α_{s1}-CN (f24–199) depends on the type of coagulant (e.g. composition) and the physicochemical composition of the cheese (e.g. pH, moisture, casein fractions) (Exterkate et al., 1997; Michaelidou et al., 1998). For instance, during cheese ripening chymosin in calf rennet can potentially cleave the fragment α_{s1}-CN (f24–199) at sites of Leu_{149}-Phe_{150}, Leu_{156}-Asp_{157}, Trp_{164}-Tyr_{165} and Leu_{101}-Lys_{102} (Exterkate et al., 1997). The porcine pepsin was reported to have a broad specificity on hydrolyzing α_{s1}-CN and preferentially cleaves the C-terminal of the fragments (Luo et al., 2018; Perna et al., 2020). The further hydrolysis of α_{s1}-CN (f24–199) induced was indeed confirmed by the high (~30%) degree of casein hydrolysis obtained in **Chapter 4**, when calf rennet and pepsin were used. The network became more deformable, which was reflected by an increase in critical strain (**Chapter 4**). This increased critical strain is attributed to the protein rearrangements. The hydrolyzed fragments started moving due to hydrophobic interactions and thus new bonds

formed. Consequently, the number of the internal bonds increased and it led to a less brittle network. Unlike the significant decrease in G' for a low degree (< 10%) of casein hydrolysis, in this case, G' did not decrease any further. The interactions among the smaller hydrolyzed fragments obtained from further breakdown of α_{s1}-CN (f24–199) were responsible for retaining the strength of the protein network (marked as green in Fig 6.2).

Regarding the hydrolysis of β-CN, G' was found to be constant even though most intact β-CN was broken down in **Chapter 3**. This is marked as green in Fig 6.2. According to the literature, hydrolysis of β-CN is usually caused by plasmin, and the cleavage is achieved at three sites (Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}), resulting in the liberation of some hydrophobic fragments (ϒ-CNs) and complementary peptides (Eigel et al., 1984; Farkye, 1995; Exterkate et al., 1997; Møller et al., 2012). Although ϒ-CNs are also considered large fragments, they have a relatively lower molecular weight than α_{s1}-CN (f24–199). This is supported by SDS-PAGE results shown in many cheese studies (Gaiaschi et al., 2001; Li et al., 2018; Chen et al., 2021): ϒ-CNs have a MW lower than 20KDa and the MW of α_{s1}-CN (f24–199) ranged from 28.3-34.8 KDa. Due to the breakdown in multiple fragments, interactions among the hydrophobic fragments (ϒ-CNs) and rearrangements among casein aggregates alter the protein network. As a result, the number of internal bonds significantly increased and the strength of the protein network was retained (**Chapter 3**).

As the focus of this thesis was on how cheese texture is linked to casein hydrolysis caused by plasmin and coagulant, the possible influence of other exogenous proteinases (from starter and secondary cultures) was excluded. Theoretically, the large fragments that contribute to the protein network can be further hydrolyzed at a later stage of cheese ripening (Fox and Stepaniak, 1993; Fox et al., 2017), which can be triggered by the other exogenous proteinases. The large peptides would be hydrolyzed into medium and small peptides, which will subsequently be transferred into tripeptides, dipeptides and free amino acids (Farkye, 2004; Ardö et al., 2017). Although

this type of proteolysis is believed to be responsible for the formation of flavor and aroma, it may also weaken the protein network, and then potentially influence texture. The related effect is marked as a dashed blue line in Fig 6.2. It should also be noted that next to the proteolysis, other factors relevant to the starter and secondary cultures may also influence the cheese texture. For example, the pH level and calcium content in the cheese will change with the growth and fermentation of starters/cultures (Upreti et al., 2006; Sheehan et al., 2007; Ayyash et al., 2012), which may eventually have a large impact on cheese texture. Further studies that involve events taking place at the later stage of ripening, such as further proteolysis, pH changes and calcium solubilization, are needed to gain further insights into cheese texture formation.

6.3.2 Relation between casein hydrolysis and cheese texture

In the above section we discussed how the protein network within the cheese matrix is affected by the interactions among intact caseins and hydrolyzed fragments. Given these insights, it is possible to reveal how the obtained network of these structural elements responds to deformation, to better understand cheese texture. Relations between the protein network and different textural parameters, obtained at different degree of deformation are discussed.

As mentioned previously, the strength of the protein network decreased with the hydrolysis of intact α_{s1}-CN. This also explains the strong relation between Young's modulus and the intact α_{s1}-CN fraction (**Chapters 3&4**). The hydrolysis of β-CN was found to have less influence on the change in Young's modulus due to the occurrence of protein rearrangements (**Chapters 3&4**). Although a correlation between Young's modulus and intact β-CN fraction ($R^2 = 0.496$, $P< 0.01$) was found in **Chapter 2**, it should be noted that the Young's modulus started to decrease when most intact β-CN had already been degraded and showed no changes afterwards. In contrast, intact α_{s1}-CN kept decreasing. The decrease in Young's modulus was still more related to the reduction of intact α_{s1}-CN fraction.

With further deformation, failure of interactions and/or bonds between the structural elements occurs, which leads to permanent changes in the protein network. This is reflected in low values of resilience and cohesion. It was found that the decrease in resilience and cohesion was associated with the hydrolysis of β-CN by plasmin (**Chapters 2&3**). Additionally, this decrease was also found to relate to a high degree of hydrolysis of α_{s1}-CN by calf rennet and pepsin (**Chapter 4**). As mentioned, the hydrolysis of β-CN and the further hydrolysis of α_{s1}-CN (f24–199) into smaller fragments enhances the formation of new bonds among hydrolyzed fragments. The newly formed bonds tend to be broken at the early stage of compression (strain < 20%), as the bonds are formed by relatively smaller fragments, which is shown in the blue part in Fig 6.3. This explains why resilience and cohesion had a strong correlation with hydrolysis of β-CN and with further hydrolysis of α_{s1}-CN (f24–199) as well. This finding is quite important for the potential of controlling the sensory attribute elasticity, as the perception of elasticity has been shown to strongly correlate to resilience and cohesion of cheese products obtained from TPA measurements (**Chapter 5**, Foegeding et al. (2003)).

The fact that resilience and cohesion can be altered by different enzymes may be beneficial for cheese varieties with eyes/holes caused by the production of CO_2 gas (Thierry et al., 2010), such as some Swiss-type cheese (e.g. Emmental) and some Dutch cheeses (e.g. Maasdam). For these cheeses, bacteria-induced fermentation (e.g. lactic acid and propionic acid fermentation) generates the characteristic eyes/holes, in addition to the typical flavor (e.g. nutty, fruity). The formation of these eyes/holes often leads to the presence of undesirable slits and cracks within the cheese matrix. It has been shown that cheeses with a higher level of cohesion and resilience are less prone to the development of slits or cracks (Cooper, 2017; Lamichhane, 2019; Özer and Kesenkaş, 2019; Turgay et al., 2020). To prevent this development, cohesion and resilience should stay low. Selecting coagulant with a low proteolytic activity, such as recombinant chymosin or coagulants with high chymosin/pepsin ratio, might be a

solution to control the degree of α_{s1}-CN hydrolysis to a low level can thus be used to improve the quality of such cheeses. In addition, milk obtained from cows at early lactation stage can be used to obtain cheese containing a low plasmin content and thus a limited degree of hydrolysis of β-CN. It has been shown that a lower activation of plasminogen to plasmin occurs during the earlier part of lactation than in the late lactation stage (Korycha-Dahl et al., 1983; Politis et al., 1989; Hameed et al., 2017).

Next to resilience and cohesion, also adhesiveness was influenced by the casein hydrolysis. The degree of casein hydrolysis (both of α_{s1}-CN and β-CN) was found to have an influence on adhesiveness (**Chapters 3&4**). The higher degree of casein hydrolysis, the more adhesive (sticky) the cheese is. The increase in adhesiveness after casein hydrolysis has been reported to be caused by the high absorption energy of products with more hydroxyl groups (Clerc et al., 2017). In addition to casein hydrolysis, the results in the thesis also show that pH and fat content are important for adhesiveness (**Chapters 3-5**). This will be discussed in the next section (6.3.3).

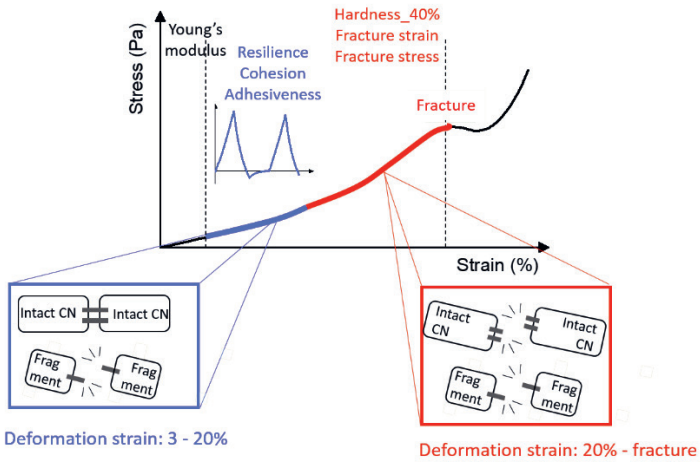

Figure 6.3. Schematic representation of the disruption of bonds under compression test.

For larger degree of deformation (marked as red in Fig 6.3), a high number of bonds was broken, leading to further permanent changes in the protein network. During this stage, interactions among intact caseins are also broken, leading to micro-cracks and

the subsequent collapse of the entire network. The cheese then fractures into pieces. Usually, the fracture strain and fracture stress are used to indicate the brittleness and hardness of the cheese. However, in the studies of this thesis, the hardness could not be accurately determined by the fracture stress, as the error bars were too large. Instead, hardness was determined as the stress at a specific strain value (40%) before the cheese fractured (**Chapters 2-4**), which has also been used by others as a measure of cheese hardness (Ak and Sundaram, 1997; Alvarez et al., 2000). It was found that both intact casein fractions and hydrolyzed products were associated with hardness. At this larger degree of deformation (40%), most bonds were broken, including the bonds among intact caseins and hydrolyzed fragments. Therefore, both the decrease of intact casein fraction and the increase of the degree of casein hydrolysis led to the decrease in hardness_40%.

In terms of fracture strain, it seems that the high moisture content (> 59%) of the model cheeses limited the effect of proteolysis on fracture properties (**Chapters 2-4**). The role of the individual casein fractions could not be completely deduced. Although it was found in **Chapter 2** that the model cheeses became more brittle with ongoing proteolysis, the link between the brittleness and the hydrolysis of specific casein fractions was not clear. Because α_{s1}-CN and β-CN were both extensively hydrolyzed in this chapter, it was thus difficult to distinguish their individual role. Interestingly, when β-CN was less hydrolyzed due to the lower plasmin content (**Chapter 3**), the fracture strain was not significantly influenced, indicating that probably the fracture strain depends on the hydrolysis of β-CN. Lamichhane et al. (2019) reported that the level of intact β-CN fraction was strongly associated with the fracture strain. In addition, they found that the fracture strain was not affected by the breakdown of α_{s1}-CN. They indicated that the initial large fragment, i.e., α_{s1}-CN (f24–199) was so large that it remained attached to the protein network. Thus, the initial breakdown of intact α_{s1}-CN had no pronounced effect on the brittleness of cheese. From these results, it seems that the hydrolysis of β-CN dominates the change in the cheese brittleness. Besides

casein hydrolysis, other parameters may have a larger influence on the fracture properties. For example, a small change in moisture content of cheese would generate a huge difference in the fracture properties, as shown in other studies (Watkinson et al., 2001; Hennelly et al., 2005; Inoue et al., 2019). This effect was also seen in **Chapter 5**, where the moisture content of commercial Gouda cheese decreased from 41.7% to 34.5% after ripening, and the corresponding fracture strain substantially decreased from 61.1% to 29.0%. However, whether this difference can be attributed to the water content only is not clear, as the extent of proteolysis in these cheeses may have influenced the fracture strain as well. Therefore, to better understand the effect of casein hydrolysis and moisture content on the fracture properties, further research is suggested to investigate their individual role. In this thesis, the effect of casein hydrolysis on fracture strain was not seen in **Chapter 3**, with model cheeses containing 61-62% moisture. However, that the hydrolysis of casein induced a decrease in fracture strain was clearly observed in **Chapter 2**, when model cheeses had a slightly lower moisture content (59-60%). Thus, when investigating the role of casein hydrolysis on fracture properties, model cheeses with lower moisture content (higher dry matter) are recommended.

6.3.3 Other factors relevant for cheese texture

Based on the main results of this thesis, the relation between casein hydrolysis and cheese texture has been further clarified. Besides casein hydrolysis, cheese texture is also affected by other factors, such as the content of moisture, fat, calcium and salt, and the pH of cheese. Especially for moisture content and pH, they can not only directly affect cheese texture, but also play a critical role in the activity of proteinases, which consequently has an indirect influence on the cheese texture as well. In the next section, the effect of these factors will be briefly discussed.

6.3.3.1 Effect of moisture content

In **Chapter 5**, it was found that the moisture content was important in determining the hardness and brittleness of cheese. With the same fat level, higher moisture cheese

was softer and less brittle. The results also indicated that the moisture content had a greater effect on hardness than the casein hydrolysis: cheese with more casein hydrolysis (longer ripening week) but lower moisture content was found to be harder, indicating that the effect of lower moisture content was larger than the effect of casein hydrolysis. In agreement with the results of cheese hardness in **Chapter 5**, Hennelly et al. (2005) found a linear relationship between moisture content and hardness (R^2 = 0.99) in model cheeses with the same protein-fat ratio but different moisture content (46-54%). The direct relation between hardness and moisture content is most likely due to the fact that increased hydration of the protein matrix directly attenuates protein-protein interactions, thus plasticizing the matrix and resulting in decreased hardness. This is supported by other studies on cheese texture as well (El-Bakry et al., 2011; Masotti et al., 2018). Next to influencing hardness (fracture stress), moisture content has also been reported to influence the fracture strain (Jack and Paterson, 1992; Everard et al., 2006; Han et al., 2011). High moisture content is usually linked to a high fracture strain (less brittle cheese). Higher moisture content allows greater movement of the casein matrix in cheeses and thus the cheese is less easily fractured (Jack and Paterson, 1992; Everard et al., 2006). This is consistent with the findings in **Chapter 5**.

When comparing the model cheeses used in the different **Chapters (2-4)** in this thesis, difference in moisture content was found ($P < 0.01$). Based on the moisture content, the model cheeses in this thesis can be grouped into three groups; a low moisture group (59-60%, **Chapter 2**), a mid moisture group (61-62%, **Chapter 3**) and a high moisture group (64-65%, **Chapter 4**). This difference might be caused by slight modifications, mainly regarding the curd cutting time and stirring duration, of the cheese-making process in the experiments discussed in the different chapters. It may also be caused by the slight variations in composition between the batches of cheese milk used, as milks were purchased in different periods of the year. It is known that season and year can influence the composition of milk (Glantz et al., 2010; Logan et al.,

2014; Li et al., 2019; 2020). Although the protein content has been standardized, the ratio between casein fractions would still be different between milks.

To investigate the effect of moisture content on proteolysis, the degree of casein hydrolysis of cheeses with similar levels of enzymes (chymosin and plasmin) but different moisture content was compared. The results are shown in Figure 6.4. It was difficult to compare the results of intact casein fractions in different chapters as they were calculated based on relative values. Thus, the protein soluble fraction was chosen to indicate the degree of casein hydrolysis instead of the intact casein fractions. It was found that the moisture content showed an effect on enzyme activity, and consequently influenced the rate of proteolysis (Fig 6.4). Higher moisture content significantly enhanced the rate of proteolysis, which eventually speeds up the development of cheese texture. Also, the higher moisture content led to a higher degree of casein hydrolysis for plasmin-dominated system (Fig 6.4a). However, this is not clear for chymosin-dominated systems due to the short storage duration (Fig 6.4b). The results provide a potential approach to adjust the rate and degree of proteolysis, only by slightly modifying the moisture content. Since a slight increase in moisture content is able to speed up the proteolysis and to increase the degree of proteolysis, the role of moisture content should not be neglected when studying the effect of enzyme activity on proteolysis in cheeses.

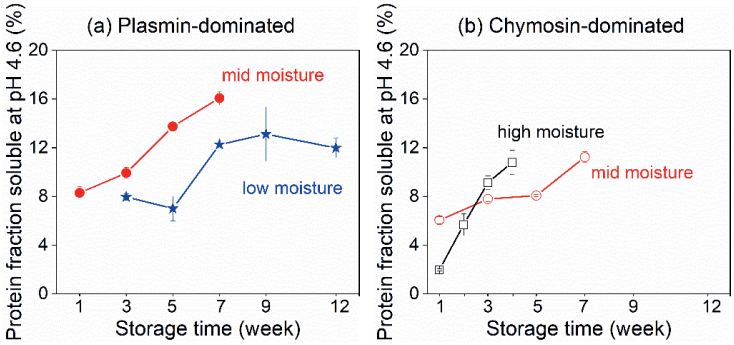

Figure 6.4. (a) Protein soluble fraction as a function of storage time for model cheeses with plasmin-dominated hydrolysis (pH 6.2, active plasmin, 20 IMCU/L chymosin addition): model cheeses with low moisture content (59-60%, Chapter 2, ★) and mid moisture content (61-62%, Chapter 3, ●). (b) protein soluble fraction as a function of storage time for model cheeses with chymosin-dominated hydrolysis (pH 5.9, inactive plasmin, 50 IMCU/L chymosin addition): model cheeses with mid moisture content (61-62%, Chapter 3, ○) and high moisture content (64-65%, Chapter 4, □).

To investigate the separate effect of moisture content and hydrolysis on cheese texture, Young's modulus and resilience were plotted as a function of protein soluble fraction for the cheese with different moisture content (Figure 6.5). Young's modulus and resilience were selected, since in this thesis (**Chapters 3&4**) it was found that they were related to the hydrolysis of α_{s1}-CN (chymosin-dominated) and β-CN (plasmin-dominated), separately. It is clearly visible that the moisture content had an effect on Young's modulus for systems with chymosin-dominated hydrolysis (Fig 6.5b). However, this was not seen for systems with plasmin-dominated hydrolysis (Fig 6.5a). This means the effect of moisture content is more pronounced when hydrolysis of α_{s1}-CN occurred. This result confirmed the important role of the breakdown of α_{s1}-CN in determining the Young's modulus. In contrast to the Young's modulus, the lower moisture content significantly reduced resilience for systems with plasmin-dominated hydrolysis (of β-CN). This again highlights that the hydrolysis of β-CN is the key to the changes in resilience of cheese. These results deliver the information that the moisture content has a different influence on specific aspects of cheese texture, depending on the hydrolysis of specific casein fractions in the cheese system. Decreasing moisture

content can be used to increase the Young's modulus of cheese with α_{s1}-CN hydrolysis, and to increase the resilience of cheese with β-CN hydrolysis. This would help to better understand the role of moisture loss in the development of texture during ripening, when the proteolysis also takes place.

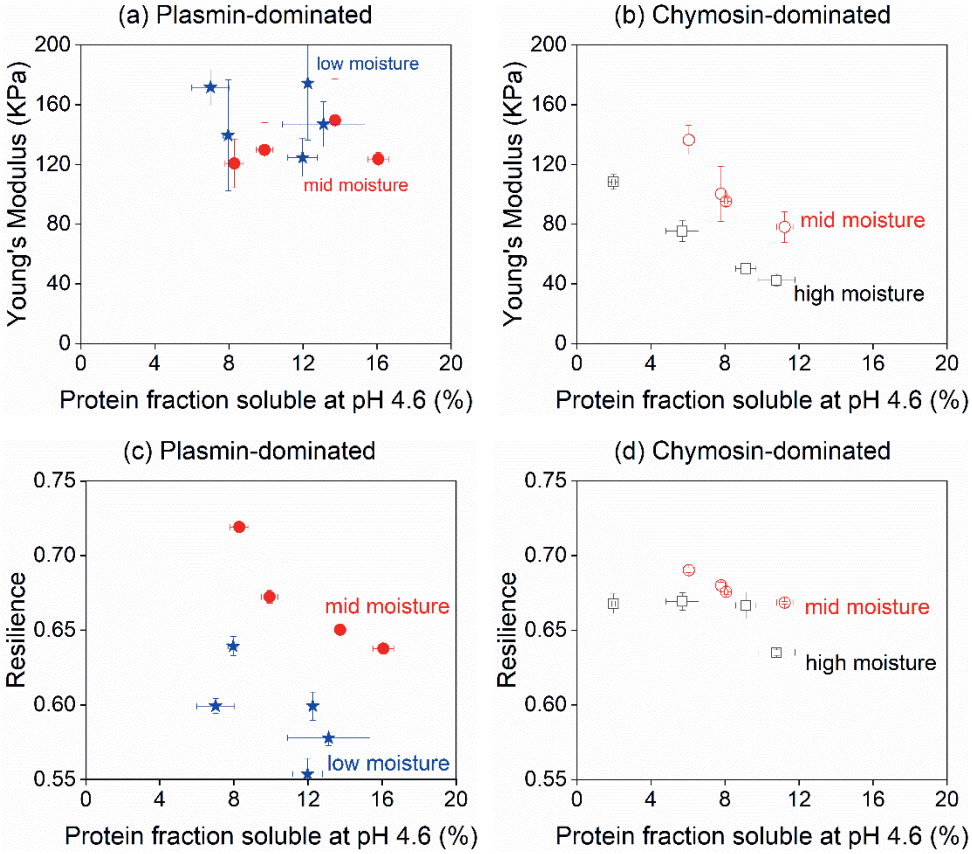

Figure 6.5. (a, c) Young's Modulus and resilience as function of protein fraction soluble at pH 4.6 for model cheeses with plasmin-dominated hydrolysis (pH 6.2, active plasmin, 20 IMCU/L chymosin addition): model cheeses with low moisture content (59-60%, Chapter 2, ★) and mid moisture content (61-62%, Chapter 3, ●). (b, d)) Young's modulus and resilience as function of protein fraction soluble at pH 4.6 for model cheeses with chymosin-dominated hydrolysis (pH 5.9, inactive plasmin, 50 IMCU/L chymosin addition): model cheeses with mid moisture content (61-62%, Chapter 3, ○) and high moisture content (64-65%, Chapter 4, □).

6.3.3.2 Effect of fat content

As the main focus of this thesis was on the effect of casein hydrolysis, skim milk was used for cheese making to eliminate any additional effect of fat on texture (**Chapters 2-4**). It is good to realize that fat has a large influence on cheese texture. As mentioned in section 6.3.2, adhesiveness can be affected by the fat content. The higher the fat content, the higher adhesiveness (more sticky) the cheese. This was clearly seen in **Chapter 5**, where two groups of Dutch-type Gouda cheese, i.e. full fat and low fat, were compared. Fat globules may be present at the surface of the cheese, and fat, due to its "sticky" nature, can adhere to the probe. Thus a high adhesiveness was shown. The same phenomenon was also reported in other studies on the effect of fat content on cheese properties (Bryant et al., 1995; Gwartney et al., 2002; Rogers et al., 2009). Although the effect of proteolysis on adhesiveness was seen for for low fat cheeses in **Chapter 5,**. all full fat cheeses with different ripening times (7, 16 and 48 weeks) showed the same high levels of adhesiveness. In this case, adhesiveness was more dominated by the fat content, rather than by the hydrolyzed peptides. These results show that the effect of casein hydrolysis thus depends a lot on the fat content of cheese.

The effect of fat content on hardness was also found in **Chapter 5**. The low fat cheese was harder than full fat cheese. This effect can be attributed to differences in the microstructure. According to literature, the network of high fat cheese is loose and open, with space occupied by the fat globules that are dispersed throughout the protein network. In contrast, low fat cheese has a more dense and compact protein matrix with less open spaces (Kerr et al., 1981; Mistry and Anderson, 1993; Bryant et al., 1995; Karami et al., 2008; Colín-Cruz et al., 2012). The dense and more compact protein matrix leads to a harder texture. It should also be noted that the low fat cheese usually has a higher moisture content compared to full fat cheese due to its higher protein content, corresponding to a higher water-holding capacity (Fife et al., 1996; Paulson et al., 1998). As we mentioned in the previous section, a high moisture content

usually corresponds to a softer cheese (lower hardness). This was not seen in **Chapter 5**. The fat content seemed to have a greater influence on cheese hardness than moisture content.

In addition to the adhesiveness and hardness, fat content also showed a great influence on the fracture properties (**Chapter 5**). Full fat cheeses were found to be more brittle than low fat cheeses in this chapter. This is based on the fact that milk fat globules act as structure breakers (Michalski et al., 2003; Everett and Auty, 2008), which leads to lower fracture strain for cheese with high fat content. The high fracture strain obtained in **Chapters 2&3** for our model cheeses may be partly attributed to the absence of fat. As fat plays a large role on the properties of cheese, effects arising from casein hydrolysis may play a less important role in cheese with high fat content.

6.3.3.3 Effect of pH

In **Chapter 3**, pH was found to have a large influence on the activity of enzymes (proteolysis). A small adjustment in pH from 6.2 to 5.9 brought significant changes in the hydrolysis of α_{s1}-CN and β-CN by chymosin and plasmin, which eventually induced differences in cheese texture. Chymosin is known to have an optimum activity at a pH ranging from 4.2 and 3.8 (Mohanty et al., 1999; Chen et al., 2021), while plasmin has an optimum activity at a pH of 7.5 (Bastian et al., 1991; Kumar et al., 2010). In this thesis (**Chapters 2-4**), pH values of 5.6 and 6.2 were chosen based on several reasons. First, to avoid the rapid drop in pH and accompanying fast acid-induced gelation, a low amount (0.3% and 0.6%) of GDL was used. In this case, gelation did not occur yet before the rennet was added. Second, the relatively high pH was chosen to potentially minimize the effect of calcium solubilization, as the solubilization of colloidal calcium phosphate (CCP) in the casein micelle occurs at a relatively low pH (Ramkumar et al., 1997; Le Graët and Gaucheron, 1999; Pastorino et al., 2003c). Such solubilization would change the properties of the micelles, which is known to also affect the textural properties of the cheese. In this case, it would be difficult to separate the effects of solubilization of CCP and casein hydrolysis on cheese texture.

As a slight modification in pH can cause considerable changes in the proteolysis of model cheese (**Chapter 3**), the findings obtained in this thesis may vary for other commercial cheeses with a lower pH. For example, the pH of Gouda, Cheddar and Parmesan cheese usually ranges from 5.1 to 5.4 (Jo et al., 2018). Further research is needed to confirm the indications reported in this thesis for cheese with varying pH, especially a lower pH. Since the usage of GDL has limitations, other alternative method should be tested to reach a lower pH (< 5.9). The approach of high-pressure injecting of a concentrated solution into cheese blocks has been previously used for modifying composition of cheese (Pastorino et al., 2003a; 2003b; 2003c). Such injecting of a concentrated solution GDL or its combination with using GDL may be helpful.

Based on the finding in **Chapter 3** that plasmin had a higher activity at pH 6.2 than at 5.9, while chymosin showed the opposite effect, model cheeses with lower pH (<5.9) are expected to show a different proteolytic pattern of the different casein fractions. With a pH lower than 5.9, the chymosin-induced hydrolysis of α_{s1}-CN will be enhanced while the plasmin-induced hydrolysis of β-CN will be slowed down. As a result, the parameters that are relevant to α_{s1}-CN fractions, such as G', Young's modulus, and hardness may increase more rapidly. Other parameters, such as critical strain, resilience and cohesion may also be altered, depending on the type of used chymosin. These parameters were found to be related to the degree of casein hydrolysis. Although the plasmin-induced hydrolysis of β-CN may be decreased at a lower pH, the degree of casein hydrolysis can still increase with the occurrence of further hydrolyzing the fragments from α_{s1}-CN. Consequently, critical strain will increase, and resilience and cohesion decrease. In addition, it was found in **Chapter 3** that the drop of pH from 6.2 to 5.9 lead to a significantly more sticky cheese. This is probably caused by the reduction of the hydration ability of casein micelles at a pH closer to their iso-electric point. It was reported that the reduction of charge through acidification from 6.7 to 5.9 led to the reduction of the amount of water bound to the micelle (Gastaldi et al., 1996; Kühnl et al., 2010). Thus, more soluble peptides diffuse in the serum-phase

water, which resulted in higher adhesiveness. Based on our results in **Chapter 3,** a higher degree of casein hydrolysis and more soluble protein at pH 5.9 is expected to show a larger influence on adhesiveness.

It is important to realize that a low pH also has effect on moisture content and that CCP starts to solubilize. Although for the experiments discussed in **Chapter 3,** the minor decrease in pH (from 6.2 to 5.9) did not alter the cheese moisture content, a further decrease in pH is expected to decrease the moisture content, as shown in other studies (Van Vliet and Walstra, 1994; Watkinson et al., 2001). This is caused by the increased rate of syneresis of milk gels at lower pH, due to the faster formation of protein-protein bonds in the milk gels as electrostatic repulsion is reduced at values closer to the iso-electric point (Lucey et al., 2000; Panthi et al., 2019). Probably, the corresponding model cheeses with a lower moisture content will be harder and more brittle. Concerning the calcium equilibrium, it was assumed that the solubilization of CCP had limited influence on cheese texture, since the pH was remained high in **Chapters 2-4**. However, if cheeses would be made at a lower pH, also this effect may have to be taken into account. According to literature, when the pH is lowered to values of 4.7, the CCP in the casein micelles start to solubilize and diffuses into the serum (Ramkumar et al., 1997; Le Graët and Gaucheron, 1999; Pastorino et al., 2003c). This subsequently decreases the interactions between caseins in the micelles, and therefore leads to a softer cheese. O'Mahony et al. (2005) proposed that the solubilization of CCP had a greater influence on the softening of Cheddar Cheeses (pH~5.2) than the hydrolysis of α_{S1}-CN. Lamichhane et al. (2019) also highlighted the role of insoluble calcium levels in the brittleness of semi-hard models cheeses (pH~5.2). Thus, the solubilization of CCP should also be taken into account when further researches are conducted at a lower pH. The determination of soluble calcium content should be done to separate the effects of solubilization of CCP and casein hydrolysis.

6.4 Understanding complex texture attributes from bolus properties

To understand sensory perception in relation to the properties of the food, the role of oral processing and bolus properties has received increasing attention (Devezeaux de Lavergne et al., 2015a; Devezeaux de Lavergne et al., 2015b; Jourdren et al., 2016; Rizo et al., 2019; Pu et al., 2021). During oral processing, cheese is broken down into small pieces and saliva is incorporated to form a bolus. The results in this thesis confirmed that for complex attributes, such as smoothness and creaminess, bolus properties became more crucial to explain these parameters than the cheese properties themselves (**Chapter 5**).

Fat content and bolus lubrication appeared to be the most important aspects in determining the complex attributes. Such lubrication aspects could be determined by measuring the friction coefficient of the bolus. However, for cheeses with significant different levels of fat content, low fat cheese with 7 weeks of ripening had a similar friction coefficient (μ= 0.52) as that of full fat cheeses. This indicates a saturation effect of fat content on the lubrication. In this case, instead of the bolus lubrication, other factors were more important to lead to different sensory perception. For example, difference in the bolus hardness arising from fat melting became crucial in explaining the varying scores in smoothness and creaminess. This means that to alter the smooth and creamy sensation, controlling the fat melting behavior during mastication would be a more efficient way, rather than modulating the bolus lubrication via fat content.

When we compare cheese with the same level of fat content, two low fat cheeses showed a significant difference in the bolus lubrication; a less lubricating bolus (μ= 0.75) was observed for cheese with a longer ripening time (16 weeks). This was attributed to the presence of hard particles in the bolus and its lower cohesion. Although the hardness of particles could not be determined in this study, it was hypothesized that particles were harder if the corresponding cheese was also harder. These results give

hints to control the sensory perception (i.e. smoothness and creaminess) of low fat cheese, from the perspective of lubricating and cohesion of the formed bolus. For example, incorporation of emulsifiers into the cheese matrix has been reported to improve the texture of reduced fat cheese (Drake et al., 1994; 1996; Euston, 2008): Cheddar-type cheeses with lecithin as a fat-substitute showed similar texture scores as for full fat cheese. Based on our results, such approach of emulsifier incorporation may also increase the lubrication of the formed bolus, and therefore lead to a smooth and creamy perception. This would be beneficial to design fat-reduced food and the development of healthier products.

In fact, the commercial cheeses studied in **Chapter 5** involved other variations in protein content, salt content, and other textural properties such as resilience (elasticity) as well. In addition to the fat content and hardness of cheese, it would also be worth to study how other properties of cheese, such as the factors mentioned above, pH and proteolysis, affect the bolus formation and the accompanying sensory perception. Since commercial cheeses are complex foods, with huge variations in physicochemical properties, it is difficult to separate the effects of different properties on texture perception. Model cheeses are thus recommended for further studies, allowing a better control over different factors. For instance, in order to investigate the effect of proteolysis on sensory perception, model cheeses with the same pH, and same content of moisture, fat and salt should be designed. Based on the aim of the intended research, the model cheeses can be further optimized. If the aim is to understand the effect of proteolysis on complex texture attributes such as smoothness and creaminess, fat should be involved in model cheeses, and low moisture content is suggested. As it has been discussed, the effect of proteolysis on the fracture properties will be more significant in model cheeses with a lower moisture content. The difference in fracture properties will lead to differences in the number of particles in the bolus. Consequently, the bolus cohesion and lubrication are altered, which will affect smoothness and creaminess.

From literature it is known that viscosity is also an important factor to determine lubrication (Martini et al., 2018). In **Chapter 5**, bolus viscosity was not investigated due to the low amount of obtained bolus per subject. To gain insights into the role of bolus viscosity, an additional experiment by using combined boluses from three subjects was conducted. The results are shown in Fig 6.6.

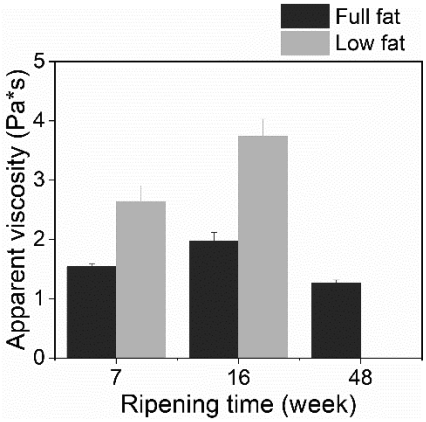

Fig 6.6. Apparent viscosity of artificial cheese boluses with different ripening times and fat content (n=3). The measurements were conducted based on the method of Chojnicka et al. (2009), using an Anton Paar rheometer with a vane geometry (ST22-4V-40).

In general, full fat cheese boluses showed a low apparent viscosity. This is in contrast with the results reported by Chojnicka et al. (2009): a gradual increase of the apparent viscosity was observed with increasing oil concentration for emulsion-filled gels with bound oil droplets. The lower apparent viscosity for full fat cheese boluses in the present study was probably related to the melting of fat during mastication. These cheeses also showed low values for the friction coefficient. The result in Fig 6.6 also showed that the boluses of two low fat cheeses had significant difference in the viscosity. With the same low fat level, the bolus obtained from cheese with longer ripening time (16 weeks) had a higher apparent viscosity. In this case, the high viscosity is possibly not related to fat content, but to other factors, such as the high number of particles in the cheese bolus. Although it has been reported in liquid samples that a

higher viscosity is usually linked to lower friction coefficients (de Wijk and Prinz, 2005; Gallier et al., 2014), the findings for our low fat cheese suggest that increased viscosity corresponds to reduced lubrication, i.e. high friction, in cheese bolus. These results offer a new perspective of manipulating the bolus lubrication by the adjustment of the bolus viscosity; a less viscous bolus seems to provide better lubrication, which may lead to a more smooth and creamy sensation. To confirm the effect of viscosity on the sensory perception of low fat cheese, further studies could be conducted. In order to archive bolus with vary viscosity, β-glucan and phytosterol are suggested to incorporated in cheese matrix. It has been reported that they are able to increase the viscosity of cream cheese and ricotta cheese (Lazaridou and Biliaderis, 2007; Schneider et al., 2009; Ningtyas et al., 2019; Nzekoue et al., 2021). Such study may also help to improve the sensory perception of fat-reduced cheese, as β-glucan and phytosterol are considered as fat replacers in cheese products (Lim et al., 2010; Bhaskar et al., 2017; Ningtyas et al., 2018).

6.5 Conclusion and outlook

We have shown that the development of cheese texture strongly relies on the hydrolysis of specific casein (α_{s1}-CN and β-CN) fractions in this thesis (**Chapters 2-4**), and that the sensory perception of complex texture attributes depends on the bolus properties, especially on bolus lubrication (**Chapter 5**). Understanding the mechanism of the development of different textural parameters offers a new perspective to control cheese texture and to design cheeses with desired textures. Nonetheless, there is still much work to be done. As we discussed in this chapter, next to casein hydrolysis, cheese texture is also affected by other factors, such as moisture and fat content, and the pH of cheese. The relevance of these factors should also be taken into consideration when extrapolating the findings obtained in model cheeses to other cheese varieties.

With regard to the sensory perception of cheese, a potential control of the complex sensory properties from bolus properties could be explored. These may be varied by choosing different fat with different melting properties and incorporating emulsifiers in cheese. In addition, it would be important to further focus on studying the links between dynamic changes in texture perception and the formation of the bolus. Temporal Dominance of Sensations (TDS) is known as an effective method to demonstrate the most "dominant" sensation over time and can be used to provide information on the sensory trajectories, including the perception of texture (Lenfant et al., 2009; Pineau et al., 2009).

The new insights obtained in this thesis may help scientists and cheese manufacturers to open new views for engineering cheese texture. Further studies are recommended to evaluate how the findings of this thesis fit other model cheeses with varying compositional properties. Such input is valuable to expand and to deepen the understanding of cheese further.

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CHEESE

Summary

Texture plays an important role in determining cheese quality and consumer preference. Understanding the textural properties of cheese allows to control cheese quality and to design products with desired texture. Numerous studies have been carried out to understand the effect of compositional properties such as moisture, fat, protein, fat content and pH on cheese texture. However, the relation between texture and specific casein fractions is not well understood yet. A better understanding of this relation is needed to unveil the mechanisms determining texture development during ripening. In addition, the texture development and final texture of the cheese influences the sensory perception of cheese. Currently, it is not well known what aspects provide complex texture attributes of cheese, such as smoothness, creaminess and fattiness, and how bolus properties play a role. The aim of this thesis was to gain insights into the relation of the hydrolysis of specific casein fractions and texture development, and the role of bolus formation in the perception of complex texture attributes.

Due to the complex structure of cheese, arising from different interactions among various compounds, it is challenging to separate the effect of casein hydrolysis on texture from other factors (i.e. fat variations). To focus on the correlations between casein hydrolysis and texture, non-fat model cheeses were thus used in **Chapters 2-4**. The hydrolysis of specific casein fractions was modulated though the adjustment of the activity of different proteinases, such as plasmin and coagulant. Compared with the coagulant, the correlation between plasmin-induced hydrolysis and cheese texture has been rarely studied. To fill this gap, the effects of the concentration of plasmin on casein hydrolysis and textural properties of model cheese were studied in **Chapter 2**. The increase in plasmin activity lead to a significantly higher degree of casein hydrolysis. 5In term of textural properties, with increased plasmin concentration, Young's modulus, hardness, fracture strain, resilience and cohesion decreased. All textural

properties showed a linear relation with the degree of casein hydrolysis. With initial breakdown of intact caseins, the textural properties showed slight changes due to rearrangements of the protein network. This explains the logarithmic correlations between textural properties and the percentage of intact casein fractions α_{s1}-CN, α_{s2}-CN and β-CN.

The role of the hydrolysis of individual casein fractions by chymosin and plasmin in the development of different textural properties were investigated in **Chapter 3**. To obtain information on the structural changes arising from casein hydrolysis, rheological measurement was also carried out. It was shown that the hydrolysis of α_{s1}-CN by chymosin led predominantly to a decrease in storage modulus (G'), and the hydrolysis of β-CN by plasmin induced an increase in the critical strain. The final strength of the protein network was influenced both by the breakdown of intact casein fractions and the formation of new-bonds among hydrolyzed products. Regarding textural properties, a decrease in Young's modulus was associated with the breakdown of intact α_{s1}-CN, while a decrease in resilience and cohesion was related to the hydrolysis of β-CN. Hardness and adhesiveness were influenced by both hydrolysis of α_{s1}-CN and β-CN.

In Chapter 4, the effect of chymosin/pepsin ratio was studied in model cheeses when plasmin activity was inhibited. It was shown that for the coagulant with a higher proportion of pepsin, less intact α_{s1}-CN fractions and a higher degree of casein hydrolysis was obtained. Correspondingly, a weaker and less brittle protein network was shown, which led to a softer, less elastic and more sticky cheese. These results were attributed to the high proteolytic activity of pepsin on hydrolyzing α_{s1}-CN. In addition, the strength of the network (G' and Young's modulus) was mainly determined by the intact α_{s1}-CN fraction. The higher degree of casein hydrolysis also led to changes in critical strain and resilience.

After exploring the relation between hydrolysis of specific casein fractions and the textural properties of model cheeses, sensory perception of commercial cheeses was examined in **Chapter 5.** The results confirmed that bolus properties were essential in explaining more complex texture attributes. As fat melted during mastication, cheeses with higher fat content provided a softer, more cohesive and better lubricating bolus, and thus the cheeses were perceived as smoother and creamier. When fat content was low, the bolus with more and harder particles showed a lower lubrication (higher μ) and a lower cohesion, and thus the cheese was perceived as less smooth and less creamy.

Finally, a general discussion was provided in **Chapter 6**. In this chapter, the relations between casein hydrolysis, protein network and cheese texture were discussed based on **Chapter 2-5**, as well as how this was related to other compositional properties such as the content of moisture and fat, and the pH of cheese. In addition, the sensory perception of cheese was also explained by taking the bolus properties into account. Overall, the findings obtained in this thesis offers a better understanding on the underlying mechanisms of texture development as a result of casein hydrolysis. It may help scientists and cheese manufacturers to better engineer cheese texture by modulating the specific casein fractions or by adjusting different proteinases. The new perspective offered on sensory perception may help to design strategies to control sensory properties of cheese.

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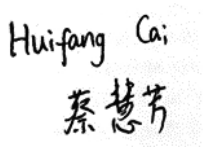
Huifang Cai

26th, July, 2022

Wageningen



Ack

About the author

Huifang Cai was born on November 1, 1993 in Lishui, Zhejiang, China. She completed her Bachelor study in Food Quality and Safety at Zhejiang Gongshang University. Her Bachelor thesis was on the study of selective and identifiable medium for *Vibrio Mimicus*, and she found keen enthusiasm in science. Therefore, she started her Master study at Zhejiang Gongshang University in 2014. She was supervised by Pro. Jianshe Chen and focused on "Rheology and tribology study of the sensory perception of oral care products". During her Master study, she joined an international exchange program and went to the Faculty of Agriculture of Kagawa University (Japan) in 2015. In 2017, she obtained the Master degree and worked at Wahaha Beverage Group (Hongsheng Group) as a sensory specialist.

In 2018, Huifang moved to the Netherlands and started her PhD project at the group of Physics and Physical Chemistry of Food and the Group of Food Quality and Design. From 2018 to 2022, she worked on the study of cheese texture, by taking the role of casein hydrolysis and bolus formation into consideration. The main results of her PhD project are present in this thesis.

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Overview of completed training activities

Discipline specific activities

Reaction Kinetics in Food Science, 10th Edition	VLAG, the Netherlands	2018
Microscopy and Spectroscopy in Food and Plant Science	VLAG&EPS, the Netherlands	2018
Dairy Protein Biochemistry	VLAG, the Netherlands	2018
Rheology Course: The do's and don'ts	VLAG, the Netherlands	2018
FOOD PROTEINS: functionality, modifications and analysis	VLAG, the Netherlands	2019
Sensory perception and food preference	VLAG	2021

Conference

Nizo protein conference[1]	Arnhem, the Netherlands	2019
16th International Symposium on Milk Genomics and Human Health[1]	Aarhus, Denmark	2019
Food Structure & Functionality Online Mini Symposium	Online	2020
IDF International Cheese Science and Technology Symposium[2]	Online	2021
35th EFFoST International Conference[1]	Lausanne, Switzerland	2021
2nd Edible Soft Matter Workshop & Conference[1]	Wageningen, the Netherlands	2022

[1] Poster presentation [2] Oral presentation

General course

Supervising BSc and MSc students	WGS, the Netherlands	2018
VLAG PhD week	VLAG, the Netherlands	2018
Brain Training	WGS, the Netherlands	2018
WGS PhD Workshop Carousel	VLAG, the Netherlands	2018

WGS PhD Workshop Carousel	VLAG, the Netherlands	2019
The Essentials of Scientific Writing & Presenting	WGS, the Netherlands	2019
Presenting with impact	WGS, the Netherlands	2018
Scientific Writing	WGS, the Netherlands	2020
Introduction to R	VLAG, the Netherlands	2020
Career Assessment	WGS, the Netherlands	2022

Other activities

Preparation of research proposal	Wageningen, the Netherlands	2018
Weekly science meeting-FPH	Wageningen, the Netherlands	2018-2022
Cheese Group meeting- FPH	Wageningen, the Netherlands	2018-2022
DST monthly meeting-FQD	Wageningen, the Netherlands	2018-2022
Scientific meetings, colloquia-FQD	Wageningen, the Netherlands	2018-2022

Teaching and supervision

Supervision of 4 BSc thesis	FPH&FQD	2018-2021
Supervision of 7 MSc thesis	FPH&FQD	2018-2021
Teaching assistant for the MSc course of "Advanced food physics-FPH 30306"	FPH	2018-2020
Teaching assistant for the BSc course of "NTU-Food Physics-CH9202"	FPH	2018-2020

Approved by Graduated school VLAG

List of publications

Huifang Cai, Etske Bijl, Elke Scholten, Guido Sala. Effect of plasmin on casein hydrolysis and textural properties of model cheeses. (Submitted)

Huifang Cai, Elke Scholten, Guido Sala, Etske Bijl. Linking casein hydrolysis by chymosin and plasmin to the physical properties of model cheeses. (Submitted)

Huifang Cai, Etske Bijl, Huabin Luo, Elke Scholten, Guido Sala. Effect of chymosin/pepsin ratio on casein hydrolysis and physical properties of model cheeses. (To be submitted)

Huifang Cai, Etske Bijl, Elke Scholten, Guido Sala. Role of bolus properties in understanding complex texture attributes of cheese. (To be submitted)

Colophon

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