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Linking casein hydrolysis by chymosin and plasmin to the rheological and textural properties of model cheese

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ABSTRACT

The texture of cheese highly depends on the structure of its network, which develops during ripening due to the enzymatic degradation of caseins. 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 and plasmin to different rheological and textural properties used to characterize cheese texture. Model cheeses with two plasmin levels (active and inactive) and three chymosin levels (20, 50, and 80 IMCU/kg milk) were prepared.

Our results showed that the hydrolysis of 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. The hydrolysis of β-CN was more associated with changes in critical strain, resilience and cohesion, which were linked 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.

1. Introduction

The textural properties of cheese highly depend on the composition and the structure of the matrix (Guichard et al., 2021; Guinee, 2016). The intact proteins, 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 and thus affects texture (Fox & McSweeney, 1996). The breakdown of casein fractions is mainly induced by chymosin from the coagulant and plasmin present in milk (Fox & 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 & Fox, 2017). In contrast, plasmin has a preference to hydrolyze β-CN and α_{s2}-CN, while α_{s1}-CN is hydrolyzed more slowly (Bastian & Brown, 1996; Korycha-Dahl et al., 1983). The extent and the pattern of hydrolysis of the different casein fractions depend thus on the specific enzymes present in the curd after 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 & Olson, 1982; Francisco-José et al., 2010; Lamichhane et al., 2019; Lamichhane et al., 2022; Wium et al., 1998). 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. 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.

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Also studies on how plasmin-induced hydrolysis affects cheese properties have been carried out. However, these works focused more on cheese functionality (i.e. stretchability and meltability) and flavor development (Fiona M et al., 1999; O'Farrell et al., 2002; Priyashantha et al., 2021; 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). Previous study on plasmin-dominated hydrolysis showed that specific textural properties (e.g. hardness, resilience, cohesion and brittleness) were highly correlated to the hydrolysis of both α_{s1}-and β-CN (Cai et al., 2023). 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.

The objective of this study was to link the hydrolysis of individual casein fractions (α_{s1}-and β- CN) by chymosin and plasmin to the rheological and textural properties of model cheese. To assess this, three levels of recombinant chymosin (20, 50 and 80 IMCU/kg milk) were tested. The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml reconstituted milk in 100 s at 30 °C (Kopelman & Cogan, 1976). According to (M.N. Raymond et al., 1973), the conversion between IMCU and kat is as follow: 1 IMCU = 23.34 nKat. 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. These two pH values were chosen to modulate the hydrolysis of different casein fractions, as chymosin and plasmin have different optimum pH values. 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.

2. Materials and methods

2.1. Materials

All model cheeses were made using the same batch of pasteurized skim milk (Jumbo, the Netherlands), which contained 36 g/kg protein and less than 1 g/kg 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.

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 (G′) (100 Pa) was reached. This was used 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 (G', describing stiffness) 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 3 g/kg or 6 g/kg GDL was added to reduce the pH to 6.2 or 5.9. After 30 min, the milk reached the target pH and chymosin (20, 50, 80 IMCU/kg milk) was added. 4.7 ml milk sample was immediately transferred to the measuring cup, and oscillatory experiments were performed by applying a constant frequency of 1 Hz and a strain of 0.01 (Bauland et al., 2022; Nassar et al., 2020; Yang et al., 2023), which was confirmed to be within the linear viscoelastic region as determined with a strain sweep test.

2.3. Model cheese production

Twelve model cheese samples were made in duplicate based on the production process described by Cai et al. (2023). Detailed information on the 12 samples is shown in Table 1.

Briefly, 5 kg skim milk (with 0.4 g/kg NaN_3) was heated to 33 °C in a water bath. Aprotinin (0 or 1.67 mg/kg) was added to the milk and mixed thoroughly for 20 s. The inhibitory effect of aprotinin on plasmin activity was confirmed with a pre-test, for both milk and model cheese during ripening (Fig. S1). Next, 3 g/kg or 6 g/kg GDL was added to the milk to reduce the pH to 6.2 and 5.9, respectively. Thirty minutes after addition of GDL, chymosin was added to coagulate the milk. After achieving a gel G' of 100 Pa, 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 30 min. The curd was then transferred into 3 cylindric cheese molds (with a diameter of 8.5 cm and a height of 9.5 cm) for shaping and pressing. Each mold was filled with approximately 400 g curd. Each curd in the mold was pressed with a weight of 2 kg for 3 h. After pressing, the obtained three cheeses were immersed in a brine with 250 g/kg 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.

2.4. Chemical properties of model cheese during storage

2.4.1. 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). All the measurements were performed in triplicate.

2.4.2. Casein hydrolysis during storage

2.4.2.1. Degree of casein hydrolysis. The degree of casein hydrolysis was

Table 1
Detailed information on the 12 studied model cheese samples.

Sample No.	pH[a]	Aprotinin (mg/kg milk)	Chymosin (IMCU[b]/kg milk)	Cutting[c] 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

[a] The pH of cheese milk was lowered to 6.2 and 5.9 by adding 3 g/kg and 6 g/kg GDL respectively.
[b] The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml of reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34 nKat.
[c] Cutting time was defined as the time at which the storage modulus reached 100 Pa.

expressed as protein fraction soluble at pH 4.6. The samples were prepared as described by O'keeffe et al. (1976) and Merheb-Dini et al. (2012). The content of the protein fraction soluble at pH 4.6 was determined using the Dumas method, using a factor of 6.38 to convert the estimated nitrogen to protein content.

2.4.2.2. Intact casein fraction by RP-HPLC. Reversed-phase high-performance liquid chromatography (RP-HPLC, Thermo ScienceTM UltiMate 3000; Waltham, USA) was used to determine the peak areas of the intact casein fractions. Samples were prepared according to the method described by Cai et al. (2023). An Aeris 3.6 μm Widepore XB-C18 column (250 × 4.6 mm, Phenomenex, Utrecht, the Netherlands) was used for analysis. 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 (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 × 4.6 mm, 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.

2.5. Rheological and textural properties of model cheese during storage

2.5.1. 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 by using a metal cutter 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 (PP25/P2/SS, diameter of 25 mm). 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 critical strain and G′ at this strain. Critical strain was defined as the strain at which the G' decreased by more than 5% from the value in the linear regime.

2.5.2. Determination of textural properties

One hour after taking the samples out of the incubator (16 °C), the cheese was cut into specimens with a 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 2 mm/s. The Young's modulus was extracted from the linear region (1–3% strain) of stress-strain curves. From the fracture point, the fracture strain and fracture stress were extracted. Even though the fracture stress is well known as an index of cheese hardness, we could not use the fracture stress to accurately determined hardness, as the error bars were too large. Therefore, we considered the stress at a strain of 40% (hardness_40%) as indicator for the hardness of the samples. This parameter has also been used by others as a measure of cheese hardness (Ak & Gunasekaran, 1997; Alvarez et al., 2000).

2.6. 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. Results and discussion

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. No significant difference in pH was found among cheeses, both in the pH 5.9 group and the pH 6.2 group. The results are shown in Tables S1 and S2 of the supplementary information. During the whole storage period, the pH values did not change significantly. Concerning the dry matter content, only the 3 cheeses with inactivated plasmin and pH 6.2 showed a slight increase, from 36.7 ± 0.8% in week 1–37.6 ± 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%).

3.2. Casein hydrolysis

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. 1. The significant differences among weeks and among cheeses can be found in Table S3 of the supplementary information.

Independently of pH and chymosin activity, cheeses with inactive plasmin showed a limited increase in the protein fraction soluble at pH 4.6, starting at 4.9–6.3% in week 1 and increasing to 10.4–12.7% in week 7 (solid lines). When plasmin was active, higher values for the protein fraction soluble at pH 4.6 were found (dashed lines). These results indicate that in our study the hydrolysis of casein by chymosin only had limited influence on the release of small (water-soluble) fragments, while in the system with active plasmin the effect was larger. 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 & Fox, 1993; Piraino et al., 2007). In our study, the small peptides had only limited contribution to the protein fraction soluble at pH 4.6. In the case of plasmin, it has been reported that three bonds (Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}) of β-CN are cleaved, resulting in the liberation of some hydrophobic γ-CNs (water insoluble), together with a larger amount of the complementary water soluble peptides β-CN f (1–28), f(1–105), f(1–107), f(29–105) and f(29–107) (Eigel et al., 1984; Exterkate et al., 1997; Farkye, 1995; Møller et al., 2012). Thus, in our study, the larger amount of soluble peptides formed from β-CN by plasmin had a larger effect on the protein fraction soluble at pH 4.6. 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 (Biswas & Metzger, 2016; Jacob et al., 2011). The required amount of chymosin for milk coagulation was substantially 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) (Irigoyen et al., 2002; Vicente et al., 2001). Thus, a low concentration of chymosin was retained in the curd after whey draining. This, together with the relatively high pH of our samples, explains why in our study the amount of soluble peptides was less influenced by chymosin.

Interestingly, in cheese with active plasmin, the chymosin content

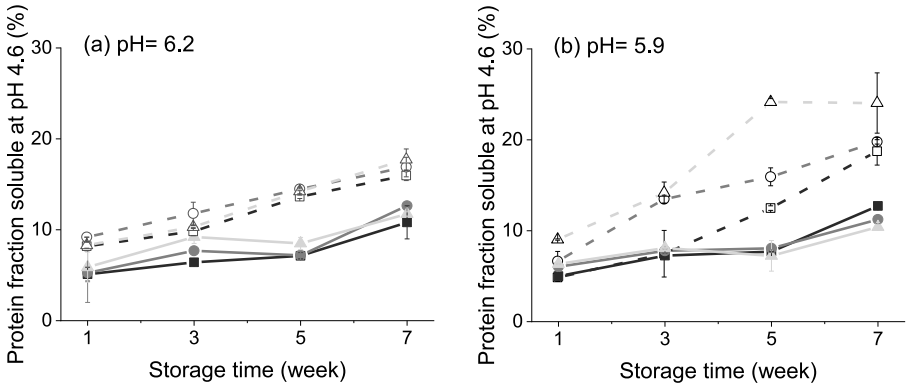

Fig. 1. Protein fraction soluble at pH 4.6 as a result of protein hydrolysis at pH 6.2 (a) and pH 5.9 (b) in model cheeses during 7 weeks of storage. Model cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 IMCU/kg milk: ■, □; 50 IMCU/kg milk: ●, ○; 80 IMCU/kg milk: ▲, △) were added. The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34nKat.

showed a significant impact on the degree of casein hydrolysis at pH 5.9 (Table S3, $P < 0.01$), while this phenomenon was not observed at pH 6.2 (Table S3, $P = 0.901$). 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.

3.2.2. Pattern of casein hydrolysis

Intact α_{s1}-CN gradually decreased during the whole storage period in all cheese samples, as can be seen in Fig. 2a&b. The decrease of the α_{s1}-CN fraction could also be observed from the RP-HPLC profiles of the casein fraction of cheese at week 1 and week 7 (Fig. S2). Chymosin is known as the main proteose responsible on the hydrolysis of α_{s1}-CN during cheese ripening (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 among 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 70–90% of α_{s1}-CN was hydrolyzed at pH 5.9, while only 40–60% was hydrolyzed at pH 6.2. Secondly, the chymosin content showed a more significant influence on the α_{s1}-CN fraction at pH 5.9 than at pH 6.2. In general, plasmin content had no significant influence on the breakdown of α_{s1}-CN, independently of the pH, as the hydrolysis showed the same trend in the system with active and inactive plasmin. This is in line with the results of Table S3. The only exception was observed in storage week 3 at pH 5.9, where the intact α_{s1}-CN fraction was lower (Fig. 2b) when plasmin was inactive. However, as the differences in hydrolyzed α_{s1}-CN between inactive and active plasmin systems was only observed at one time point and disappeared at longer storage time points, this difference can be considered to be minor.

For cheeses with inactive plasmin, β-CN stayed mostly intact both at pH 6.2 and pH 5.9 (solid lines in Fig. 2c&d), and rapidly decreased when plasmin was active (dashed lines in Fig. 2c&d). The degradation of β-CN was affected by the small difference in pH: for a higher pH (6.2), a faster

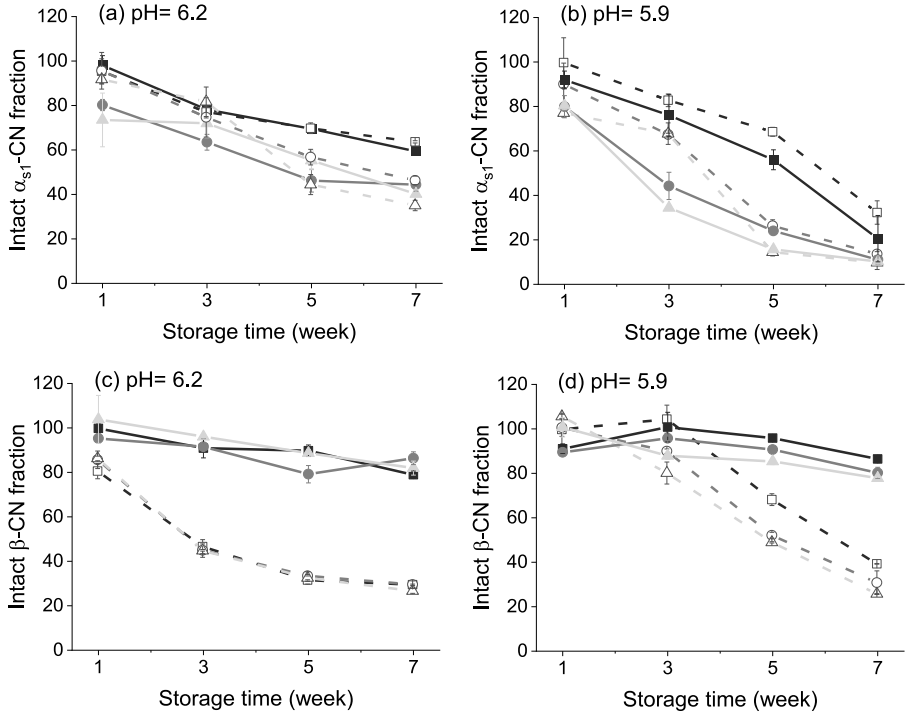

Fig. 2. Intact α_{s1}-CN (a, b) and β-CN (c, d) as a result of protein hydrolysis at different pH (left: 6.2, right: 5.9) in model cheeses during 7 weeks of storage, determined by RP-HPLC. Model cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 IMCU/kg milk: ■, □; 50 IMCU/kg milk: ●, ○; 80 IMCU/kg milk: ▲, △) were added. The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34nKat.

degradation was found, due to the higher activity of plasmin. This is also shown in Fig. S2, which presents RP-HPLC profiles of casein fractions of cheese at week 7. However, after 7 weeks of storage, the level of intact β-CN at pH 5.9 and 6.2 was similar, with values of 70–73% and 61–73%, respectively. We also found a synergistic effect between chymosin and plasmin on the level of breakdown of β-CN at pH 5.9. This is in line with the high level of protein fraction soluble at pH 4.6. According to literature, the cleavage site sensitive to chymosin, Leu192−Tyr193, is located at the hydrophobic C-terminal region of β-CN (Møller et al., 2012). The release of two γ-CNs, β-CN f(106–209) and f(108–209), will have 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 (Fig. 1b).

3.3. Rheological and textural properties of cheese during storage

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. Critical strain and G′ at critical strain of model cheeses are shown in Fig. 3. The critical strain refers to the strain at which permanent damage or fracturing of the microstructure starts to occur (Fox et al., 2017), whereas G' represents a measure for the rigidity of the network.

Our results showed that at pH 6.2, when plasmin was inactive, critical strain and G′ at critical strain remained constant (solid lines in Fig. 3a&c). With active plasmin, G′ was slightly higher (dashed lines in Fig. 3c), indicating a stronger network compared to cheese with inactive plasmin. These results will be discussed in more detail in the next sections. 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. 2d). This indicates that a higher chymosin activity slightly reduced the strength of the protein network. At pH 5.9, a significant influence of chymosin content on the critical strain was shown only when plasmin was active (see dashed lines in Fig. 2b). This may be associated with the synergistic effect of plasmin and chymosin on the hydrolysis of β-CN.

3.3.2. Textural properties

To further reveal changes in textural properties during storage, in this section the results of large deformation compression tests and TPA are given.

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 (Fig. 4a), while at pH 5.9 (higher activity of chymosin) it slightly decreased during storage (Fig. 4b). Although cheese with active plasmin had a stronger network (higher G′ in Fig. 3), this was not seen from the result of Young's modulus, as the cheese with active and inactive plasmin had similar values for this parameter, both at pH 5.9 (Figs. 4a) and 6.2 (Fig. 4b). As the activation of plasmin did not lead to significant changes, chymosin likely dominated the changes of the Young's modulus.

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. At pH 6.2, hardness_40% was higher in cheese with inactive plasmin (solid lines, Fig. 5a) and decreased with increasing chymosin content (dashed lines, Fig. 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. 5b) and active (dashed lines, Fig. 5b) plasmin. These results show that both plasmin and chymosin affected hardness_40%, but that chymosin played a more pronounced role.

Even though changes in the protein network due to casein hydrolysis led to differences in Young's modulus and hardness, the effect on fracture properties was limited. No effects of plasmin and chymosin on fracture stress and strain were observed. All cheeses showed similar fracture strain around 61.8 ± 3.6%, and a fracture stress around 300.0 ± 97.7 KPa, as seen in Fig. S3 in the supplementary information. The

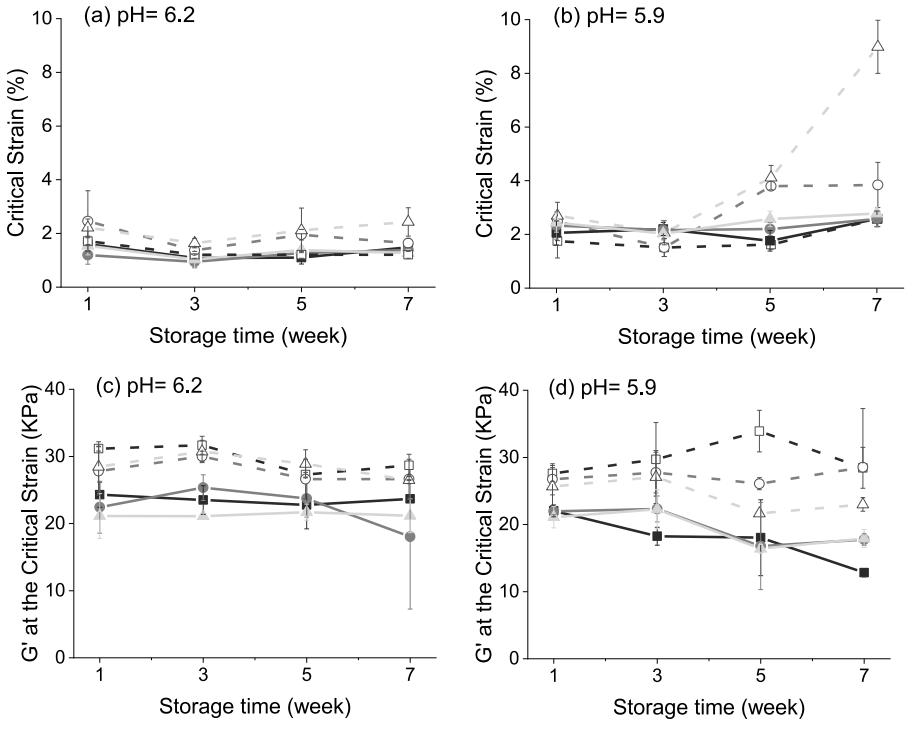

Fig. 3. Critical strain (a, b) and G′ at critical strain (c, d) of model cheeses during 7 weeks of storage, determined by strain sweep tests. Left: pH = 6.2; right: pH = 5.9. Model cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 IMCU/kg milk: ■, □; 50 IMCU/kg milk: ●, ○; 80 IMCU/kg milk: ▲, △) were added. The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34nKat.

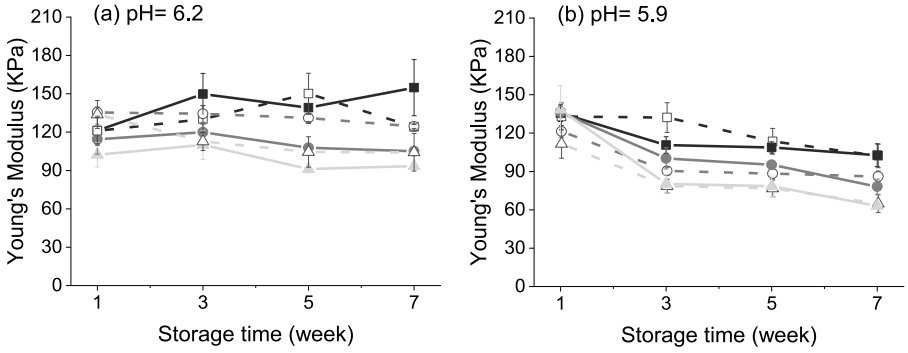

Fig. 4. Young's Modulus of model cheeses during 7 weeks of storage at pH 6.2 (a) and at pH 5.9 (b), determined by a compression test. Model cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 IMCU/kg milk: ■, □; 50 IMCU/kg milk: ●, ○; 80 IMCU/kg milk: ▲, △) were added. The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34nKat.

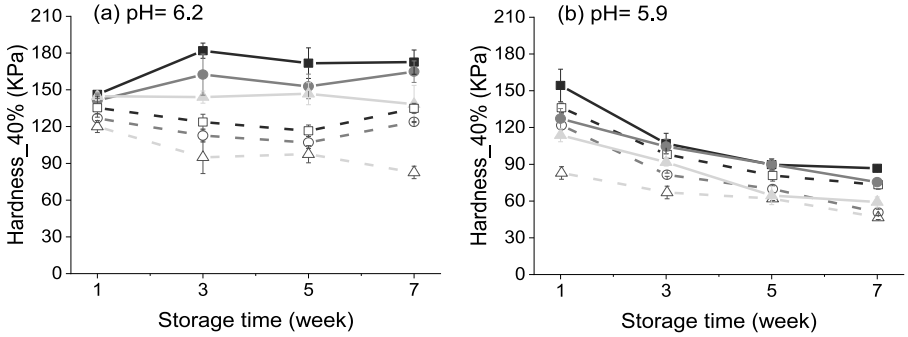

Fig. 5. Hardness_40% of model cheeses during 7 weeks of storage determined by a compression test. (a) pH = 6.2; (b) pH = 5.9. Model cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 IMCU/kg milk: ■, □; 50 IMCU/kg milk: ●, ○; 80 IMCU/kg milk: ▲, △) were added. The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34nKat.

high values for fracture strain can probably be attributed to the high moisture content (62.2 ± 0.9%) of the model cheese used in our study. It has been reported that higher moisture content allows greater movement of the casein matrix in cheeses (Everard et al., 2006; Jack & Paterson, 1992). Such greater movement would not allow the model cheeses to easily fracture, which could explain the high fracture strain.

3.3.2.2. Textural properties obtained from TPA test. Besides the compression test, a TPA test was also conducted to gain more information on different textural properties, such as resilience, cohesion and adhesion. The results are shown in Fig. 6. Both at pH 6.2 and 5.9, when plasmin was inactivated, resilience and cohesion remained constant during storage (solid lines in Fig. 6a–d). When plasmin was active, resilience and cohesion decreased rapidly (dashed line in Fig. 6a–d). Although at pH 6.2 no effect of chymosin content was seen, an increase in chymosin content led to a slight additional decrease in cohesion and resilience at pH 5.9. We thus concluded that both chymosin and plasmin influenced resilience and cohesion, and apparently plasmin had much larger effect.

Concerning adhesiveness, the effect of pH was obvious (see Fig. 6e&f). At pH 5.9, adhesiveness significantly increased, whereas only limited changes were observed at pH 6.2. The same effect of pH 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. At pH 5.9, both chymosin and plasmin (Fig. 6f) 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, plasmin played a key role in the decrease of resilience and cohesion during storage, while chymosin had more effect on the Young's modulus. Adhesiveness was mainly correlated with the pH, even though both chymosin and plasmin were responsible for an increase in adhesiveness. Hardness_40% was highly dependent on both chymosin and plasmin. However, parameters related to larger deformation, such as fracture strain and fracture stress, were not affected by the enzymes in our study.

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 obtained at this pH value (Figs. 7 and 8). An overview of all results is provided in Figs. S4–9 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. 7c–d) and β-CN (Fig. 7e–f), independently of plasmin activity. Therefore, we think that the decrease in G′ and Young's modulus was dominated by the breakdown of α_{s1}-CN, which is also mostly independent on plasmin activity. The same phenomenon was seen at pH 6.2, although the difference was less pronounced (Figs. S6–S7). In general, the 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 is supposed to decrease with a reduction of intact casein. This was clearly shown for hydrolysis of α_{s1}-CN. As no clear effect of plasmin activity was seen, we assume that the hydrolysis of β-CN showed less impact on these properties. However,

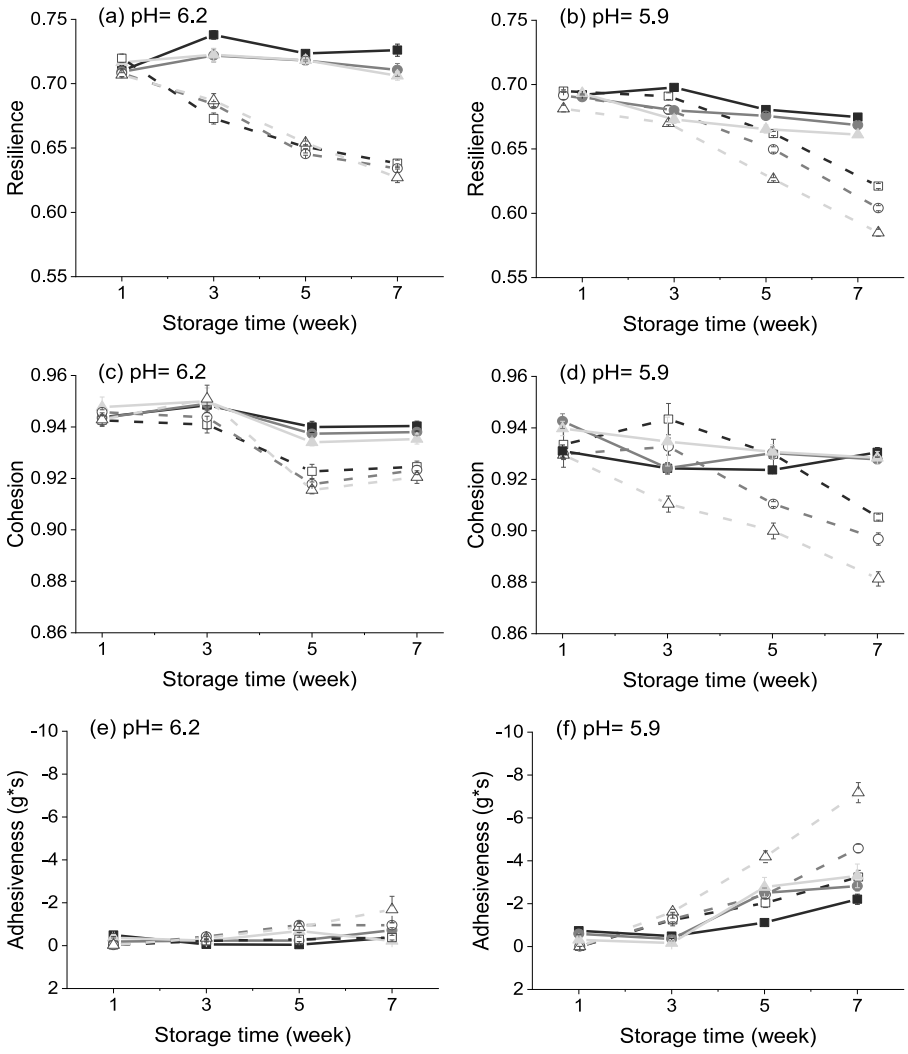

Fig. 6. Resilience (a, b), cohesion (c, d) and adhesiveness (e, f) of model cheeses during 7 weeks of storage, determined by a TPA test. Left: pH = 6.2, right: pH = 5.9. Model cheeses were made with (inactive plasmin, solid line) or without (active plasmin, dashed line) aprotinin. Different concentrations of chymosin (20 IMCU/kg milk: ■, □; 50 IMCU/kg milk: ●, ○; 80 IMCU/kg milk: ▲, △) were added. The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml of reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34nKat.

we did expect changes in the structure, as the hydrolysis of β-CN by plasmin has been discussed to lead to a rearrangement of the protein network as a result of hydrophobic interactions among γ-CNs (Lucey et al., 2003). In samples with active plasmin, new bonds among such hydrolyzed products were thus expected to be formed during storage. The fact that no changes in the strength of the network was seen can thus be explained by a counteracting effect of a decrease in network strength by β-CN hydrolysis, and an increase in network strength by new bond formation. 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), is a large fragment and cannot easily move within the network. Therefore, the strength of the protein network decreased when intact α_{s1}-CN was broken down.

Our results showed that the hydrolysis of β-CN played a crucial role in the changes of three parameters, critical strain, resilience and cohesion (Fig. 8). In contrast to G′ and the Young's modulus, these parameters were not clearly correlated with intact α_{s1}-CN fraction in cheeses with inactive plasmin (black squares in Fig. 8d–f). However, they significantly changed with the occurrence of β-CN hydrolysis by plasmin (white squares in Fig. 8g–i). In addition, the synergistic effect of chymosin and plasmin is again visible here, as also the α_{s1}-CN with active plasmin (white squares) showed a higher correlation (Fig. 8d–f). In the presence of plasmin, chymosin is able to further hydrolyze β-CN (Fig. 2d), and the higher level of hydrolyzed products lead to more rearrangements in the casein network.

Resilience and cohesion are associated with the occurrence of permanent network damage, which is caused by the breakage of bonds under deformation. The correlation between resilience and cohesion with β-CN can be explained by the fact that the network became weaker upon hydrolysis of this casein fraction, and the newly formed bonds after rearrangement were insufficient to resist deformation. This may be explained by the fact that the fragments of β-CN (γ-CNs) are small, and were perhaps not retained within the network. So, upon hydrolysis of β-CN, the network tended to break more easily, and, therefore, a strong correlation with resilience and cohesion is seen (Fig. 8h–i). However, these parameters were not related to α_{s1}-CN (Fig. 8e–f), as the fragments produced from the hydrolysis of α_{s1}-CN were relatively large.

We saw that adhesiveness and hardness_40% were related to both the hydrolysis of the α_{s1}-CN fraction and β-CN fraction (Figs. S8–9). The strong correlation with adhesiveness can be explained by the degree of soluble protein faction. It has been discussed by others that the hydrolysis products potentially enhance the adhesiveness of casein products (Bye, 1990). After hydrolysis, the hydrolyzed products gain more hydroxyl groups, which were reported to enhance the adsorption capacity of cheese surface (Clerc et al., 2017), and can help to adhere to the probe of the Texture Analyzer. This explains the high adhesion. 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

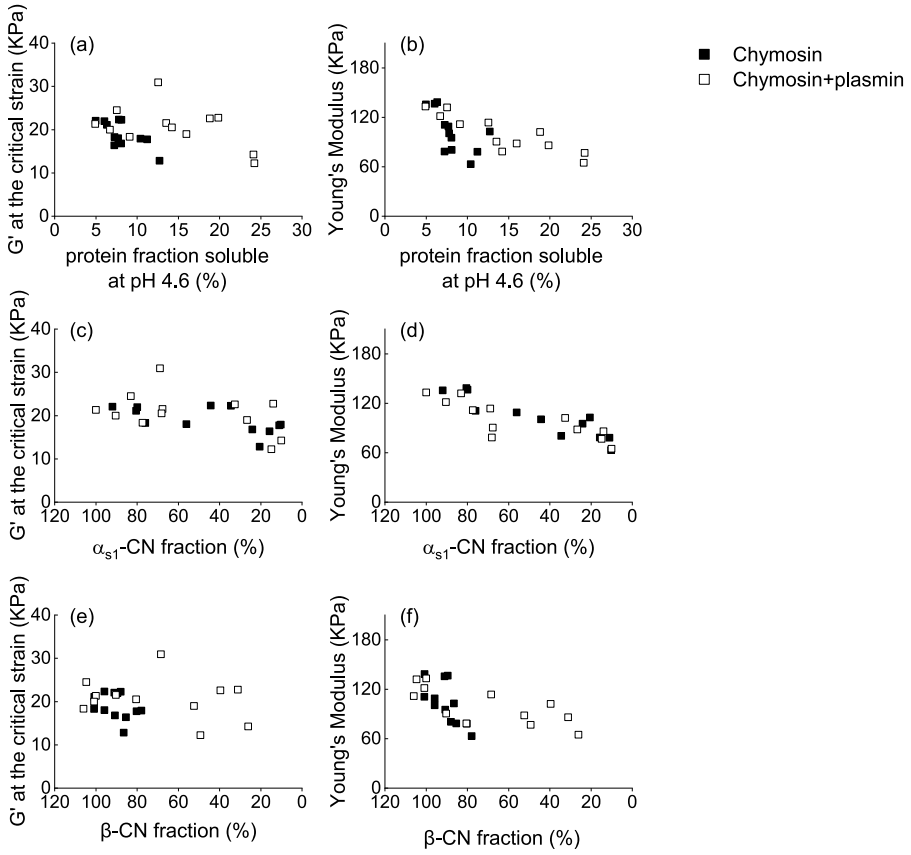

Fig. 7. G′ at critical strain (a, c, e) and Young's modulus (b, d, f) as a function of protein fraction soluble at pH 4.6 and intact α_{s1}-and β-CN, in model cheeses made at pH 5.9, with inactive (■, chymosin) and active plasmin (□, chymosin + plasmin). The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml of reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34nKat.

adhesiveness of cheese during storage (results of pH 6.2 can be found in Figs. S5–6 in supplementary information). That the synergistic effect between chymosin and plasmin led 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. S4g). Thus, the hydrolyzed products obtained from both the α_{s1}-CN and β-CN fraction by plasmin and chymosin seem to have an important role in determining adhesiveness.

Also for hardness_40%, the hydrolysis of both α_{s1}-CN and β-CN played a role (Figs. S8–9). When plasmin was inactive, hardness_40% rapidly decreased with decreasing intact α_{s1}-CN fraction (black squares in Fig. S8d). When plasmin was active, the hydrolysis of β-CN led to lower hardness_40% as well (white squares in Fig. S9d). This indicates that both the breakdown of α_{s1}-CN and β-CN contributed to the cheese softening during storage, while the effect from α_{s1}-CN was more dominant. This large effect of α_{s1}-CN can be explained by the chemical composition of this type of casein, as α_{s1}-CN has a high number of phosphate groups and two phosphate centers (phosphorylated residues in a short sequence) (Bijl et al., 2019; Holt, 2004). These allow α_{s1}-CN to generate strong bonds with colloidal calcium phosphate nanoclusters, which aids in the formation of the core of casein micelles (Dalgleish & Corredig, 2012; Walstra, 1999). During cheese making these micelles will aggregate and form the main structure of the network in the cheese matrix. Changes in α_{s1}-CN, therefore, showed a large influence on this parameter. When α_{s1}-CN is hydrolyzed, the network with colloidal calcium phosphate may be broken, which leads to a large decrease in the cheese hardness. Apparently, as β-CN is also related to hardness, also these hydrolyzed products have an influence on the core of the protein network. However, the influence of such hydrolyzed products is smaller than those formed from α_{s1}-CN, due to the lower number of phosphate groups and the presence of one binding point instead of two for β-CN.

4. Conclusion

Overall, the hydrolysis of specific casein fractions (α_{s1}-and β-CN) affected the textural properties of model cheese samples differently. Due to the production of hydrophobic fragments from β-CN, rearrangements of the protein network were enhanced and thus an increase in critical strain was shown. Consequently, textural parameters related to rearrangements of the protein network, such as resilience and cohesion, were associated with the breakdown of β-CN by plasmin. The hydrolysis of α_{s1}-CN directly led to a decrease in the strength of the protein network, and therefore G′ and Young's modulus decreased. Due to a synergistic effect, the hydrolysis of β-CN was enhanced when both plasmin and chymosin were present. In this case, a significant increase in critical strain was observed, as well as a faster decrease in resilience and cohesion. Adhesiveness and hardness were affected by the hydrolysis of both the α_{s1}-and β-CN fraction, although the hydrolysis of α_{s1}-CN played a greater role than β-CN in the case of hardness. 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.

CRediT authorship contribution statement

Huifang Cai: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **Elke Scholten:** Conceptualization, Methodology, Data curation, Writing – review & editing, Supervision.

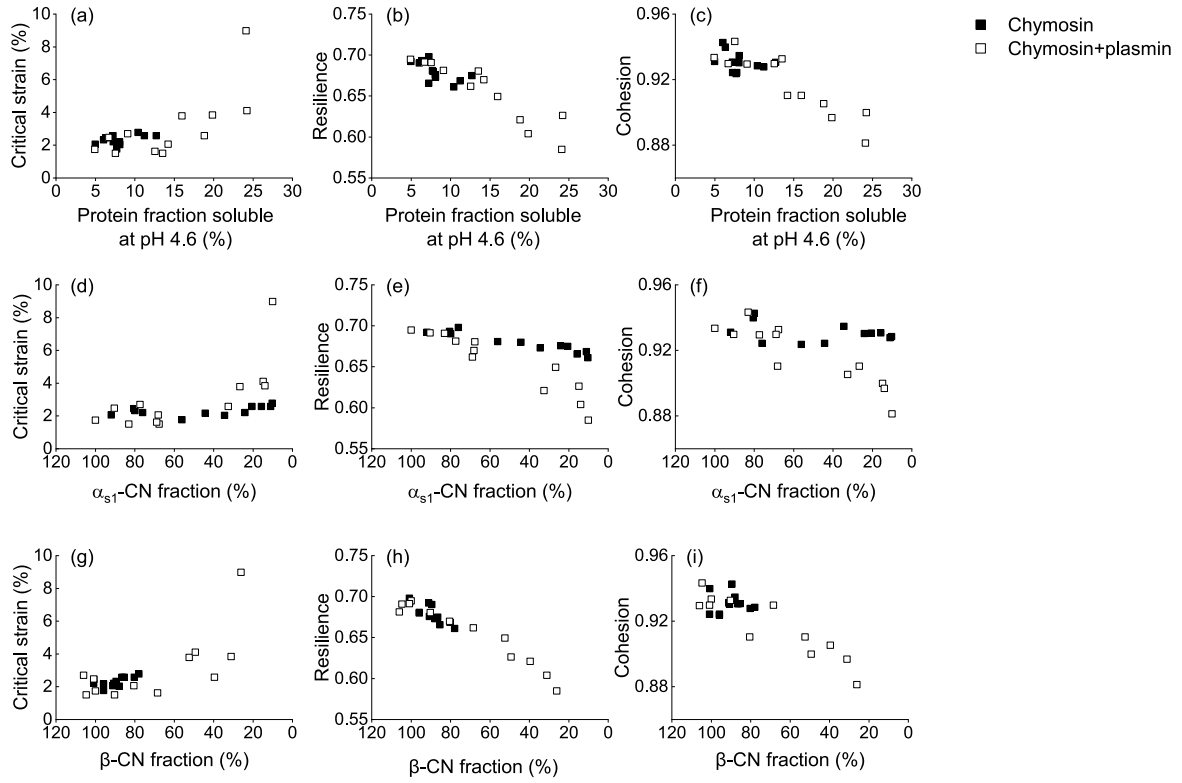

Fig. 8. Critical strain (a, c, e), resilience (b, e, f), and cohesion (c, f, i) as a function of protein fraction soluble at pH 4.6 and intact α_{s1}-and β-CN, in model cheeses made at pH 5.9, with inactive (■, chymosin) and active plasmin (□, chymosin + plasmin). The chymosin activity is expressed in IMCU, i.e. the amount of enzyme which induces gelation of 10 ml of reconstituted milk in 100 s at 30 °C. 1 IMCU = 23.34nKat.

Guido Sala: Conceptualization, Methodology, Writing – review & editing, Supervision. **Etske Bijl:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision.

Declaration of competing interest

The work described has not been published previously, and it is not under consideration for publication elsewhere. Authors declare that they have no conflict of interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115158.

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