MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Study on the interactions between milk protein and digestive enzymes (pepsin)

A thesis presented in partial fulfilment of the requirements for the degree of

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KŌKIRI – TE HĀ O TE KAI

Abstract

Pepsin, as a main digestive enzyme in the gastric environment, plays a key role in milk protein digestion. Pepsin and κ-casein at pH > 5, leading to coagulation of casein micelles. The hydrolysis of κ-casein in skim milk was investigated using reverse phase high performance liquid chromatography (RP-HPLC). The coagulation behavior and coagulum structure were observed using oscillatory rheology, microscopy, small and ultra-small angle neutron scattering. The effects of important variables, including temperature, pH, divalent cations, ionic strength, whey protein concentration, and pre-heat treatment of milk, were studied on the hydrolysis kinetics and coagulation process.

The pepsin-induced hydrolysis of κ-casein was described using a combined kinetic model of first-order hydrolysis and pepsin denaturation. The hydrolysis rate was independent of the divalent cations, ionic strength, and whey protein concentration, but was highly dependent on the temperature, pH, pre-heat treatment, and milk species.

Coagulation of the casein micelles occurred when a critical amount of κ-casein had been hydrolyzed (H_{ct}). The H_{ct} was independent of pepsin concentration (ranging from 0.275 to 5.50 U/mL milk) at a given pH, but it depended on the pH, temperature and calcium concentration: a lower H_{ct} was observed at lower pH (5.3); at higher temperature (43 °C); and at a higher addition of $CaCl_2$ 17.5 (m*M*). Sheep milk coagulated at a lower H_{ct} and goat milk coagulated at higher H_{ct} compared to cow milk. In addition, the rheological properties and the microstructures of the curd were significantly different depending on all the variables.

A human gastric simulator (HGS) was used to investigate the *in vitro* dynamic gastric digestion of different processed milk. The 4 °C milk exhibited slower coagulation with a faster breakdown and hydrolysis of the caseins by pepsin, when compared to that at 37 °C and 50 °C. Addition of $CaCl_2$ enhanced coagulation of casein micelles and resulted in more cohesive curd, which decreased the emptying rate of caseins.

Overall, this research provides fundamental information on the kinetics of hydrolysis of κ-casein and how it affects the coagulation behavior of casein micelles under various conditions in milk systems. The different gastric digestion behaviors of different processed milks are explained. The outcomes from this study have the potential to design dairy products with specific coagulation and digestion behaviors to meet the specific digestion requirements of consumers.

Graphical abstract

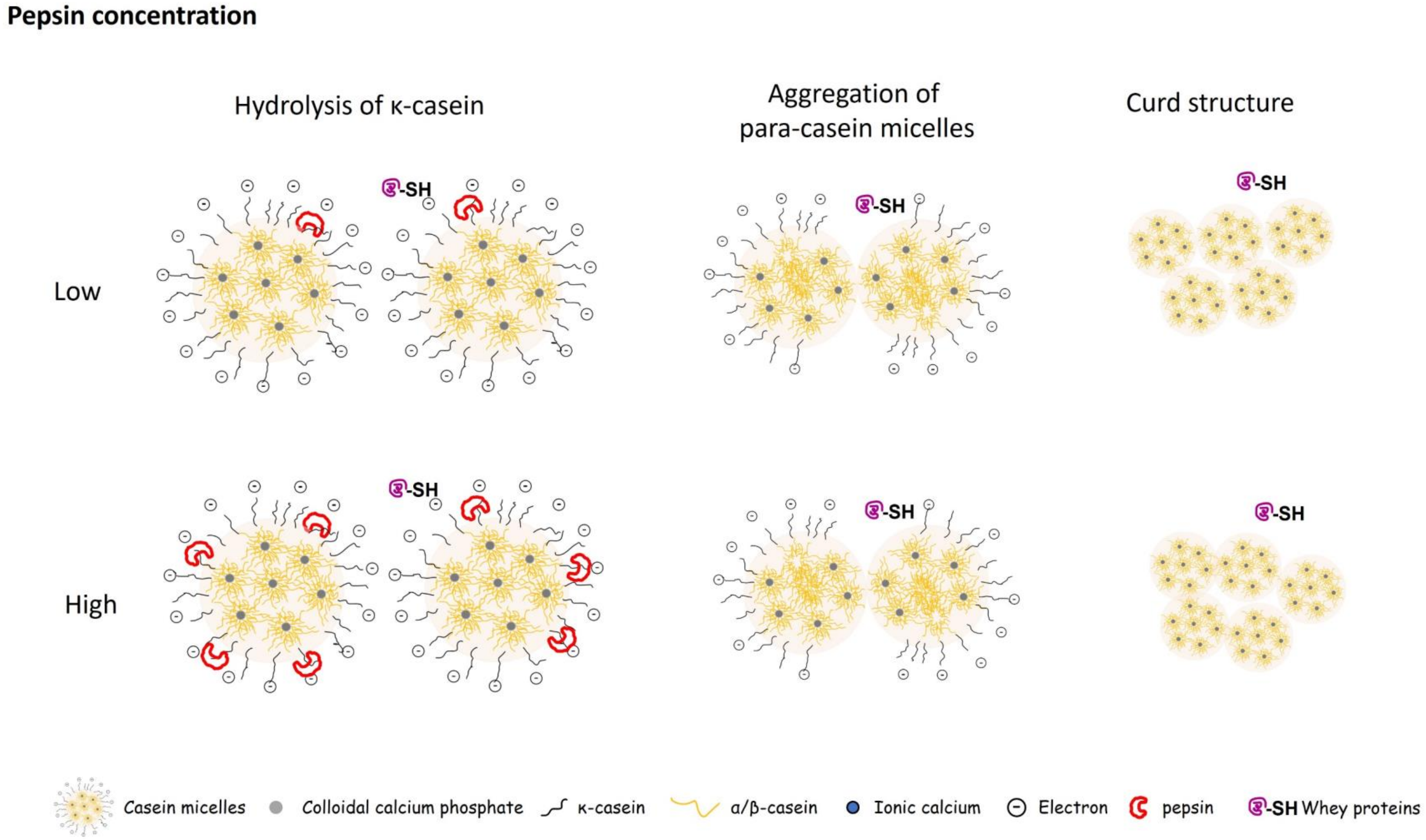

Figure 1 Schematic representation of the effect of pepsin concentration on the hydrolysis of κ-casein, the aggregation of para-casein micelles and the curd structure. The higher pepsin concentration (in range of 0.275–5.50 U/mL) resulted in faster hydrolysis of κ-casein, but did not impact the aggregation of para-casein micelles nor curd structure.

Graphical abstract

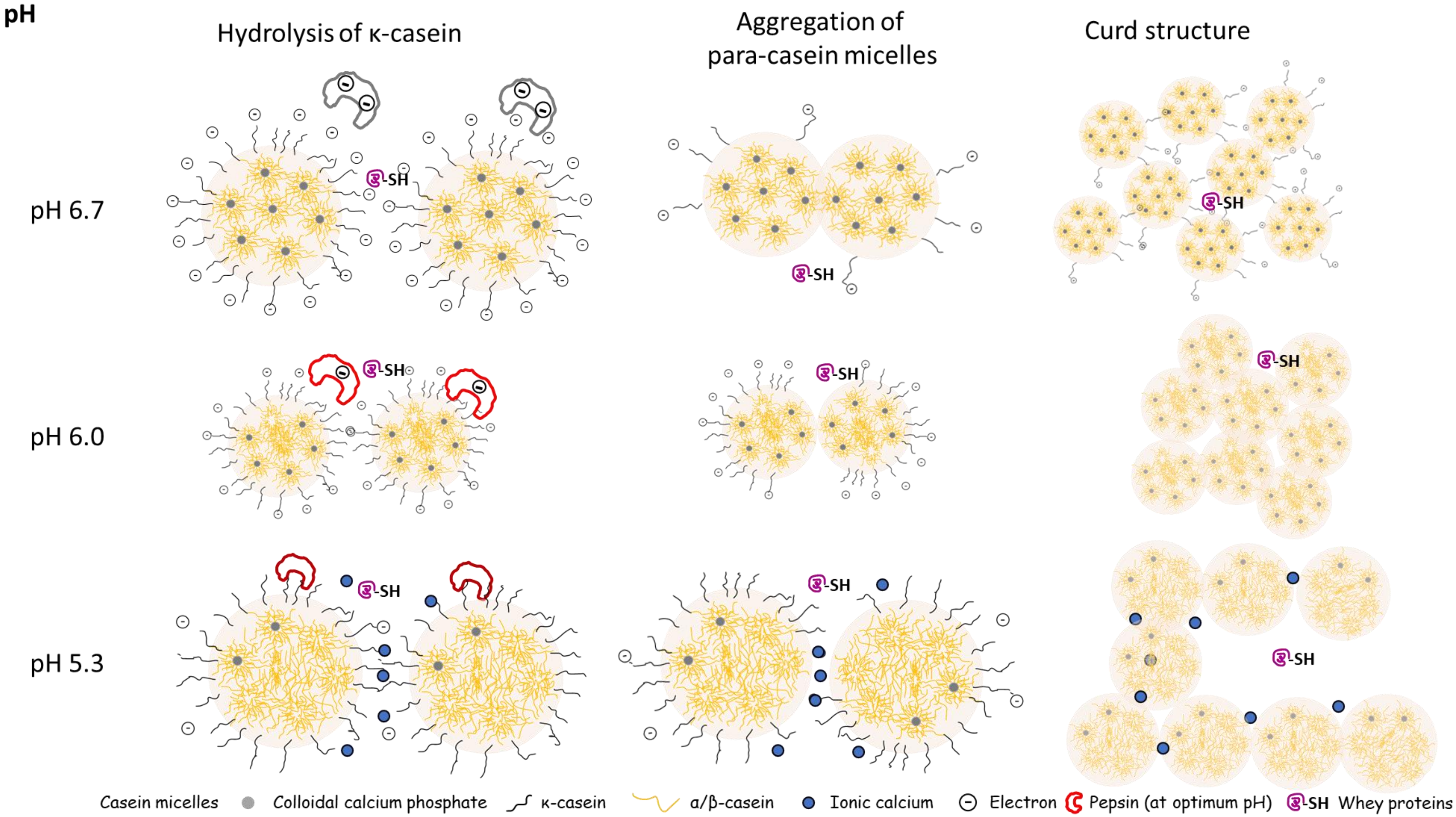

Figure 2 Schematic representation of the effect of pH (in range of 5.3–6.7) on the hydrolysis of κ-casein, the aggregation of para-casein micelles and the curd structure. The optimal pH for κ-casein hydrolysis by pepsin was identified as pH 6.0. Aggregation at lower pH required a reduced hydrolysis degree. The network of curd at lower pH exhibited significant rearrangement.

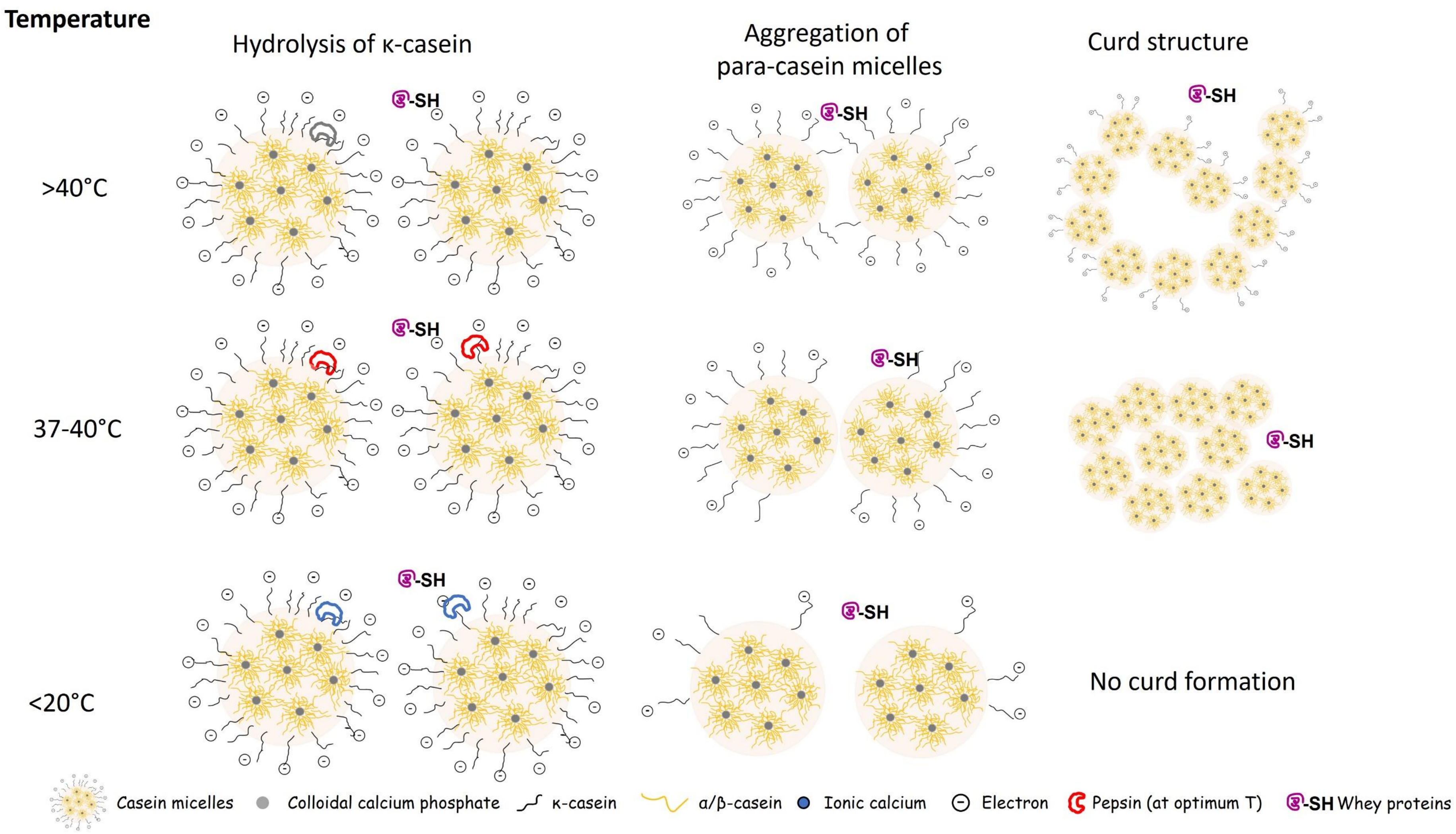

Figure 3 Schematic representation of the effect of temperature on the hydrolysis of κ-casein, the aggregation of para-casein micelles and the curd structure. The optimal temperature for κ-casein hydrolysis by pepsin was ~ 37−40 °C. A less extensive degree of hydrolysis of κ-casein was required for coagulation at higher temperatures. Below 25 °C, no coagulation of casein occurs even when κ-casein was totally hydrolyzed.

Graphical abstract

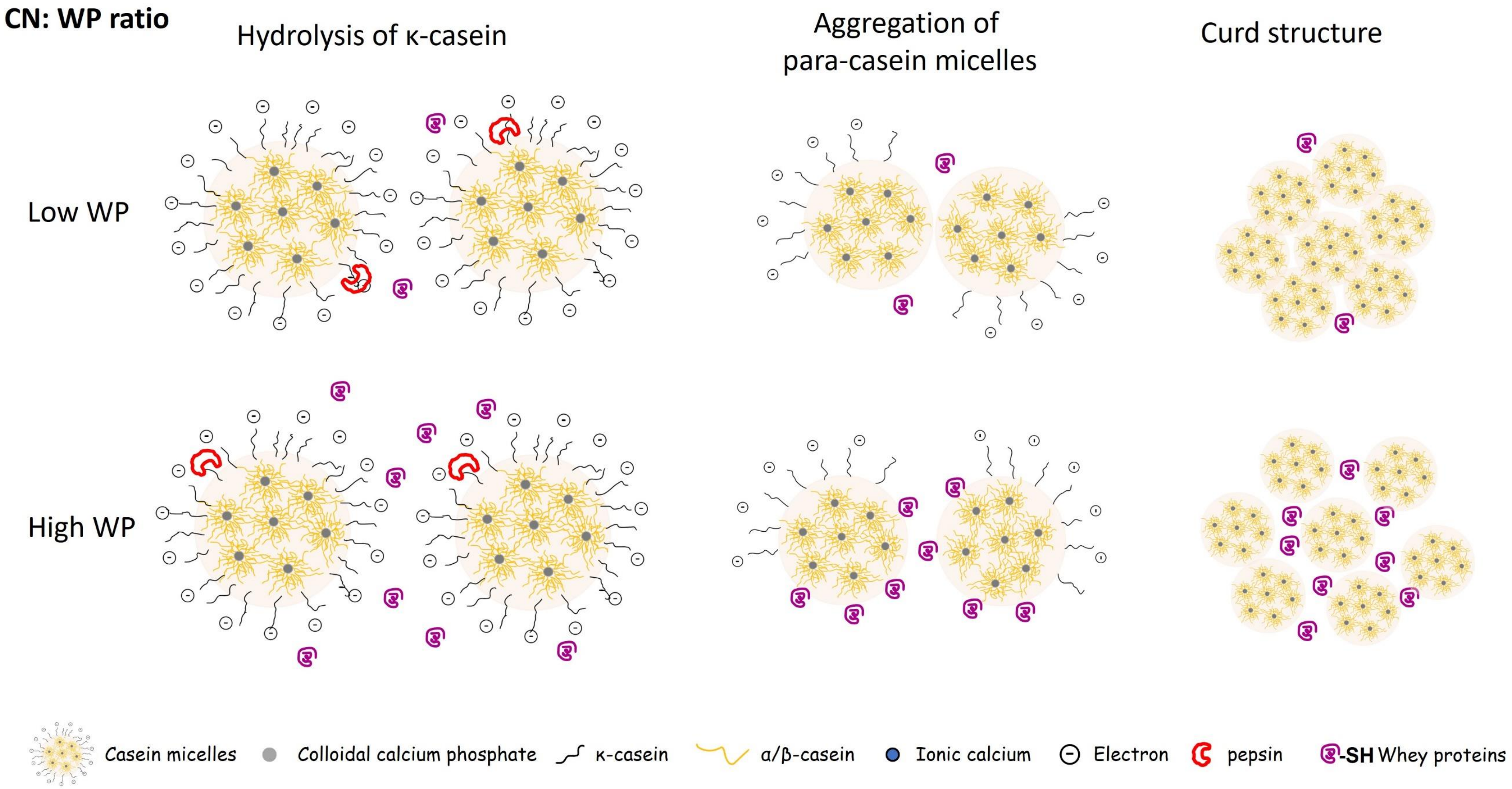

Figure 4 Schematic representation of the effect of casein-to-whey protein (CN:WP) ratio on the hydrolysis of κ-casein, the aggregation of para-casein micelles and the curd structure. A higher concentration of whey protein did not impact the hydrolysis of κ-casein by pepsin but impairs the aggregation of casein micelles and resulted in sparse and weak structures.

Graphical abstract

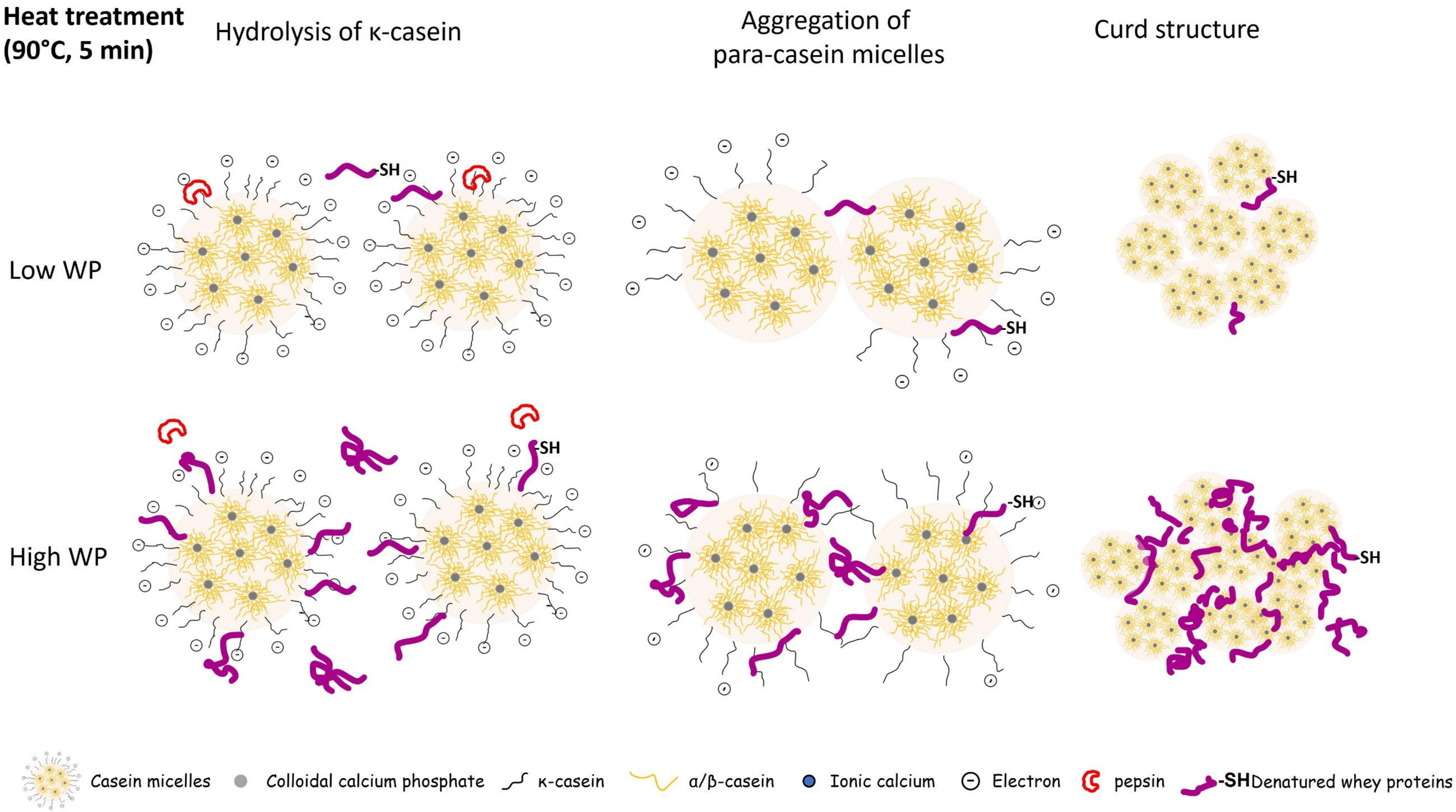

Figure 5 Schematic representation of the effect of heat treatment (90 °C, 5 min) on the hydrolysis of κ-casein, the aggregation of para-casein micelles and the curd structure of the skim milk. The denatured proteins adsorbed onto the surface of the casein micelles and impaired the hydrolysis rate of κ-casein in skim milk and was incorporated into the protein network.

Graphical abstract

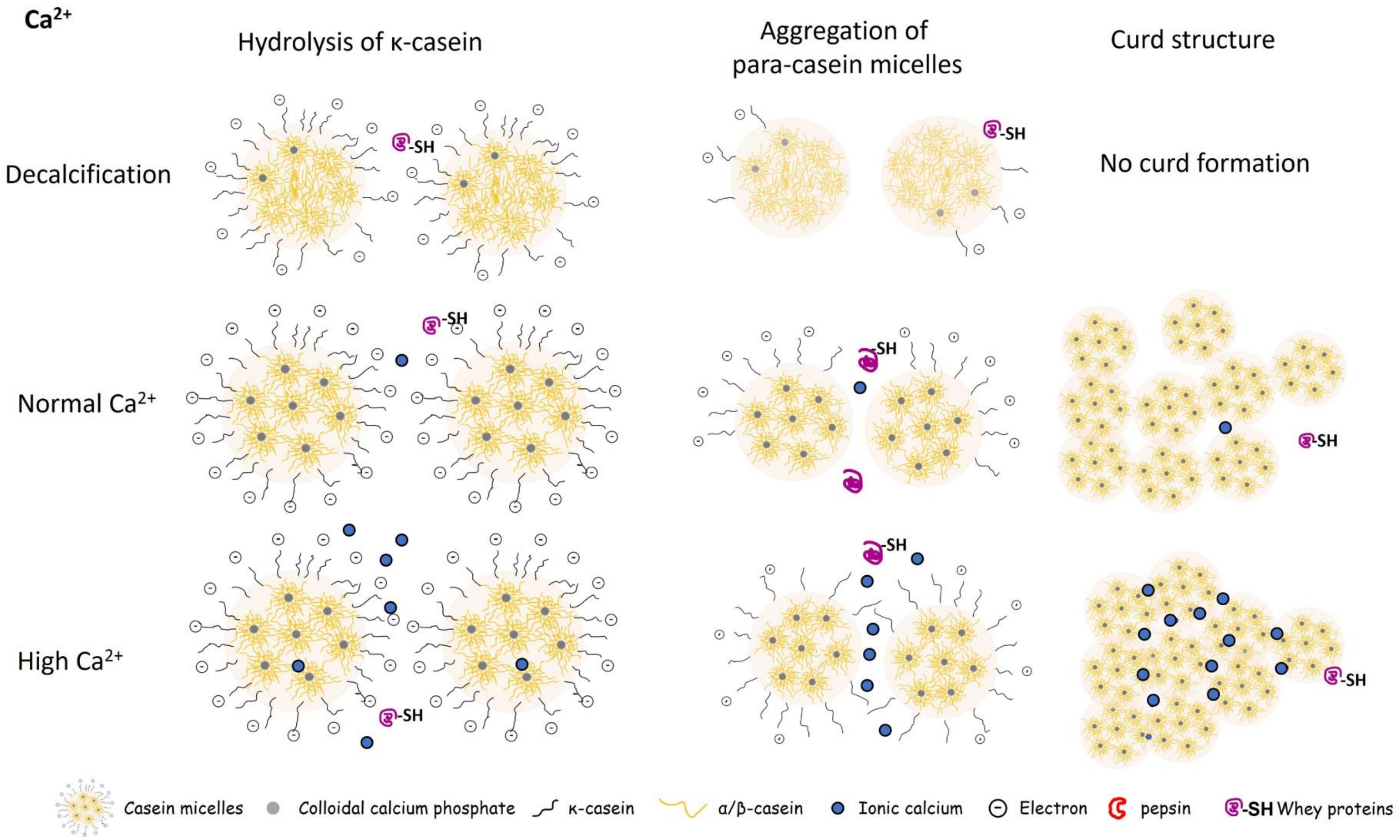

Figure 6 Schematic representation of the effect of calcium concentration on the hydrolysis of κ-casein, the aggregation of para-casein micelles and the curd structure. The addition of ionic calcium did not impact the hydrolysis of κ-casein by pepsin, but it facilitated the coagulation of para-casein micelles through the calcium bridge, resulting in a stronger network.

Graphical abstract

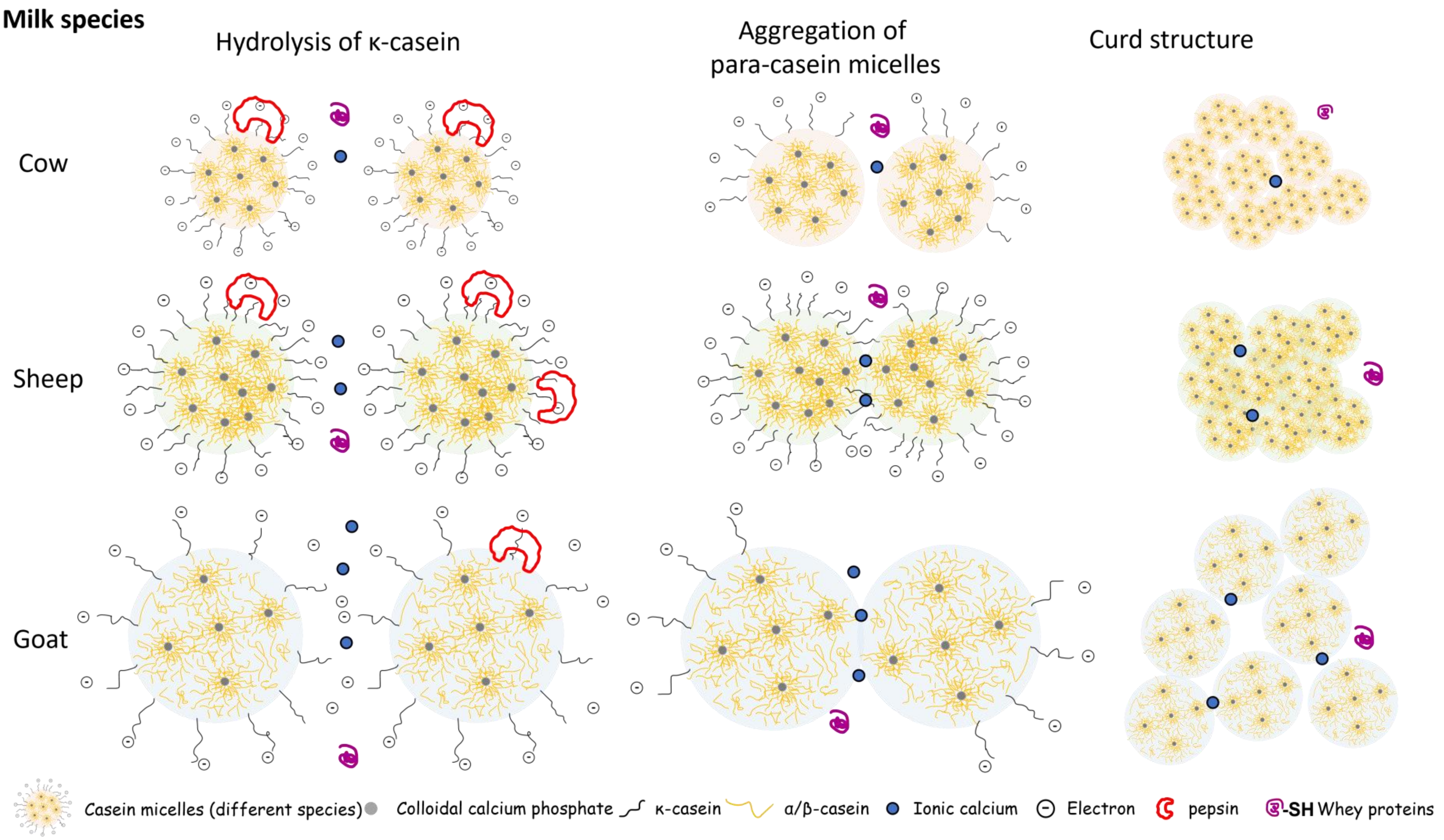

Figure 7 Schematic representation of the hydrolysis of κ-casein, the aggregation of para-casein micelles and the curd structure in cow, sheep, and goat skim milk. Hydrolysis rate of κ-casein was found to be most pronounced in sheep milk, followed by cow milk and goat milk. Sheep milk coagulated with the lowest κ-casein hydrolysis requirement and exhibited a dense microstructure. In contrast, goat milk coagulated with the highest extent of κ-casein hydrolysis and exhibited a porous microstructure.

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List of publications, proposals, and awards

Publications

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Role of Ca 2+ in the pepsin-induced coagulation and in the dynamic in vitro gastric digestion behavior of casein micelles. Food & Function, 14(15), 6985-6997. https://doi.org/10.1039/D3FO01126G

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Effect of ingestion temperature on the pepsin-induced coagulation and the in vitro gastric digestion behavior of milk. Food Hydrocolloids. https://doi.org/10.1016/j.foodhyd.2023.108550

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, EP., & Singh, H. (2023). Pepsin-induced coagulation of casein micelles: Effect of whey proteins and heat treatment. Food Chemistry. 402, 134214. https://doi.org/10.1016/j.foodchem.2022.134214

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2022). Kinetics of pepsin-induced hydrolysis and the coagulation of milk proteins. Journal of Dairy Science. 105(2), 990-1003. https://doi.org/10.3168/jds.2021-21177

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Gastric digestion of proteins: the role of pepsin (In preparation).

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Pepsin-induced hydrolysis and coagulation of proteins in goat, sheep and cow milk (In preparation).

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). (Ultra) Small-angle neutron scattering investigation of acid- and/or pepsin-induced milk coagulation and degradation of milk curd (In preparation).

Awards & Granted proposals

Second Place Poster presentation, titled ‘Role of Ca^{2+} in the dynamic in vitro gastric digestion behavior of casein micelles’, 7th International Conference on Food Structures, Digestion and Health, 2023

Australian Nuclear Science and Technology Organization (ANSTO) AINSE Post Graduate Research Award (PGRA)

ANSTO Proposal 9635: SAXS, SANS and USANS investigation of the interactions between milk protein and digestive enzyme (pepsin)

Conference presentations

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. Oct 2023). Role of Ca^{2+} in the pepsin-induced coagulation and in the dynamic in vitro gastric digestion behavior of casein micelles. Oral presentation at *13th NIZO Dairy Conference*. Papendal, The Netherlands.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (Jul 2023). Effect of ingestion temperature on the pepsin-induced coagulation and in-vitro gastric digestion behavior of milk. Poster at *The New Zealand Institute of Food Science and Technology (NZIFST) Conference.* Dunedin, New Zealand.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (Nov 2022). Pepsin-induced coagulation of casein micelles: Effect of whey proteins and heat treatment. Oral presentation at *Riddet Institute Conference and Student Colloquium*. Napier, New Zealand.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (Oct 2022). Pepsin-induced coagulation of casein micelles: Effect of pH, whey proteins and heat treatment. Oral presentation at *16th International Hydrocolloids Conference (IHC)*. Guelph, ON, Canada.

Yang, M. (Oct 2022) *ANSTO-HZB Neutron School 2022.* Sydney, Australia.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (Apr 2021). Kinetics of pepsin-induced hydrolysis and the coagulation of milk proteins. Poster at *Riddet Institute Conference and Student Colloquium.* Wellington, New Zealand.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (Jul 2021). Interaction between milk protein and digestive enzyme (pepsin). 3 min pitch & Poster at *The New Zealand Institute of Food Science and Technology (NZIFST) Conference.* Palmerston North, New Zealand.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (Nov 2021). Interaction between milk protein and digestive enzyme (pepsin). Poster at *Food Structures, Digestion & Health.* Online.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (Feb 2021). Interaction between milk protein and digestive enzyme (pepsin) by using Small Angle Scattering techniques. Oral presentation at *Australian Centre for Neutron Scattering Early Career Researchers (ACNS) CLIP DAY.* Online.

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List of Abbreviations

ACH	Acid-induced coagulation of heated milk
ANOVA	Analysis of variance
ACU	Acid-induced coagulation of unheated milk
$[Ca^{2+}]$	Ionic calcium
CCH	Combination coagulation of heated milk
CCP	Colloidal calcium phosphate
CCU	Combination coagulation of unheated milk
CLSM	Confocal laser scanning microscopy
CMP	Caseinomacropeptide
CN:WP	Casein:whey protein
D_2O	Deuterium oxide
G'	Storage modulus
G''	Loss modulus
GDL	Glucono-δ-lactone
HGS	Human gastric simulator
HPH	High pepsin treated heated milk
HPU	High pepsin treated unheated milk
MC	Micellar casein
PCH	Pepsin-induced coagulation of heated milk
PCU	Pepsin-induced coagulation of unheated milk
pI	Isoelectric point
Q_{10}	Temperature coefficient
RP-HPLC	reverse phase high performance liquid chromatography
SANS	Small angle neutron scattering
SAXS	Small angle X-ray scattering
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SMUF	Simulated milk ultrafiltrate
SSF	Simulated salivary fluid
TEM	Transmission electron microscopy
UHT	Ultra-high temperature
USANS	Ultra small angle neutron scattering
WHC	Water-holding capacity
WPI	Whey protein isolate
α-La	α-lactalbumin
β-Lg	β-lactoglobulin

Various terms or descriptors, some of which are used interchangeably, are employed to describe the coagulation of milk; there are some simple definitions according to Fox et al. (2017b), as follows:

Aggregation: the joining of particles (e.g., para-casein micelles) by various types of electrostatic or hydrophobic bonds; the aggregates are visible by electron microscopy.

Coagulation: the collision and joining of aggregates, especially under non quiescent conditions, to form flocs, visible to the naked eye.

Gelation: the aggregation of particles (e.g., micelles or aggregates of micelles) to form particulate strands, in which the particles undergo limited touching, and eventually form a gel network.

In summary, aggregation involves the joining of individual particles, coagulation occurs when aggregates collide and join to form larger flocs, and gelation is the process of particles forming a gel network with limited touching and a three-dimensional structure. These terms describe different stages and aspects of particle interactions and the formation of structures in a system. However, it is important to note that these stages are not always strictly distinct and can overlap to some extent. The solid mass is often called **'clot', 'curd', 'gel'** or **'coagulum'**, which are used interchangeably. The stress required to cause a given strain or deformation is defined as **gel (curd) firmness**.

Chapter 1 Introduction

Studies on milk digestion, especially milk proteins, have attracted considerable attention in recent years (Mulet-Cabero, Mackie, Brodkorb, & Wilde, 2020a; Tunick et al., 2016; Wada & Lönnerdal, 2014; Ye, Cui, Dalgleish, & Singh, 2016b). Both *in vivo* and *in vitro* studies have reported on milk coagulation phenomena in the gastric environment before transit into the small intestine (Huppertz & Chia, 2021; Roy, Ye, Moughan, & Singh, 2020b; Ye, 2021). During gastric digestion, the overall action of pepsin on milk proteins (in particular, casein micelles) consists of several distinct phases (**Figure 1-1**). (1) After milk is consumed, it enters the stomach where a small amount of gastric fluid is present, thus the pH of the stomach content can reach values > 6.0. Pepsin specifically hydrolyzes the Phe^{105}–Met^{106} bond of κ-casein at this relatively high pH, yielding the C-terminal (glycosylated) caseinomacropeptide and para-κ-casein. (2) Aggregation of casein micelles takes place by hydrophobic association together with electrostatic effects (Horne & Lucey, 2014), which occurs when a minimum amount of the caseinomacropeptide has been removed (a ‘critical’ degree of hydrolysis). (3) Additional secretion of acidic gastric juice continually lowers the pH, and dissolution of the colloidal calcium phosphate (CCP) induces the rearrangement of the coagulum. (4) Further proteolysis occurs where both caseins and whey proteins are hydrolyzed into peptides at pH < 4.

Many aspects of pepsin-induced milk coagulation broadly resemble the enzymatic coagulation of milk during cheese manufacture and acid coagulation of milk during yoghurt manufacture (Huppertz & Chia, 2021). Despite the overall mechanisms of pepsin-induced casein micelles coagulation being similar to chymosin-induced coagulation (Kageyama, 2002), there are limited studies investigating the mechanism of pepsin-induced hydrolysis of κ-casein and its connection to coagulation of casein micelles in the stomach environment.

The overall aim of this study is therefore to study the effect of different environmental conditions (e.g., pH, pepsin concentration, temperature), processing treatments (e.g., heat treatment, calcium enhancement), and milk from different species (e.g., cow, goat, and sheep) on the pepsin-induced hydrolysis of κ-casein, coagulation of casein micelles and proteolysis of the milk proteins. Since the progressive proteolysis of milk proteins during digestion highly depends upon the coagulum structure, understanding the specific hydrolysis reactions of κ-casein by pepsin and the consequent coagulation process are critical. Understanding the kinetics of the specific hydrolysis of κ-casein and the coagulation behavior and their inter-relationship will provide new insights into milk protein digestion.

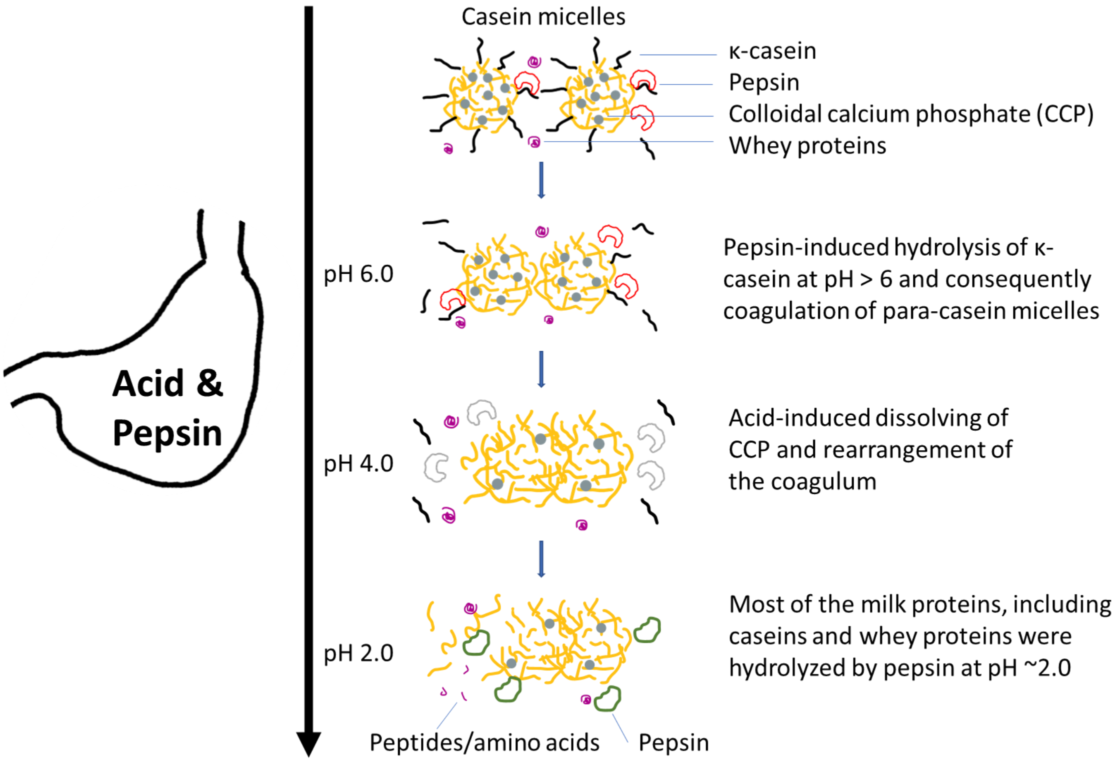

Figure 1-1 Mechanism of milk protein digestion in the gastric environment.

Although the hydrolysis phase and aggregation phase partially overlap (Frederiksen et al., 2011), they differ kinetically. The hydrolysis degree of κ-casein in this study was determined using reverse phase high performance liquid chromatography (RP-HPLC), and the coagulation process and coagulum properties of casein micelles were monitored by small and large amplitude oscillatory shear, and by microscopy (CLSM: confocal laser scanning microscopy, and SEM: scanning electron microscopy). The structure of the coagulum in **Chapter 7** and **Chapter 8** was probed using small/ultra-small neutron angle scattering (USANS/SANS). In addition, the effect of curd structure on the proteolysis of protein during gastric digestion was studied in a dynamic *in vitro* gastric model – the human gastric simulator (HGS) – in **Chapter 4** and **Chapter 6**.

Over the years, chymosin-initiated hydrolysis of κ-casein and the aggregation of the casein micelles in cow milk (referred to as "milk" in the thesis, unless otherwise specified) have been extensively studied (Dalgleish, 1980, 1988; van Hooydonk, 1987). Chaplin and Green (1980) demonstrated that chymosin hydrolyzes the Phe^{105}–Met^{106} bond following the Michaelis–Menten equation, although, according to van Hooydonk (1987), a first-order rate equation describing enzymatic hydrolysis provides a better fit to the data. However, the kinetics of κ-casein hydrolysis catalyzed by pepsin in milk systems have not been fully elucidated. To better understand this, **Chapter 3** examined the pepsin-induced hydrolysis of κ-casein and coagulation of casein micelles as different concentrations of pepsin (0.110–5.50 U/mL milk) in skim milk, with pH varying from 5.3 to 6.7.

As temperature is known to influence pepsin activity (Zhao, Budge, Ghaly, Brooks, & Dave, 2011) and enzyme-induced coagulation behavior (Dalgleish, 1983; Horne & Lucey, 2014; Nájera, De Renobales, & Barron, 2003), the temperature of the milk could influence the structure of the curd/clot. It has been reported that the temperature coefficient [Q_{10}, defined as the rate ratio of a given process taking place at temperatures differing by 10 units (°C or K)] of chymosin-induced hydrolysis of κ-casein was around 2.0; whereas a greater effect of temperature was found for the aggregation phase, with a Q_{10} of ~ 16 (Fox, Guinee, Cogan, & McSweeney, 2017a). However, the effect of the temperature of milk on digestive properties has not been fully explored. Therefore, **Chapter 4** investigated the pepsin-induced hydrolysis of κ-casein and aggregation characteristics of skim milk (pH ~ 6.0, which is the optimum pH for the hydrolysis of κ-casein) at different temperatures. In addition, the different digestive properties of milk ingested at 4 °C, 37 °C, or 50 °C were investigated using an HGS (Kong & Singh, 2010). This study provided better understanding of the effect of temperature on the coagulation and digestion of milk in the gastric environment.

Whey proteins, primarily β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), have globular structures and are heat-sensitive to unfolding (Anema & Li, 2003). In an *in vitro* digestion model, infant formula powder with an 80:20 casein:whey protein (CN:WP) ratio showed more extensive coagulation of casein micelles compared to a 40:60 ratio (Phosanam, Chandrapala, Huppertz, Adhikari, & Zisu, 2021). Ye, Cui, Dalgleish, & Singh (2017) found more fragmented and crumbly structures of curd formed from heated milk (both pasteurized and ultra-high temperature (UHT) processed) than for fresh milk after digestion in an HGS. However, the effect of whey protein concentration and heat treatment on the pepsin-induced hydrolysis of κ-casein and subsequent coagulation has not been fully explored. In **Chapter 5**, micellar casein (MC) powder and whey protein isolate (WPI) were dissolved in simulated milk ultrafiltrate (SMUF) at ratios of 4:0, 4:0.6, 4:1 and 4:2 (at a constant casein concentration) with or without heating at 90 °C for 5 min. The effect of whey protein concentration and heat treatment on the hydrolysis of κ-casein and the coagulation behavior of casein micelles was investigated at a constant pepsin concentration (1.1 U/mL milk) at pH 6.0.

To improve the nutritional and functional properties of milk, enrichment with calcium salts has been a common practice (Gastaldi, Pellegrini, Lagaude, & de la Fuente, 1994; Ramasubramanian, D’Arcy, Deeth, & Oh, 2014; Vyas & Tong, 2004). The addition of $CaCl_2$ leads to an increase in the calcium content in all forms (free/ionized calcium, calcium complexed with inorganic anions, and colloidal calcium) accompanied by a decrease in the milk pH (Bringe & Kinsella, 1986). The reported effects of $CaCl_2$ addition on the enzymatic hydrolysis of casein micelles are contradictory. In **Chapter 6**, the effect of added $CaCl_2$ on the hydrolysis of κ-casein and the coagulation behavior of MC solution was investigated at a constant pH 6.0, where NaCl was used as a positive control in an assessment of the effects of an increase in ionic strength that occurs with $CaCl_2$ addition. Furthermore, to better understand the effects of Ca^{2+} and ionic strength on the digestion behavior of MC solutions, samples were examined in an HGS.

Milk from non-cow species such as buffalo, goat and sheep have been widely accepted by consumers, and constitutes an important part of milk consumption by humans (Claeys et al., 2014; Roy, Ye, Moughan, & Singh, 2020a). During *in vivo* and *in vitro* gastric digestion, regardless of the dairy species (i.e., cow, goat and sheep), the milk coagulates and separates into a clot and a liquid phase (Li et al., 2022b; Roy et al., 2022; Roy, Ye, Moughan, & Singh, 2021). However, the coagulation and digestion behavior are different among these species, e.g., the firmness of the sheep skim milk clot is relatively higher than those of the cow and goat milk clots at the end of gastric digestion (Li et al., 2022b). **Chapter 7** was designed to find out the underlying reasons. At the same pepsin-to-κ-casein ratio and pH, the pepsin-induced hydrolysis of κ-casein and coagulation of proteins in raw fresh milk (cow, sheep and goat) were examined. In addition, small-angle neutron scattering (SANS) was employed to explore the coagulation process in different milk types on the nanoscale.

In **Chapter 8**, SANS and USANS were used to monitor the real-time structural evolution of acid and/or pepsin-induced milk coagulation on the nano- and micro-scale. Combined with rheological measurements and confocal laser scanning microscopy (CLSM) observations, the effect of heat treatment (85 °C, 30 min) on acid (glucono-δ-lactone GDL)-, pepsin- and a combination of acid and pepsin-induced coagulation of cow skim milk were investigated. It was shown that at a high concentration of pepsin (2000 U/mL), both protein coagulation and hydrolysis occur. This study shows the potential of using time resolved USANS and SANS to investigate the microstructural evolution of milk proteins in the gastric environment.

The overall goal of this project was to understand the mechanism of the pepsin-induced coagulation of milk. The relevant literature background is presented in **Chapter 2** and the key objectives have been addressed in **Chapter 3** to **Chapter 8**:

1) To determine the kinetics of hydrolysis of κ-casein by pepsin in skim milk and characterize the coagulation process under various environmental conditions (i.e., pepsin concentration, milk pH, temperature).
2) To investigate the effect of milk composition (i.e., whey protein concentration, calcium concentration), pre-treatment (i.e., heat treatment) and milk species (i.e., comparison between cow, sheep and goat milk) on the kinetics of pepsin hydrolysis of κ-casein, and the coagulation of casein micelles.
3) To understand the impact of the coagulum structure on the further proteolysis of milk proteins during gastric digestion to fill the knowledge gap about the effect of temperature and calcium concentration on *in vitro* digestion behavior of milk.
4) To better understand the changes in protein structure during coagulation and general hydrolysis process using small and ultra-small angle neutron scattering (SANS and USANS) techniques.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Mengxiao Yang
Name and title of main supervisor:	Professor Aiqian Ye
In which chapter is the manuscript/published work?	Chapter 2

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Mengxiao Yang: conceptualization, visualization, literature search, Writing – original draft.
Aiqian Ye: Conceptualization, Funding acquisition, Supervision, Resources, Writing – review & editing. Zhi Yang: Supervision, Writing – review & editing. David W. Everett: Supervision, Writing – review & editing. Elliot Paul Gilbert: Supervision, Writing – review & editing. Harjinder Singh: Supervision, Resources, Writing – review & editing.

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Chapter 2 Literature review - Interactions between milk proteins and pepsin[1]

Abstract

This literature review comprehensively covers the interactions between milk proteins and pepsin under gastric conditions, focusing on pepsin-induced milk coagulation, including the hydrolysis of κ-casein by pepsin and the consequent coagulation of para-casein micelles. It highlights the role of pepsin as the primary enzyme responsible for initiating the coagulation process through its interaction with casein micelles. Additionally, the effects of different processing pretreatments, such as heat treatment of different milk species, on the interaction between milk proteins and pepsin are discussed. Because the mechanism of pepsin-induced milk coagulation resembles rennet-induced coagulation, studies on the latter topic are included in this review. Moreover, the methodologies for monitoring aggregation and gelation of milk proteins are summarized. Overall, this review brings together published information on the intricate interactions between milk proteins and pepsin, thus enhancing our understanding of milk coagulation under gastric conditions.

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[1] Part of the contents presented in this chapter will be submitted for publication: Gastric digestion of proteins: the role of pepsin (In preparation).

2.1. Introduction

Dietary proteins are crucial for muscle development, immune function, enzyme and hormone production, tissue maintenance, nutrient transport, satiety, and overall health (Shang, Chaplot, & Wu, 2018). The human body can only utilize proteins when they are hydrolyzed by proteases and peptidases to free amino acids, dipeptides, or tripeptides through digestion (Wu, 2021). Three distinct phases of protein digestion can be identified in the gastrointestinal tract: the stomach phase, the pancreatic phase and the small intestinal phase (Erickson & Kim, 1990). The first and important step in protein digestion occurs in the stomach, where pepsin is the principal enzyme involved in protein digestion (Silk, Grimble, & Rees, 1985; Whitecross, Armstrong, Clarke, & Piper, 1973). It has been agreed that the nutritive value of dietary protein is not only related to the amino acid composition, but also depends upon the digestibility coefficients and the absorption of the hydrolysis products in the human gastrointestinal tract (Villamide, Nicodemus, Fraga, & Carabaño, 2010; Wu, 2021). The products generated during protein digestion largely depend upon the structural properties of food, including protein solubility and accessibility of digestive enzymes, protein source and processing history of food products (Kaur et al., 2022), e.g., it has been reported that insoluble proteins are more resistant to pepsin. In addition, the structure formed during gastric digestion also plays an important role in nutrient delivery (Ye, 2021). As a specific example, bovine milk coagulates under gastric conditions whereas an oat milk beverage does not show significant changes in structure and physical stability during gastric digestion with pepsin (Wang, Ye, Dave, & Singh, 2022).

Bovine milk is an excellent source of nutrients for humans that provides proteins, fats, vitamins and minerals etc. (Haug, Høstmark, & Harstad, 2007). It has been reported that in most ruminant milk, nitrogen is distributed in forms of caseins, whey proteins, milk fat globule membrane proteins and non-protein fractions (Swaisgood, 1982). Studies on milk digestion, especially milk protein digestion, have attracted extensive attention in recent years (Huppertz & Chia, 2021; Miranda & Pelissier, 1983; Mulet-Cabero et al., 2020a; Tunick et al., 2016; Wada & Lönnerdal, 2014; Ye, 2021; Ye et al., 2016b). Different hydrolysis and digestion behaviors have been observed under the gastric environment due to the structural difference between casein and whey proteins (Wang, Ye, Lin, Han, & Singh, 2018). It has been reported that the caseins, together with CCP, assemble into the colloidal particles known as casein micelles that coagulate by pepsin action (Tam & Whitaker, 1972) and by low pH (Dalgleish & Corredig, 2012). In contrast, whey proteins remain soluble and pass rapidly through the stomach (Wang et al., 2018). With the different food matrices formed during gastric digestion, diffusion of digestive enzymes is impacted, i.e., relative inhibition of pepsin diffusion within a solid matrix compared to a liquid one (Luo, Borst, Westphal, Boom, & Janssen, 2017). This, in turn, impacts the passage of gastric chyme, degradation of proteins/peptides, and absorption of free amino acids in the small intestine (Moughan, 2009). Therefore, casein micelles remain in the stomach for longer, whereas whey proteins have a faster delivery of amino acids to the circulatory system (Boirie et al., 1997). Consequently, this initial step of digestion, the interaction between proteins and pepsin, is important for subsequent nutrient release.

In this review, the interactions between pepsin and different milk proteins will be discussed with an emphasis on the coagulation behavior of casein micelles. The impact of important variables, such as pH, temperature, and processing treatments of milk, are discussed in relation to the reaction kinetics and to the implications for the coagulation process. The impacts of differently structured milk coagulum/curd on the delivery of nutrients for absorption are summarized as well.

2.2. Gastric environment

The stomach secretes an average of 2–3 L of gastric fluids each day, which contains water, electrolytes, hydrochloric acid, enzymes, mucus, and intrinsic factor (a glycoprotein), etc. (Arora, Ali, Ahuja, Khar, & Baboota, 2005; Bornhorst & Singh, 2014). In a fasting state, gastric pH is about pH 3.5–5.0 for infants and about 1.5–2.0 for a healthy adult. Generally, after consuming a meal, the pH of the gastric content increases to about pH 5.5–7 and then drops down with further gastric emptying, depending upon the nature and the amount of the ingested food (Huppertz & Chia, 2021; Ye, 2021).

2.2.1. Pepsin

One of the important components in the stomach are the digestive enzymes that enable efficient digestion of food proteins. There are five groups of pepsinogens: pepsinogens A, B, and F, progastricsin, and prochymosin, which are precursors of pepsins A, B, and F, gastricsin, and chymosin, respectively (**Table 2-1**) (Kageyama, 2002).

Table 2-1 Classification of pepsinogens [adapted from Kageyama (2002), with permission by Springer Nature].

Group	Active form	EC number
pepsinogen A	pepsin A	3.4.23.1
pepsinogen B	pepsin B	3.4.23.2
progastricsin	gastricsin	3.4.23.3
prochymosin	chymosin	3.4.23.4
pepsinogen F	pepsin F	

A comparison of the primary structures of typical mammalian pepsinogens and pepsin has been reported by Kageyama (2002). For ease of use, most *in vitro* studies have utilized commercial porcine pepsin A (EC 3.4.23.1), referred to as ‘pepsin’ throughout the rest of this thesis. Pepsin has a broad specificity with a preference to hydrolyze bonds adjacent to aromatic and hydrophobic amino acids (peptide sequence that contains phenylalanine, tyrosine, tryptophan and leucine residues) (Dunn, 2002; Inouye & Fruton, 1967), with an optimum pH of around 2.0 (Kondjoyan, Daudin, & Santé-Lhoutellier, 2015; Pletschke, Naudé, & Oelofsen, 1995).

It has been reported that the amino acid sequence homology is 57.5% between chymosin and pepsin, and the active site sequences Phe^{33}-Ser^{38} and Asp^{216}-Thr^{219} of chymosin are fully conserved in pepsin (Phe^{31}-Ser^{36} and Asp^{215}-Thr^{218}) (Plowman & Creamer, 1995). Both of these can rapidly hydrolyze the Phe^{105}-Met^{106} bond of κ-casein at pH above 5, and prior to the availability of cloned enzymes, porcine pepsin was often admixed with bovine chymosin for cheesemaking (Kageyama, 2002; Plowman & Creamer, 1995). The gastric pH is critical for pepsin, which impacts both the active site and activity, especially with milk proteins as a substrate. The pepsin activity against typical substrates (hemoglobin and milk) at different pH is shown in **Table 2-2**.

Table 2-2 Pepsin activity against typical substrates [a] [adapted from Kageyama (2002), with permission by Springer Nature].

Enzyme	**Hydrolytic activity (%) [b] Hemoglobin**	**Milk clotting activity (%) [c]**
Porcine pepsin A	55–59	25–66
Porcine pepsin B	1–3	0.4–1
Human gastricsin	100	43
Bovine chymosin	7–14	100

[a] The variation in some values is due to differences reported within the literature.

[b] Hemoglobin-digestive activity was determined at pH 2.0 (Foltmann, 1992; Nielsen & Foltmann, 1995; Tang, Wolf, Caputto, & Trucco, 1959). The highest value in each column is taken as 100%.

[c] Assay at pH 6.3 (Foltmann, 1992; Tang et al., 1959). The value of porcine pepsin B is estimated from a ‘caseogram’ assay (Foltmann, Szecsi, & Tarasova, 1985). The highest value is taken as 100%.

2.3. Milk proteins

It has been reported that in most ruminant milk, nitrogen is mainly present in caseins (around 80%), whey proteins (around 20%), milk fat globule membrane proteins (typically 1–

2%), and non-protein fractions. Because of their different physicochemical properties, these proteins have different digestion behaviors under a gastric environment (Boirie et al., 1997; Wang et al., 2018; Ye, 2021).

Whey proteins mainly consist of β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) (ratio ~ 3: 1) (Visser, Slangen, & Rollema, 1991). Both of these have well-defined secondary and tertiary structures containing intermolecular disulfide bonds, and are prone to heat-induced unfolding and denaturation near 80 °C (Boland, 2011).

In most milk species, caseins comprise four gene products, i.e., α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein (Visser et al., 1991). The individual casein molecules lack secondary and tertiary structure but, together with CCP, are able to assemble into large structures called casein micelles with κ-casein located at the surface (Dalgleish & Corredig, 2012; Fox, 1993). Over the last few decades, different models have been proposed to describe the internal and external structure of casein micelles, including a submicelle model (Walstra, 1990), dual binding model (Horne, 2006) and nanocluster model (Dalgleish, 2011; de Kruif & Holt, 2003).

2.4. Interaction between whey protein and pepsin

In solution, native α-La is very susceptible to hydrolysis by pepsin (Nik, Wright, & Corredig, 2010). The rate of proteolysis of α-La increases during gastric digestion, resulting in the production of lower molecular-weight products. However, α-La became resistant to pepsin hydrolysis upon adsorption at the oil–water interface due to alterations in tertiary conformational structure (Nik et al., 2010).

β-Lg has three-dimensional structure consisting of an eight-stranded antiparallel β-hydrophobic barrel unit with an α-helix, accounting for its resistance to acidic environments and to pepsin in the gastrointestinal tract (Chobert, Briand, Grinberg, & Haertlé, 1995;

Dalgalarrondo, Dufour, Chobert, Bertrand-Harb, & Haertlé, 1995; Reddy, Kella, & Kinsella, 1988; Schmidt & Poll, 1991). The relative resistance of β-Lg to acid hydrolysis, as well as to proteases, allows a portion to remain intact after digestion, subsequently absorbed through the intestinal mucosa and presented to immunocompetent cells, thus becoming one of the main allergens in cow milk (Wal, 2001). It has been previously shown that heating at 80–90 °C induces structural changes in β-Lg that improves susceptibility to pepsin (Reddy et al., 1988). Reduction of disulfide bonds in β-Lg can also increase its susceptibility to pepsin action (Kananen et al., 2000). Furthermore, adsorption-induced changes facilitate the hydrolysis of peptides by pepsin (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009). When presented as an adsorbed layer on emulsion surfaces, the change in conformation exposes peptic hydrolysis sites, thus significantly decreasing the resistance of β-Lg to pepsin (Nik et al., 2010; Peram, Loveday, Ye, & Singh, 2013; Sarkar, Goh, Singh, & Singh, 2009).

2.5. Interaction between caseins (without micellar structure) and pepsin

It was reported that hydrolysis of individual caseins normally occurs at pH < 4 (Tam & Whitaker, 1972). α_{S1}-Casein and α_{S2}-casein are rapidly hydrolyzed by pepsin during gastric digestion (Lisson, Lochnit, & Erhardt, 2014), and no measurable amount of intact protein could be found at the end of this phase (Astwood, Leach, & Fuchs, 1996; Dupont et al., 2010; Fu, Abbott, & Hatzos, 2002). This is expected, as α_{S1}- and α_{S2}-caseins are poorly structured proteins with no defined tertiary structure, therefore very susceptible to proteases such as pepsin, trypsin and chymotrypsin (Astwood et al., 1996; Swaisgood, 2003). According to Lisson et al. (2014), after gastric digestion, almost no breakdown could be observed in region 54–89 of α_{S1}-casein, while more pepsin-generated peptides were detected in the rest of the protein sequence. For α_{S2}-casein, with the exception of regions 34–59 and 198–207 which barely provided any fragments, peptides were distributed along the whole protein sequence (Lisson et al., 2014). In addition, there are preferential hydrolysis sites for pepsin, for example, glutamic acid at position 192 in α_{S1}-casein B variant and phenylalanine in α_{S2}-casein variant B.

When β-casein from each of the variants (A1, A2, B, and I) was subjected to *in vitro* digestion using pepsin at pH 2.0, Petrat-Melin et al. (2015) observed very little intact β-casein presented by SDS-PAGE after 60 min of pepsin digestion (with little difference between different variants), in good accordance with Dupont et al. (2010). According to Jinsmaa & Yoshikawa (1999) and Haq, Kapila, &Kapila (2015), pepsin hydrolyzes the leucine^{58}-valine^{59} peptide bond of β-casein, and leucine aminopeptidase removes the valine from the N terminus. His^{67} in β-casein variant A1 residue being more permissive than the Pro^{67} in β-casein variant A2 residue toward pepsin cleavage after the Ile^{66} residue (Thiruvengadam, Venkidasamy, Thirupathi, Chung, & Subramanian, 2021). Cleavage here would create a peptide with Ile in the C-terminal position. This difference is responsible for releasing the amino terminus to form β-casomorphin-7 in β-casein variant A1 (Thiruvengadam et al., 2021).

There are nine variants of κ-casein, containing galactose, N-acetylgalactosamine, and N-acetylneuraminic acid or sialic acid, which occur as either trisaccharides or tetrasaccharides attached to threonine residues in the C-terminal region (Vreeman, Visser, Slangen, & Van Riel, 1986). In the presence of pepsin, the hydrolysis of κ-casein is highly pH dependent. Tam & Whitaker (1972) have investigated the rates and extents of hydrolysis of κ-casein by pepsin at pH 3.0, 3.5, 5.5 and 6.0. The reaction between pepsin and κ-casein at pH 6.0 differs with that at pH 3.0 (**Figure 2-1**): pepsin hydrolyzes κ-casein into para-κ-casein and CMP (caseinomacropeptide, and also referred to as glycomacropeptide or caseinoglycopeptide) (Manso & Lopez-Fandino, 2004) at pH 6.0, whereas pepsin hydrolyzes κ-casein into peptides or amino acids at pH 3.0 (Tam & Whitaker, 1972). It has been reported that at pH 6.0, pepsin had the fastest initial rates of hydrolysis on κ-casein, followed by α-casein and β-casein (Tam

& Whitaker, 1972). However, the extent of hydrolysis of κ-casein at pH 6.0 was lower than that at pH 3.0. The intact κ-casein acts as a steric stabilizing ‘hairy’ layer on the casein micelle surface, providing both steric and electrostatic repulsion between micelles. After a specific proportion of κ-casein proteins are hydrolyzed (usually around 90%) to form CMP (while other caseins are not hydrolyzed at pH 6.0), the destabilized casein micelles coagulate. This will be discussed further in **Section 2.6**.

$$\kappa\text{-casein} \xrightarrow[\text{pH 6}]{\text{pepsin}} \text{para-}\kappa\text{-casein} + \text{CMP}$$

$$\kappa\text{-casein} \xrightarrow[\text{pH 3}]{\text{pepsin}} \text{peptides/amino acids}$$

Figure 2-1 Summary of the hydrolysis of κ-casein by pepsin at pH 6 and pH 3.

2.6. Interaction between casein micelles and pepsin

The stability of the casein micelle is dependent upon the presence of κ-casein on the surface where it functions as an interface between the hydrophobic caseins of the micelle interior and the aqueous environment. Since κ-casein can be specifically hydrolyzed by pepsin at relative high pH (> 5) (Kageyama, 2002; Plowman & Creamer, 1995), with the decreases in pH during gastric digestion, several changes occur in milk protein in the gastric environment:

1) At pH > 6, hydrolysis of the Phe^{105}-Met^{106} bond in κ-casein results in removal of the protective effect of κ-casein on casein micelle (**Figure 2-2**).
2) After a certain proportion of κ-casein ‘hairs’ are clipped off as CMP, the destabilized casein micelles coagulate.
3) When pH decreases further to 4-5, the dissociation of CCP in casein micelles results in a structural rearrangement within the curd.
4) At pH < 4, subsequent proteolysis of all casein proteins leads to the release of peptides into the serum phase.

As the structured casein curd can impact upon subsequent proteolysis of proteins at low pH, the pepsin-induced hydrolysis of κ-casein (1) and coagulation of casein micelles (2) will be discussed below.

1
Pyro Glu-Glu-Gln-Asn-Gln-Glu-Gln-Pro-Ile-Arg-Cys-Glu-Lys-Asp-Glu-Arg-Phe-Phe-Ser-Asp-

21
Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-Leu-

41
Asn-Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu-Ile-Asn-Asn-Gln-Phe-Leu-Pro-Tyr-Pro-Tyr-

61
Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser-

81
Asn-Thr-Val-Pro-Ala-Lys-Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg-His-Pro-His-

101 105↓106
Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro-

121 Ile (Variant B)
Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr- -Glu-Ala-Val-Glu-
Thr (Variant A)

141 Ala (Variant B)
Ser-Thr-Val-Ala-Thr-Leu-Glu- -SerP - Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn-
Asp (Variant A)

161 169
Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val.OH

Figure 2-2 Amino acid sequence of κ-casein showing the enzyme hydrolysis site (downwards arrow) [adapted from Fox et al. (2017b), with permission by Cambridge University Press].

2.6.1. Measurement of pepsin-induced milk coagulation properties

2.6.1.1. Determination of degree of hydrolysis of κ-casein by pepsin

The hydrolysis phase of κ-casein and aggregation phase of para-casein micelles differ kinetically and are not fully separated processes over time (Fox et al., 2017a; Frederiksen et al., 2011; van Hooydonk, De Koster, & Boerrigter, 1987). Until now, there has been no simple and 'real-time' method reported in the literature for analyzing the enzymatic process. Determination of the coagulation time is one way to provide estimates of enzyme activity, but it cannot provide detailed information on the kinetics of the enzymatic reaction (Dalgleish, 1993a). To quantitatively determine the extent and rate of pepsin action on κ-casein, the most common method is to measure the extent of hydrolysis of κ-casein. This can be quantified by either measuring the fraction of κ-casein, or by analyzing the products of the reaction (the para-κ-casein or CMP).

In early studies, κ-casein was quantified by using anion exchange column chromatography or by electrophoresis (Vreeman et al., 1986; Vreeman, Both, Brinkhuis, & van Der Spek, 1977). Para-κ-casein has been determined by electrophoretic separation on cellulose acetate strips (Chaplin & Green, 1980) or on polyacrylamide gels (Dalgleish, 1979). Carlson, Hill Jr, & Olson (1987a) and Dalgleish (1979) investigated proteolysis of κ-casein by quantification of para-κ-casein stain intensities in gel-electrophoresis-separated milk samples using cellulose acetate strips as the support medium. Electrophoretic techniques, however, are laborious and not very suitable for quantitative analysis (van Hooydonk & Olieman, 1982).

The classical methods for measuring the CMP are based on the determination of soluble nitrogenous constituents in trichloroacetic acid solutions (Abd El-Salam, El-Shibiny, & Buchheim, 1996). A rapid and sensitive high-performance liquid chromatography (HPLC size exclusion chromatography) method was first established by van Hooydonk & Olieman (1982) to quantify CMP release. A reverse-phase high-performance liquid chromatography (RP-HPLC) method (López-Fandiño, Olano, San José, & Ramos, 1993; Olieman & Van Riel, 1989; Regester & Smithers, 1991; Visser et al., 1991) was employed to determine not only the formation of CMP or para-κ-casein, but also the disappearance of κ-casein (Humphrey & Newsome, 1984). Fast protein liquid chromatography (FPLC) is another option for estimation without extensive prior treatment of the samples (Dalgleish, 1986). Based on the determination of κ-casein in aqueous solutions, a new electrochemical technique - Fourier transform continuous cyclic voltammetry (FFTCCV) - was reported to be an efficient method for studying enzymatic hydrolysis processes (Shayeh, Sefidbakht, Siadat, & Niknam, 2017).

It was demonstrated by Chaplin and Green (1980) that chymosin hydrolyzes the Phe^{105}–Met^{106} bond following the Michaelis–Menten equation, although according to van Hooydonk (1987), a first-order rate equation was a better fit of the enzymatic hydrolysis data (the comparation of the fits are shown in **Figure 2-3**). However, there are limited studies investigating the kinetics of κ-casein hydrolysis catalyzed by pepsin in milk systems. Carlson et al. (1987a) briefly compared the hydrolysis of κ-casein by pepsin with that by rennet, but the effects of the concentration of pepsin and the pH on κ-casein hydrolysis have not been studied in detail. According to Carlson et al. (1987a), pepsin-induced hydrolysis of κ-casein follows a combined kinetic model of first-order hydrolysis and putative pepsin denaturation:

$$ln\left(1-\frac{H_t}{100}\right)=\frac{K_{enz}\cdot C}{K_{den}}\cdot\left[exp(-K_{den}\cdot t)-1\right]$$ (**Equation 2-1**)

which is similar to chymosin hydrolysis of κ-casein (van Hooydonk, 1987).

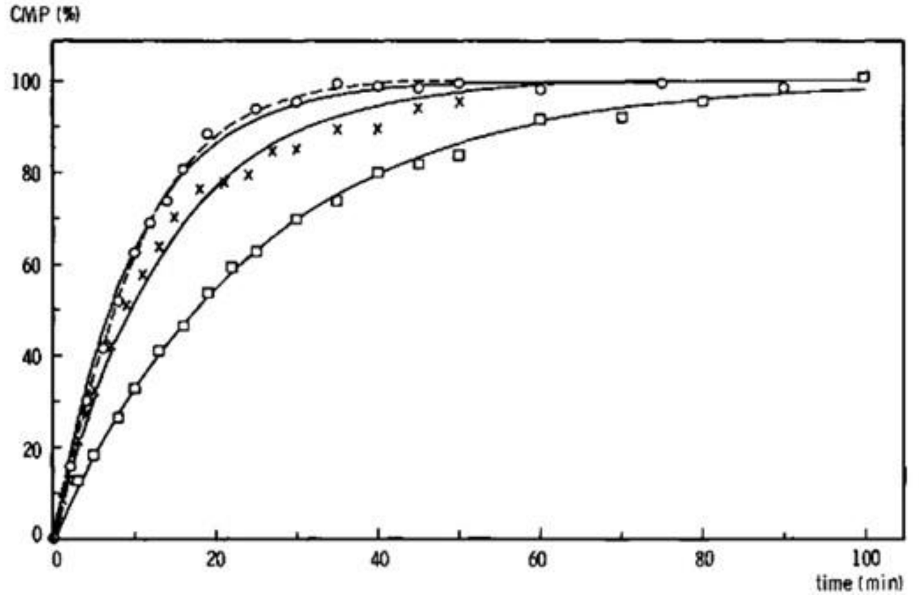

Figure 2-3 Release of CMP as a function of time after the addition of rennet at different pH values (rennet concentration 0.01%, temperature 30°C. Solid curves are first-order fits, dashed curve is a Michaelis-Menten fit [reprinted from van Hooydonk (1987), with permission by Springer Nature].

2.6.1.2. Methods of assessing milk aggregation

During aggregation, the skim milk curd is primarily formed from the casein micelles. For coagulated whole milk, fat globules are entrapped in the casein network (Fox, McSweeney, Cogan, & Guinee, 2004; Joshi, Jhala, Muthukumarappan, Acharya, & Mistry, 2004). There are several important parameters in describing the aggregation phase: coagulation time, curd firmness and curd microstructure. A considerable number of studies have determined milk coagulation properties using different types of measuring equipment, which have been summarized by Troch et al. (2017) and Lucey (2002). Coagulation can be measured by means of mechanical and/or optical devices (Beux, Pereira, Cassandro, Nogueira, & Waszczynskyj, 2017). Mechanical instruments, such as the Formagraph or its new version Formoptic, Computerized Renneting Meter and Lactodynamography have been developed (Cipolat-Gotet, Cecchinato, De Marchi, Penasa, & Bittante, 2012; Kübarsepp, Henno, Kärt, & Tupasela, 2005). In addition, a dynamic rheometer has been extensively employed to monitor mechanical properties that occur as milk proteins gel after the addition of enzymes. Some of the techniques that are commonly used to study the coagulation process are briefly discussed below.

Dynamic oscillatory low-strain rheology

Since the 1980s, several controlled strain and controlled stress rheometers have been increasingly used as research tools for the continuous measurement of the viscoelastic properties of milk as a function of time following enzyme addition. Dynamic measurements are performed by applying a low amplitude oscillating shear stress (σ) or shear strain (γ), depending upon the type of rheometer, to the milk sample. Either by using a parallel stainless steel plate geometry (Yang et al., 2023d) or a cylinder geometry (cup and bob) (Lucey, Teo, Munro, & Singh, 1997), the following rheological parameters can be obtained: storage or elastic shear modulus, G', is given by the equation: $G' = (\sigma_0 / \gamma_0) \cos \delta$; viscous or loss modulus, G'' is given by the equation: $G'' = (\sigma_0 / \gamma_0) \sin \delta$, where σ_0 is maximum stress amplitude, γ_0 is maximum shear strain amplitude, and δ is the phase angle between shear stress and shear strain oscillations. The degree of solidness or fluidity is quantified by the loss-tangent value ($\tan \delta$), equal to the inverse tangent of the ratio of G'' to G'. G'' and δ are useful parameters for monitoring the viscoelastic changes in the gel during ageing but are not directly related to gel strength. In contrast, G', is a direct measure of curd firmness. Guinee, O'Callaghan, Pudja, &O'Brien (1996) fitted the G'-time curve into a three-coefficient sigmoidal equation described by Scott-Blair model. Various rheological parameters that are used to characterize milk coagulation are listed below (with some modifications) (Fox et al., 2017a; Guinee et al., 1996; Lucey, Johnson, & Horne, 2003; Tunick, 2000):

- Coagulation time, defined as the time at which G' reaches a threshold value, G_g, arbitrarily set at 0.2 Pa or 1 Pa, or intersection of the tangent of the G' versus time curve with the time=0 axis.
- The firmness at a fixed time, e.g., 30, 60 or 120 min, G'_{30}, G'_{60}, G'_{120}.
- Maximum firmness, $G'_{\max}$.
- Maximum curd firming rate, defined as the maximum slope of the G'/time graph.

Microscopy

Microscopy allows direct visualization of the microstructure of curd resulting from pepsin-induced protein aggregation and helps to understand relationships between rheological properties and microstructural characteristics. The microstructure of casein micelles and curd formation can be observed using light microscopy (LM), confocal laser scanning microscopy (CLSM), electron microscopy such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Auty, Twomey, Guinee, & Mulvihill, 2001; Bowland & Foegeding, 2001; El-Bakry, Duggan, O’Riordan, & O’Sullivan, 2011a; El-Bakry, Duggan, O’Riordan, & O’Sullivan, 2011b; Ong, Dagastine, Kentish, & Gras, 2012, 2013a; Romeih, Moe, & Skeie, 2012). A comprehensive summary that highlights major historical developments and characterization of techniques to assess cheese and curd microstructure have been reported (Everett, 2007; Everett & Auty, 2008).The resolutions of different types of microscopy are shown in **Figure 2-4** together with the size of the constituents of milk. CLSM has been extensively used to examine the structure of milk aggregate, with a resolution of approximately 0.2 μm. The ability to optically penetrate below the surface of the sample minimizes surface-related artifacts caused by sample cutting. With an option to fluorescently label various components, proteins and fat can be distinguished, and interactions in samples can be visualized. CLSM can also assemble a three-dimensional image from two-dimensional micrographs below the sample surface using computerized image analysis (Ong et al., 2012). However, there are some potential structural artefacts that can occur during sample preparation (e.g., freezing, drying, and staining) for microscopy (Chodankar, Aswal, Kohlbrecher, Vavrin, & Wagh, 2009).

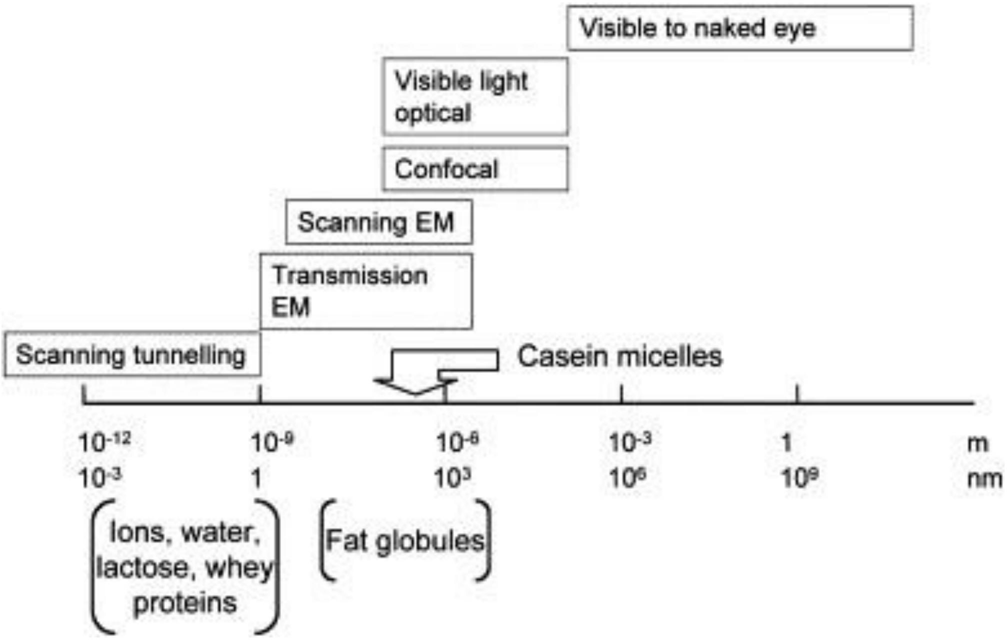

Figure 2-4 Resolution of different types of microscopies and the size of milk constituents [Reprinted from (Everett & Auty, 2008), with permission by Elsevier].

Nuclear magnetic resonance (NMR)

Non-invasive methods, such as high-resolution magic angle spinning nuclear magnetic resonance (NMR) can quantify the water and fat content, and their diffusion rate within a curd structure (Budiman, Stroshine, & Cornillon, 2002; Callaghan, Jolley, & Humphrey, 1983; Rosenberg, McCarthy, & Kauten, 1992; Ruan, Chang, Chen, Fulcher, & Bastian, 1998; Shintu & Caldarelli, 2005; Sone et al., 2022).

Diffusing-wave spectroscopy (DWS)

Diffusing-wave spectroscopy (DWS) is an optical technique derived from dynamic light scattering that measures the dynamics of scattered light in the limit of strong multiple scattering. Thus, DWS can be used to study particle motion, such as colloids, microemulsions, and other systems in concentrated fluids which are characterized by strong multiple scattering. DWS has been used for estimating mean particle size and degree of mobility in acid and rennet induced milk gelation (Alexander, Corredig, & Dalgleish, 2006; Alexander & Dalgleish, 2004; Sandra, Alexander, & Dalgleish, 2007).

Small angle scattering

Neutron and X-ray scattering methods are valuable tools for structural studies of food colloids (Gilbert, 2019). Small-angle neutron scattering (SANS), ultra small-angle neutron scattering (USANS) and small angle X-ray scattering (SAXS) have been used to elucidate the sizes and morphology of the fat globules and casein micelles in commercial milk (Adams et al., 2019; de Kruif, 2014). Besides, small-angle neutron scattering (SANS) has been shown as a powerful technique to probe the internal structure of casein micelles and CCP features in casein micelles (Bayrak et al., 2021; Yang et al., 2023d). Scattering curves can be fitted by a hierarchical structural model with features corresponding to dense protein overlap regions (a), CCP clusters (b), large heterogeneous regions of 'incompressible' protein and water channels (c) and the overall micelle size (d) as shown in **Figure 2-5** (Ingham et al., 2016).

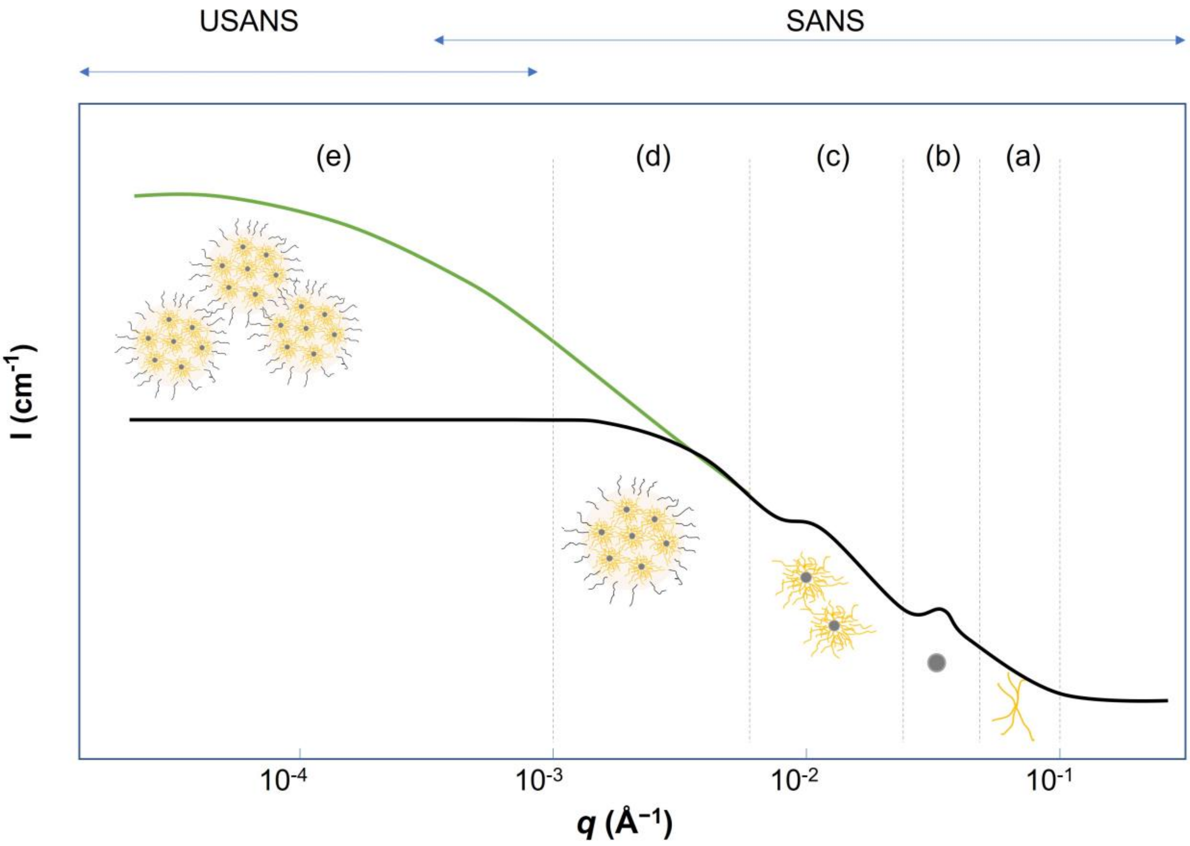

Figure 2-5 Schematic representation of the models applied to segments of a combined small angle neutron scattering (SANS) and ultra-small angle neutron scattering (USANS) curve at different size scales for milk (black curve) and milk curd (green curve) [adapted from Ingham et al. (2016), Callaghan-Patrachar, Peyronel, Pink, Marangoni, & Adams (2021) and Bayrak et al. (2021)].

The high penetrating power and non-destructive nature of neutrons make SANS and USANS robust techniques to probe protein structural evolution during aggregation *in situ* (Callaghan-Patrachar et al., 2021; Chodankar et al., 2009; Li et al., 2018; Yang et al., 2023d). USANS has been used to study rennet-induced (Callaghan-Patrachar et al., 2021) and plant protease-induced (tamarillin from tamarillo fruit) coagulation of cow milk (Li et al., 2018), as well as the GDL-induced gelation of skim cow and goat milk and infant formulae made from these types of milk (Wang et al., 2019). Once aggregation occurs, the aggregates can be observed (e) in the green curve in **Figure 2-5**. According to Yang et al. (2023d), the microstructural evolution kinetics probed by USANS correlates well with the development of gel strength (*G′*) determined by rheological measurements. Pasquier et al. (2019) reported the first small-angle neutron scattering (SANS) study to monitor structural evolution of different vegetable (cruciferin and napin) protein gels during simulated *in vitro* gastric and intestinal digestion. Using SANS and USANS, Bayrak et al. (2021) and Bayrak et al., (2023) investigated the structural changes of casein gels during simulated gastric digestion with/without pepsin in real time.

2.6.2. Factors influencing pepsin-induced milk coagulation

In most *in vivo* or *in vitro* studies, the pepsin to protein ratios are typically far in excess (pepsin secretion in adults is estimated between 20 and 30 kUnits of enzyme activity/24 h at 37 °C) (Moreno, 2007). The concentration of pepsin steadily increases with the secretion of simulated gastric fluid, reaching up to 2000 U/mL for *in vitro* gastric environment simulation (Brodkorb et al., 2019) In the meantime, pH decreases to different extents, depending upon the buffering capacity of ingested food. The temperature of a human gastric environment is around 37 °C at a fasting state, but it could change depending upon the temperature of ingested food (Sun, Houghton, Read, Grundy, & Johnson, 1988). Under these dynamic changes in the gastric environment, the hydrolysis of κ-casein by pepsin is too rapid to be determined [particularly due to the rapid pH decrease to < 4, which accelerates the general hydrolysis of all proteins (Tam & Whitaker, 1972; Ye, 2021)]. Therefore, to investigate the kinetics of the hydrolysis of κ-casein and the consequent coagulation of milk proteins, it is necessary to use low pepsin concentrations [< 23.71 (U/mg)/100 mL milk] under controlled pH and temperatures (Roy et al., 2020b).

Pepsin concentrations

According to Carlson et al. (1987a), at a fixed pH (> 5), the rate of the hydrolysis of κ-casein is positively related to the pepsin concentration. This is expected as pepsin hydrolysis of κ-casein follows a pseudo steady state Michaelis-Menten kinetics model and first-order deactivation of the enzyme (Carlson et al., 1987a). There are few studies investigating the effect of pepsin concentration on the coagulation of milk proteins, whereas the effect of chymosin or rennet (a complex mixture containing chymosin and pepsin) concentration on milk coagulation has been extensively reported. An inverse dependence of the coagulation time of milk on the concentration of enzyme (either pepsin or chymosin) has been reported (Carlson et al., 1987a; Dalgleish, 1993a; Foltmann, 1959; Hyslop, Richardson, & Ryan, 1979; Lopez, Lomholt, & Qvist, 1998). At fixed pH values (> 5), the degree of hydrolysis of κ-casein that is required for milk protein coagulation is independent of the rennet concentration. However, results from several studies differ in relation to the effect of enzyme level on the curd firming rate and curd firmness, with some studies showing increases in the latter parameters and others showing no effect or slight decreases (Fox et al., 2017a). The amount of enzyme retained in the curd is proportional to the amount of enzyme added to the milk (at least for calf rennet) and this has a major effect on the rate of proteolysis at longer reaction times (Fox et al., 2017a).

pH

Milk typically has a pH of ~ 6.7 and will vary by the action of the starter culture, pre-ripening of the milk, addition of acidulants (Ong et al., 2012) or the presence of gastric juice. The pH of milk affects both the pepsin activity and the physicochemical properties of milk (Carlson et al., 1987a; Ong et al., 2012; van Hooydonk, 1987; Zoon, 1988). The effect of milk pH on the properties of enzyme (chymosin and pepsin)-induced coagulation has been reported by various groups (Carlson et al., 1987a; Daviau, Famelart, Pierre, Goudédranche,

& Maubois, 2000; Esteves, Lucey, Wang, & Pires, 2003; Mishra, Govindasamy-Lucey, & Lucey, 2005; Ong et al., 2012; van Hooydonk, Hagedoorn, & Boerrigter, 1986; Zoon, 1988).

At a certain pepsin concentration, it has been reported that the optimal pH for chymosin-induced hydrolysis of κ-casein is pH 6.0 (van Hooydonk, 1987). However, the sensitivity of hydrolysis of κ-casein to pH depends upon the enzyme used. It has been reported that the porcine pepsin is particularly sensitive to pH changes (Fox et al., 2017a), but limited information has been published. The coagulation time and the degree of hydrolysis required for coagulation to occur decreases markedly with a decrease in pH as was observed in chymosin-induced milk coagulation (Carlson, Hill Jr, & Olson, 1987b, 1987d; van Hooydonk, 1987). This could be due to partial charge neutralization of the negatively charged para-casein micelles and an increase in the concentration and activity of calcium ions in the milk serum at lower pH (Choi, Horne, & Lucey, 2007; van Hooydonk, 1987; Zoon, 1988). Milk curd is more prone to microsyneresis at lower pH (comparing pH 5.3 and 6.65) (Mellema, Walstra, van Opheusden, & van Vliet, 2002), with large pores, observed by cryo-SEM. However, information about the effect of pH on milk coagulation behavior during gastric digestion is still limited, i.e., milk at a different initial pH may be digested differently under *in vitro* gastric conditions.

Temperature

The temperature coefficient (Q_{10}), has been used as a measure of the temperature dependence of chymosin-induced hydrolysis of κ-casein and the coagulation of para-κ-casein (van Hooydonk, 1987). It has been reported that chymosin-induced hydrolysis of κ-casein occurs, even at 0 °C, and the reaction rate increased when temperature was increased from 7 to 37 °C, with a Q_{10} at ~ 2.0. A greater effect of temperature has been found in the aggregation phase, with a Q_{10} of ~ 16 (Fox et al., 2017a). However, aggregation occurs very slowly, or not at all, at temperatures < 15 °C (Fox et al., 2017a). In another study, it was reported that the rate of curd formation increases progressively from 20 to 42 °C but slows down at much higher temperatures (> 42 °C) because of denaturation of chymosin (Nájera et al., 2003). Panthi et al. (2019). Ong, Dagastine, Auty, Kentish, &Gras (2011a) reported that temperature had a significant effect on milk curd microstructure. The optimum temperature for coagulation of milk by calf rennet at pH 6.6 is 45–48 °C but differs when using other proteinases (chymosin, *Rhizormucor pusillus* and *Rhizomucor miehei* proteinases) (Fox et al., 2017a). However, the effect of temperature on pepsin-induced hydrolysis of κ-casein and coagulation has not been fully explored. As temperature can impact upon pepsin activity (Zhao et al., 2011) and milk coagulation behavior (Dalgleish, 1983; Horne & Lucey, 2014; Nájera et al., 2003), milk temperature could have a profound influence on the structural organization of the pepsin-induced curd.

Concentration of Ca^{2+}

To improve the nutritional and functional properties of milk, enrichment with calcium salts has been a common practice (Gastaldi et al., 1994; Ramasubramanian et al., 2014; Vyas & Tong, 2004). The addition of $CaCl_2$ naturally contributes to an increase in the calcium content in all forms and is accompanied by a decrease in milk pH (Bringe & Kinsella, 1986). The reported effects of $CaCl_2$ addition on the enzymatic hydrolysis of casein micelles are contradictory. According to Bauland et al. (2020) and Holt (1997), the kinetics of κ-casein hydrolysis is negatively correlated with the concentration of Ca^{2+} in the soluble phase of milk at pH 6.6. Bringe & Kinsella (1986) found that the hydrolysis rate increased with the addition of $CaCl_2$ up to 8 m*M* and then decreased with a further addition of $CaCl_2$ at pH 6.7. Several studies concluded that neither dialysis nor the addition of calcium salts directly affected the hydrolysis of κ-casein at controlled pH where the pH was adjusted to the same level (> 6) before the addition of rennet (Sandra & Corredig, 2013; Sandra, Ho, Alexander, & Corredig, 2012; Walstra & Jenness, 1984a).

It was reported that an increase in ionic calcium concentration ([Ca^{2+}]) promoted coagulation (Bringe & Kinsella, 1986) by decreasing repulsion between para-casein micelles and promoting the formation of calcium bridges between casein micelles (Dalgleish, 1983; Sandra et al., 2012; van Hooydonk, 1987). This is concomitant with a decrease in coagulation time (Hardy & Fanni, 1981; Kowalchyk & Olson, 1978; Marshall & Green, 1980) and an increase in firming rate (Bringe & Kinsella, 1986), final curd firmness, and yield stress (Gastaldi et al., 1994). Several *in vitro* gastric digestion studies reported the effect of calcium concentration on the coagulation behavior of milk under gastric conditions: milk with a higher micellar calcium phosphate content showed a faster rate of coagulation and firmer curd (Huppertz & Lambers, 2020); whereas casein micelles with partial decalcification formed softer gastric clots (Wang et al., 2023; Zhang et al., 2023).

Whey proteins

The interaction between whey proteins and pepsin was discussed in Section **2.4**. In cow milk, the ratio of casein:whey protein is around 4:1 (Haug et al., 2007). Casein micelles are the main proteins associated with enzyme-induced milk coagulation; few studies have reported on the effect of whey proteins on this process. According to de Koning, Boer, Both, & Nooy (1981); van den Berg (1979), whey proteins appear to largely act as an inert filler and disturb the formation of the milk coagulum. Gamlath, Leong, Ashokkumar, & Martin (2018) stated that whey proteins present a physical barrier to para-casein aggregation, resulting in an increased coagulation time and decreased firming rate. These authors also reported that the enzymatic action of chymosin can be inhibited by the presence of whey proteins at fixed casein concentrations. Although the overall mechanisms of pepsin-induced casein coagulation are similar to chymosin-induced coagulation, there are limited studies that have investigated the effect of whey proteins on pepsin-induced κ-casein hydrolysis and the subsequent coagulation process.

Heat treatment

Heat treatment is an essential step in the processing of milk, which is typically applied for enhancing the desirable properties of dairy products, such as taste and texture, or to extend shelf-life and ensure safety (Chavan, Chavan, Khedkar, & Jana, 2011). α-La reversibly denatures at a lower temperature (63 °C) than β-Lg (73 °C) (McMahon, Yousif, & Kaláb, 1993; Rüegg, Moor, & Blanc, 1977). The major portion of the irreversibly denatured protein is β-Lg (Dannenberg & Kessler, 1988; Havea, Singh, Creamer, & Campanella, 1998; Parris, Anema, Singh, & Creamer, 1993; Patel, Singh, Anema, & Creamer, 2006). β-Lg aggregates with κ-casein through thiol/disulfide interchange, hydrophobic interactions, electrostatic and ionic interactions, leading to the formation of whey protein/κ-casein complexes (Guyomarc'h, Law, & Dalgleish, 2003; Jang & Swaisgood, 1990; Singh & Creamer, 1991). Overall, at a constant time of heat treatment (10, 15 or 30 min), increasing the temperature in the range 70–95 °C increases the extent of denaturation of whey proteins (Dannenberg & Kessler, 1988).

Some previous studies suggest that whey protein/κ-casein complexes inhibit the enzymatic action of chymosin on κ-casein (Guyomarc'h et al., 2003; Jang & Swaisgood, 1990). However according to Vasbinder, Rollema, & de Kruif (2003) and Anema, Lee, & Klostermeyer (2007), heat treatment of milk has little effect on the hydrolysis reaction, regardless of whether the whey proteins are associated with the casein micelles or in the serum as non-sedimentable aggregates. The controversial findings from these studies could be caused by the use of different methods, or the measurement of different reaction products, i.e., para-κ-casein or CMP. Association of denatured whey proteins on the surface of casein micelles upon heating has a more predominant effect on the secondary (aggregation) phase of milk coagulation. It is generally accepted that due to calcium precipitation and/or the steric hindrance of potentially aggregating para-casein micelles by whey protein/κ-casein complexes, the aggregation of skim milk is retarded by heat treatment (Dalgleish, 1993a; Lucey, 1995; Vasbinder et al., 2003). However, some studies report that the impact of heat treatment on milk coagulation can vary depending upon the pH conditions: after heat treatment, the pH-unadjusted sample (pH 6.6) failed to form a firm coagulum, whereas acidified samples (pH 6.4) formed curd with more total nitrogen compared to the unheated sample. (Reddy & Kinsella, 1990; Singh, Shalabi, Fox, Flynn, & Barry, 1988). Several dynamic *in vitro* and *in vivo* gastric digestion studies have demonstrated that heat treatments significantly influence the gastric coagulation and casein micelle emptying in milk. It has been reported that the denaturation of whey proteins and their association with casein micelles (e.g., UHT and 90°C for 20 minutes) result in the formation of more fragmented clots, speeding up gastric emptying and casein hydrolysis (Mulet-Cabero et al., 2019; Ye et al., 2016a, 2016b, 2017, 2019b). Additionally, pasteurization and UHT treatment have been observed to soften the consistency of the gastric clot (Mulet-Cabero et al., 2019; Roy, Ye, Moughan, & Singh, 2021; Ye et al., 2019).

Fat

Fresh whole milk contains fat globules with a size from approximately 0.1 to 10 μm (Mulder & Walstra, 1974). There is little information available on the presence of fat globules on enzyme-induced hydrolysis of κ-casein. Native fat globules are known to weakly interact with casein protein structure (Michalski, Cariou, Michel, & Garnier, 2002). The ratio of fat globules to casein micelles is one of the main factors influencing the curd firmness of whole milk (Walstra, Walstra, Wouters, & Geurts, 2005). It has been suggested that the presence of fat globules may weaken the structure of chymosin-induced milk protein clots by interrupting the matrix and creating 'soft spots' (Walstra & Jenness, 1984b). According to Guinee et al. (1997), increasing fat levels in the range 0.1–10 % (w/w) at a constant protein content (3.3 % (w/w)) leads to reductions in gelation time and higher values of maximum curd firming rate and final curd firmness.

Homogenization

Two-stage high pressure homogenization is commonly used to reduce the size of fat globules in milk, in which the first stage reduces the size of fat globules and the secondary stage disrupts clusters that may be formed (Guinee et al., 1997). After the homogenization process, milk fat globules are broken into smaller lipid droplets, greatly slowing down the rate of cream separation, thereby increasing stability and shelf life of milk. During homogenization, casein micelles are attached to the surface of fat globules and act as stabilizing agent for the increased lipid-water interfacial area (Dalgleish, 1993a). The change in the distribution of κ-casein and the structures of the adsorbed casein micelles may induce changes in milk protein interactions with pepsin.

It was reported that the pepsin hydrolysis reaction on κ-casein was faster in homogenized milk than in untreated skim milk and whole milk (Borad, Kumar, & Singh, 2017; Guerzoni et al., 1999; Humbert, Driou, Guerin, & Alais, 1980). One possible explanation could be the formation of casein micelles layers at the fat globule/water interface after homogenization increases the ‘effective’ surface area of casein micelles in milk. This would increase the susceptibility of κ-casein to enzymatic hydrolysis (Kelly, Huppertz, & Sheehan, 2008).

Homogenization decreases the size of milk fat globules and adsorbs more milk proteins at the surface of the globules, therefore the contribution by fat globules to the network of milk clots is expected to be different (Guinee et al., 1997; Lopez & Dufour, 2001; Michalski et al., 2002; Robson & Dalgleish, 1984). Micelles in homogenized milk aggregate faster than in skim milk as a consequence of the higher hydrolysis rate of κ-casein (Humbert et al., 1980). According to Ong, Dagastine, Kentish, & Gras (2010), homogenized fat globules act as "active fillers" in the milk gel, i.e., they interact with the casein network and participate in the rennet-induced coagulum structure. In contrast, native fat globule membranes from unhomogenized milk are not able to interact with casein micelles during coagulation and are mostly entrapped within serum part of the milk coagulum. In both *in vitro* or *in vivo* digestion studies, homogenization has been shown to lead to softer and more fragile curds (Anthony & Scholtes, 1936; Doan & Dizikes, 1942; Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019; Ye et al., 2017). A similar effect of homogenization on the structure of milk gel was also observed for curds formed during cheesemaking. Cheese curds formed from homogenized milk exhibit less syneresis and are more brittle compared to those formed from unhomogenized milk (Kelly et al., 2008). This was attributed by Kelly et al. (2008) to the larger number of homogenously distributed fat globules in curds leading to a smaller mean free distance between casein micelle particles. The presence of these fat globules at much smaller length scales hinders the fusion and syneresis of the casein matrix over larger length scales, thus leading to increased moisture retention and more brittle curds (Green, Marshall, & Glover, 1983).

Milk species

Milk from different species vary in composition, structure, and physicochemical properties, which result in different coagulation and digestion behavior. It has been reported that milk from different species also responds differently to the action of pepsin (Roy et al., 2020b), GDL (Yang et al., 2023d), and a combination of both pepsin and GDL (Roy et al., 2020b). The Phe^{105} and Met^{106} peptide bond is the most chymosin-sensitive bond of κ-casein in cow milk, whereas this bond is not intrinsically essential for chymosin action in other milk species. There are numerous Phe and a substantial number of Met residues in all milk proteins. In porcine and human κ-caseins, the chymosin-sensitive bond is Phe-Ile, whereas in rat and mouse κ-caseins, it is Phe-Leu; yet, these proteins are readily hydrolyzed by calf chymosin, although more slowly than cow κ-casein (Fox et al., 2017a). In contrast, porcine milk is coagulated faster than bovine milk by porcine chymosin, indicating that unidentified subtle structural features influence chymosin action (Fox et al., 2017a). The amino acid sequence comparison of the chymosin-sensitive region of κ-caseins from cow, buffalo, goat, camel and pig is shown in **Figure 2-6**.

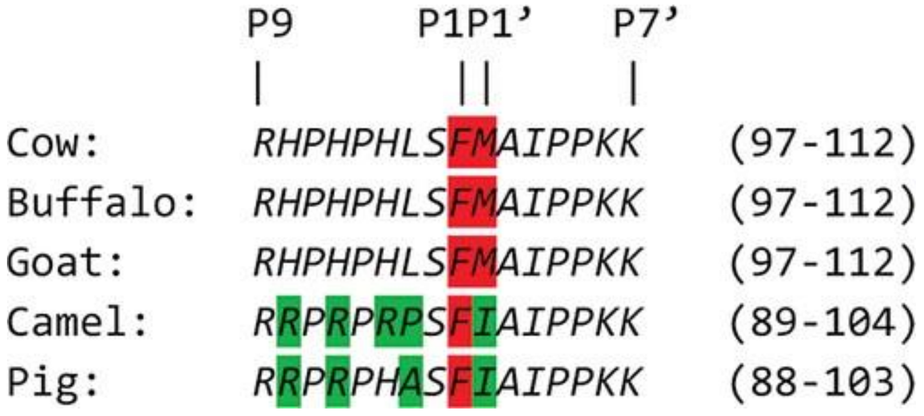

Figure 2-6 The amino acid sequences of cow, buffalo, goat, camel, and pig κ-casein near the Phe^{105}-Met^{106} hydrolysis site. The residues on either side of the scissile bond are highlighted in red. Residues in camel and pig κ-casein that differ from the related residues in cow, buffalo, and goat κ-caseins are highlighted in green [reprinted from Palmer et al. (2010), with permission from American Chemical Society].

A range of factors, such as casein micelle size, CCP content, proportions of different caseins and whey proteins, mineral content, genetic variants, and interactive effects among these are all responsible for the different coagulation behavior observed in different types of milk (Roy et al., 2020b). Goat, sheep, and cow milk are similar in casein micelle structure, but differ in composition, size, extent of hydration and mineralization (Selvaggi & Tufarelli, 2012). Compared to cow milk, a shorter coagulation time and weaker curd consistency of goat milk during chymosin-induced coagulation was observed, which was explained by the lower casein content, lower α_{s1}-casein content and larger casein micelle size (Park, Juárez, Ramos, & Haenlein, 2007; Remeuf & Lenoir, 1986). With a higher β/α_s-casein ratio, sheep milk is very sensitive to chymosin and tends to coagulate faster than cow or goat milk (Muir, Horne, Law, & Sweetsur, 1993; Park, 2007). In addition, sheep milk generally produces firmer curds and has a slower rate of syneresis due to its higher casein and colloidal calcium contents (Muir et al., 1993; Storry & Ford, 1982). During *in vivo* and *in vitro* gastric digestion, regardless of dairy species (i.e., cow, goat and sheep), milk coagulates and separates into curd and liquid phases (Roy et al., 2022; Roy et al., 2021). However, the coagulation and digestive behavior were different among these milk species, e.g., the firmness of the sheep skim milk curd was higher than those of the cow and goat milk curds at the end of gastric digestion (Li et al., 2022b; Roy et al., 2022).

2.7. Interactions between milk protein curd and pepsin

Further hydrolysis of proteins in the curds highly depends upon curd structure and the coagulation behavior of casein micelles during gastric digestion (Ye, 2021). It has been reported that, in general, protein hydrolysis is much slower in a dense, structured clot than in a loose clot with larger voids. A soft curd forms from heated milk (72 °C for 15 s or 140 °C for 4 s), thus, casein proteins were hydrolyzed faster than in a hard clot formed from unheated milk (Miranda & Pelissier, 1983; Ye et al., 2016b, 2017; Ye et al., 2019b). High-pressure processing (HPP) results in a looser and more fragmented clot structure in milk, leading to faster hydrolysis of the caseins (He, Yang, Yuan, Singh, & Ye, 2022). Pepsin-induced hydrolysis of milk curds also depends upon milk species. Goat milk-based infant formula forms smaller protein aggregates, leading to faster hydrolysis of proteins compared with those from a cow milk formula (Maathuis, Havenaar, He, & Bellmann, 2017; Ye, Cui, Carpenter, Prosser, & Singh, 2019a).

The structured curd formed during milk digestion, and the matrix and type of curd structure before ingestion also affects the digestion of milk proteins. It has been reported that by varying the ionic strength, a ‘hard’ whey-protein gel structure slows down disintegration of the protein matrix, whereas a ‘soft’ whey-protein gel structure can be totally broken down after 4 h of digestion (Guo, Ye, Lad, Dalgleish, & Singh, 2014; Luo, Ye, Wolber, & Singh, 2021). Qazi, Ye, Acevedo-Fani, & Singh (2021) reported a faster gastric emptying of peptides produced from an acid-induced (1.6% w/w GDL) curd, whereas the restructuring of the rennet-induced curd during gastric digestion reduced the flux of proteins and peptides into the digesta.

2.7.1. Methods for measuring the general proteolysis of milk proteins

Previous studies on protein digestion fall into four categories: *in vivo* human studies, *in vivo* animal studies, static *in vitro* studies, and (semi)-dynamic *in vitro* studies (van Lieshout, Lambers, Bragt, & Hettinga, 2019). Over the last 40 years, a number of *in vitro* digestion models ranging from single static systems to multi-compartmental dynamic systems have been developed and widely used as an efficient tool to study food digestion (Li, Yu, Wu, & Chen, 2020). Digestion models from different research groups include the INFOGEST static model (Brodkorb et al., 2019), dynamic gastric model (DGM) (Mercuri, Lo Curto, Wickham, Craig, & Barker, 2008), and the human gastric simulator (HGS) (Kong & Singh, 2010).

The unique coagulation behavior of milk proteins under digestive conditions has attracted extensive attention. During digestion, multiple aspects are elucidated such as: protein digestibility, fat digestibility or nutrient interactions. *In vitro* experiments provide the opportunity to characterize various properties of milk curds during digestion, e.g., the weight, texture, microstructure of the curd It has been reported that coagulum weight (both wet weight and dry matter) decreases with progressive digestion, but that wet weight decreases more rapidly (Ye, Cui, Dalgleish, & Singh, 2016a; Ye et al., 2016b). Microscopically, the coagulum network appears to become more dense and the coalescence of fat globules was observed with an increase in digestion time (Ye et al., 2016a, 2016b). Textural and rheological properties of the clots change with digestion time as well (Li et al., 2022b; Roy et al., 2021).

One important parameter to evaluate digestion behavior is the degree of hydrolysis (DH), which is defined as the proportion of hydrolyzed peptide bonds in a protein hydrolysate. The most commonly used methods for determining DH includes the pH-stat, trinitrobenzenesulfonic acid (TNBS), o-phthalaldehyde (OPA), trichloroacetic acid soluble nitrogen (SN-TCA), and formol titration methods (Rutherfurd, 2010). SDS-PAGE and Kjeldahl methods have been applied to determine the protein/peptide/amino acid/nitrogen distribution, and the rate of protein hydrolysis can thereby be determined (Wang et al., 2022; Ye et al., 2016a, 2016b). Liquid chromatography with tandem mass spectrometry (LC-MS-MS) could also be useful to determine the protein molecular weight, analyze peptides and protein modification sites, and identify proteins (Vogeser & Parhofer, 2007).

2.8. Conclusions

Previous research on gastric digestion of milk has been mostly carried out in an *in vitro* dynamic human gastric simulator or *in vivo* in a rat or pig stomach. It has been shown that during gastric digestion, milk separates into curd and liquid phases. To elucidate the distinct coagulation behavior and curd properties, many studies have referenced enzyme-induced milk coagulation (mostly chymosin or rennet). Enzyme-induced coagulation involves two phases: the highly specific hydrolysis of κ-casein by an enzyme and the subsequent aggregation of the para-casein micelles. The effects of many variables, such as temperature, pH, concentration of divalent cations, ionic strength, casein concentration, and milk species have been summarized and discussed in relation to the enzymatic reaction kinetics and the aggregation processes. Although pepsin has a high similarity with chymosin in enzymatic action, the gastric environment differs from that used in cheese production. There is a gap and inconsistency in the literature on the mechanisms of enzymatic and aggregation reaction kinetics between milk proteins and pepsin. As the main digestive enzyme in stomach, the proteolytic activity of pepsin determines the gastric emptying rate of milk proteins. Various characterization techniques, including microscopy and rheology, have been extensively utilized for studying the milk coagulation processes. Small angle scattering techniques such as SAXS, SANS, and USANS have the ability to monitor milk protein structural evolution during digestion *in situ*, from which the mechanisms of pepsin-induced coagulation and curd degradation are expected to be resolved. These results can be applied to not only predict the digestion behavior of milk in the gastric environment, but also to design dairy products with specific digestion requirements.

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In which chapter is the manuscript/published work?	Chapter 3

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Mengxiao Yang: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft.
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Chapter 3 Kinetics of pepsin-induced hydrolysis and the coagulation of milk protein[2]

Abstract

Hydrolysis-induced coagulation of casein micelles by pepsin occurs during the digestion of milk. In this study, the effect of pH (6.7–5.3) and pepsin concentration (0.110–2.75 U/mL) on the hydrolysis of κ-casein and the coagulation of the casein micelles in bovine skim milk was investigated at 37 °C using reverse phase high performance liquid chromatography (RP-HPLC), oscillatory rheology, and confocal laser scanning microscopy (CLSM). The hydrolysis of κ-casein followed a combined kinetic model of first-order hydrolysis and putative pepsin denaturation. The hydrolysis rate increased with increasing pepsin concentration at a given pH, was pH-dependent, and reached a maximum at pH ~ 6.0. Both the increase in pepsin concentration and decrease in pH resulted in a shorter coagulation time. The extent of κ-casein hydrolysis required for coagulation was independent of the pepsin concentration at a given pH and, because of the lower electrostatic repulsion between para-casein micelles at lower pH, decreased markedly from ~ 73% to ~ 33% when pH decreased from 6.3 to 5.3. In addition, the rheological properties and the microstructures of the coagulum were markedly affected by the pH and the pepsin concentration. The knowledge obtained from this study provides further understanding on the mechanism of milk coagulation, occurring at the initial stage of transiting into gastric conditions that is high pH and low pepsin concentration.

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3.1. Introduction

Milk is an excellent source of nutrients for humans, providing proteins, fats, vitamins, and minerals (Haug et al., 2007). When milk transits into the gastric environment, the gastric juice secretion is promoted, leading to the gradual decrease in milk pH from 6.7 to ~ 2.0 and the gradual increase in pepsin concentration from 0 to ~ 2000 U/mL (Gao, Mitsui, Fujiki, Ishiguro, & Kondo, 2002; Minekus et al., 2014; Ye et al., 2016a, 2017). Both in vitro and in vivo studies on the digestion of milk have found that the milk proteins coagulate at early stage during gastric digestion, even though the pepsin concentration is low (< 5 U/mL) and at pH > 6. As the coagulation occurs when the milk pH is still > 6, which is far from the isoelectric point of casein proteins (pI 4.6), it is generally believed that this is induced by pepsin hydrolysis of the casein micelles (Boirie et al., 1997; Huppertz & Chia, 2021; Ye et al., 2016a). The coagulum plays an important role in determining the gastric emptying rate, the release of nutrients, and the composition of the digesta emptied into the small intestine (Miranda & Pelissier, 1981; van Lieshout et al., 2019; Ye et al., 2017).

It is well known that κ-casein on the surface of the casein micelles provides steric and electrostatic stabilization (Payens, 1979). The action of pepsin on the casein micelles involves three distinct phases (Huppertz & Chia, 2021): (1) specific hydrolysis of the Phe^{105}–Met^{106} bond of κ-casein by pepsin at pH > 5 yielding the CMP and para-κ-casein; (2) aggregation of casein micelles by hydrophobic association together with ionic electrostatic effects (Horne & Lucey, 2014), which occurs when a minimum amount of the caseinomacropeptide has been removed (a ‘critical’ degree of hydrolysis); (3) progressive proteolysis where both caseins and whey proteins are hydrolyzed into peptides at pH < 4 (Ye, 2021).

As the first two overlapping phases broadly resemble the mechanisms occurring in cheese manufacture; the coagulation of milk induced by pepsin could be similar to that induced by chymosin. Over the years, chymosin-initiated hydrolysis of κ-casein and the aggregation of the casein micelles in milk have been extensively studied (Dalgleish, 1980; van Hooydonk, 1987). Chaplin & Green (1980) demonstrated that chymosin hydrolyzes the Phe^{105}–Met^{106} bond following the Michaelis–Menten equation, although, according to van Hooydonk (1987)and Jensen et al. (2015), a first-order rate equation describing enzymatic hydrolysis provides a better fit to the data. However, the kinetics of κ-casein hydrolysis catalyzed by pepsin in milk systems have not been fully elucidated. Carlson et al. (1987a) briefly reported on hydrolysis of κ-casein by pepsin with that by rennet, but the effects of the concentration of pepsin and the pH on κ-casein hydrolysis have not been studied in detail.

After a minimum amount of κ-casein has been hydrolyzed, the casein micelles begin to aggregate (Dalgleish, 1979) leading to changes in the physicochemical properties of the milk which can be monitored using various techniques (Everett & Auty, 2008), such as confocal laser scanning microscopy (Auty et al., 2001), oscillatory rheology (Lucey et al., 2003), and diffusing wave spectroscopy (Sandra et al., 2007). Under in vitro gastric digestion conditions, it was reported that the coagulation of milk occurs at pH > 6.2, and a firm milk clot with a smooth surface is formed (Ye et al., 2017). In a recent study, the evolution of storage modulus (G') was monitored to investigate milk coagulation induced by glucono-δ-lactone (GDL, employed to obtain a gradual decrease in pH) and porcine pepsin (Roy et al., 2020b). These researchers found a decrease in the coagulation time (from 74.4 to 27.6 min) and an increase in the coagulation pH (from 4.7 to 5.2) when the pepsin concentration increased from 5.03 to 23.71 (U/mg)/100 mL in milk. However, the rheological properties and the structural characteristics of the milk coagulum that is solely formed by pepsin-induced coagulation have not yet been investigated.

Therefore, to determine the relationship between hydrolysis and coagulation, this study examined the effects of the concentration of pepsin (0.110–2.75 U/mL milk) and pH (5.3–6.7) on protein hydrolysis and the aggregation characteristics of skim milk. The amount of para-κ-casein released by hydrolysis was determined by RP-HPLC, and the rheological properties and microstructural characteristics of coagulum were examined using oscillatory rheology and CLSM, respectively. Using the above approach, an enhanced understanding was gained of the action of pepsin on κ-casein in fresh bovine skim milk.

3.2. Materials and methods

3.2.1. Materials

Fresh bovine whole milk was obtained from Dairy Farm 4 (Massey University, Palmerston North, New Zealand) and was skimmed by centrifugation with a swing bucket rotor (Thermo Fisher Scientific Multifuge Heraeus 3SR+ centrifuge, Thermo Electron LED GmbH, Langenselbold, Germany) at 3000 *g* and 4 °C for 15 min. The skimmed milk contains 0.12% fat, 4.31% protein and 4.67% lactose as determined by a Milkoscan FT120 (Foss Electric, Hillerød, Denmark). Sodium azide was added at a concentration of 0.02% (wt/vol) to act as a bacteriostatic agent. The pH of the skim milk samples was adjusted to 6.7, 6.3, 6.0, 5.7, or 5.3 at 20 °C by the gradual addition of 1 *M* HCl under vigorous stirring conditions. The samples were stored at 4 °C, and small pH adjustments were made to achieve the desired pH value, as required, before further experiments.

Pepsin (EC 3.4.23.1) from porcine gastric mucosa with an enzymatic activity of 550 U/mg protein was obtained from Sigma-Aldrich (St. Louis, MO). Porcine pepsin (50 mg) was first dissolved in Milli-Q water (1 mL; 27,500 U/mL) and was then further diluted in Milli-Q water to obtain five pepsin concentrations: 2750, 1100, 550, 275, and 110 U/mL. The diluted pepsin solutions were added to the skim milk samples at a ratio of 1 µL per 1 mL of milk and resulted in final pepsin concentrations of 2.75, 1.10, 0.550, 0.275, and 0.110 U/mL milk. Hydrolysis and aggregation occurred within 2 h within this range of pepsin concentrations.

All other chemicals (analytical grade) were also obtained from Sigma-Aldrich unless otherwise specified.

3.2.2. Measurement of κ-casein hydrolysis

RP-HPLC was used to quantify para-κ-casein. A 2 µL aliquot of diluted pepsin solution was added into the skim milk samples (2 mL), which was then immediately transferred into 10 different test tubes, with 0.2 mL per tube, in a water bath at 37°C. HPLC buffer solution (0.8 mL: 6 *M* guanidinium hydrochloride, 0.1 *M* bis-Tris, 19.5 m*M* dl-dithiothreitol, and 5.37 m*M* sodium citrate, pH 7) was subsequently added into each tube at different time points (1, 2, 5, 10, 20, 30, 50, 70, 90, and 120 min) to stop the pepsin reaction. Each sample was shaken for 10 s, incubated for 1 h at room temperature and centrifuged before HPLC injection.

RP-HPLC analysis, based on previous methods established by Bonfatti, Giantin, Rostellato, Dacasto, & Carnier (2013) and Day, Williams, Otter, & Augustin (2015) with some modifications, was carried out using a Nexera-X2 ultra-HPLC instrument equipped with an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). Separation was carried out using a Phenomenex Aeris WIDEPORE XB-C18 column (100 mm × 4.6 mm, 3.6 μm particles). The column temperature was maintained at 45°C and the detection wavelength was 214 nm. Chromatographic runs were carried out with an injection volume of 10 μL at a flow rate of 0.8 mL/min, with (A) 0.1% (vol/vol) trifluoroacetic acid, and (B) acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid as solvents. The following solvent gradients were then applied: 0–2.5 min, isocratic conditions, 10% B; 2.5–22 min, 10–49% B; 22–23 min, 49–10% B; 23–30 min, isocratic conditions, 10% B. The amount of para-κ-casein was quantified using LabSolutions Main (Shimadzu Corporation, Kyoto, Japan) software based on peak areas.

3.2.3. Measurement of protein coagulation

The coagulation process was carried out in a stress-controlled rheometer (MCR301 Anton Paar, Graz, Austria) equipped with Couette geometry (Anton Paar; CC27, with 28.93 mm cup diameter and 26.64 mm bob diameter). Milk samples (17 mL) were equilibrated at 37°C for 15 min, then pepsin solution (17 µL) was added into the samples which were stirred for 30 s before being loaded into the rheometer. A time sweep measurement was carried out at a constant frequency of 0.1 Hz with a strain of 1% (within the linear viscoelastic region as determined by strain sweep measurements) and the storage modulus (G') was recorded at 37°C for 120 min. The pH measurements started simultaneously after the addition of pepsin in a sample held under the same conditions outside the rheometer, and the pH was recorded every 1 min.

3.2.4. Microstructural characterization of coagulum

The microstructure of the pepsin-induced coagulum was examined using confocal laser scanning microscopy. Fast Green fluorescent dye (3 µL; 1% wt/vol) was added to skim milk (100 µL) and stirred for 30 s. After pre-warming to 37°C, pepsin was added to the milk, reaching a final pepsin concentration of either 0.550 or 1.10 U/mL milk. The milk–pepsin mixture was then transferred to the cavity of a microscope glass slide and covered with a glass cover slip. The coagulum was examined using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with the temperature set at 37°C and with a 63× magnification lens. The micrographs were captured at different time points after pepsin addition (16, 20, 30, 40, 60, 80, and 120 min).

3.2.5. Statistical analysis

Samples were prepared in triplicate and rheological measurements of each sample were carried out in triplicate. Ten time points were examined by RP-HPLC and four of them (1, 10, 50, and 120 min) were examined in triplicate. The ANOVA tests were carried out using

Prism 8 (GraphPad Software Inc., San Diego, CA) to determine the significance of the differences. Differences were significant at $P < 0.05$.

3.3. Results and discussion

3.3.1. Kinetics of κ-casein hydrolysis by pepsin

The degree of hydrolysis of the κ-casein was determined by comparing the peak area of para-κ-casein released at each time point with the peak area of the completely hydrolyzed milk (a sample treated with 2.75 U/mL pepsin at pH 6.0 for 8 h) (Gastaldi et al., 2003; Liu, Juliano, Williams, Niere, & Augustin, 2014). This is expressed as:

$$H_{\mathrm{t}} = 100 \times P_{\mathrm{t}} / P_{\mathrm{tot}} \qquad \textbf{(Equation 3-1)}$$

where H_{t} is the percent hydrolysis of κ-casein at time t, P_{t} is the peak area of para-κ-casein released at time t, and P_{tot} is the peak area of para-κ-casein for a completely hydrolyzed sample. The κ-casein peaks almost completely disappeared after holding for a period of 8 h at pH 6.0 in the presence of 2.75 U/mL pepsin (**Figure S 3-1**), and this was designated as a sample where complete hydrolysis had taken place. In the presence of pepsin at concentrations of 0.110–2.75 U/mL, the degree of hydrolysis of κ-casein in skim milk at pH 6.7–5.3 increased with increasing reaction times, as plotted in **Figure 3-1 a–e**.

Several models were applied to fit the hydrolysis data. In **Figure 3-2**, the black dots represent hydrolysis of κ-casein in skim milk at pH 6.0 when the pepsin concentration was 0.55 U/mL. Both the Michaelis–Menten fitted curve (green dashed curve in **Figure 3-2**) and the first-order equation fitted curve (blue dashed curve in **Figure 3-2**) describes the data over the first 20 minutes of hydrolysis well; however, the deviation was considerable after ~ 80% hydrolysis. Therefore, these models were not considered further, and a pseudo-first-order equation was applied instead (Jensen et al., 2015):

$$H_t = H_{120} \cdot (1 - \exp(-K_2 \cdot t)) \qquad \textbf{(Equation 3-2)}$$

where K_2 (min^{-1}) is the reaction rate constant and H_{120} is the degree of hydrolysis of κ-casein at 120 min. With a calculated K_2 of 0.08 min^{-1} and an H_{120} of 87%, the fitted curve from equation (2) describes the experimental data points well, as shown by the black dashed curve in **Figure 3-2**. H_{120} was lower than 100%, which means that κ-casein was not completely hydrolyzed at 120 min. According to Carlson et al. (1987a), the denaturation of pepsin can occur in parallel with the hydrolysis of κ-casein due to a pH greater than 5, and denaturation could be complete before there is complete hydrolysis of the κ-casein. This enzyme denaturation also occurs in chymosin-catalyzed proteolysis of κ-casein (van Hooydonk, 1987), who proposed an overall reaction that was derived from a combination of first-order proteolysis and first-order enzyme denaturation:

$$ln\left(1-\frac{H_t}{100}\right)=\frac{K_{enz}\cdot C}{K_{den}}\cdot\left[exp(-K_{den}\cdot t)-1\right] \qquad \textbf{(Equation 3-3)}$$

where H_t is the percent hydrolysis of κ-casein at time t, K_{den} (min^{-1}) is the reaction rate constant for the denaturation reaction, C (U/mL) is the pepsin concentration, K_{enz} [min^{-1}/(U/mL)] is the reaction rate constant for the enzymatic reaction per unit quantity of enzyme solution, and $K_{\text{enz}} \cdot C$ is defined as the overall reaction rate constant K (min^{-1}). This equation describes the experimental data well, in which proteolysis and denaturation take place simultaneously, as shown in **Figure 3-2** (red curve). Both **Equation 3-2** and **3-3** were used for fitting the data in **Figure 3-1**, yielding the parameters shown in **Table 3-1**. However, the K_2 values cannot reflect the initial hydrolysis velocities [V_{i} (%/min) as determined by linear regression of the first four time points of the degree of hydrolysis (**Figure 3-1 a**) as, for example, at pH 6.7, the V_{i} value for the sample at a pepsin concentration of 2.75 U/mL was higher than that of 1.10 U/mL whereas the K_2 value for the sample at a pepsin concentration of 2.75 U/mL was 0.292±0.028, lower than 0.403±0.045 for 1.10 U/mL. Therefore, **Equation 3-3** was selected in this study as the best method to calculate the degree of hydrolysis of κ-casein at specific times during coagulation. For this reason, the degrees of hydrolysis are reported by the red curves in **Figure 3-1**.

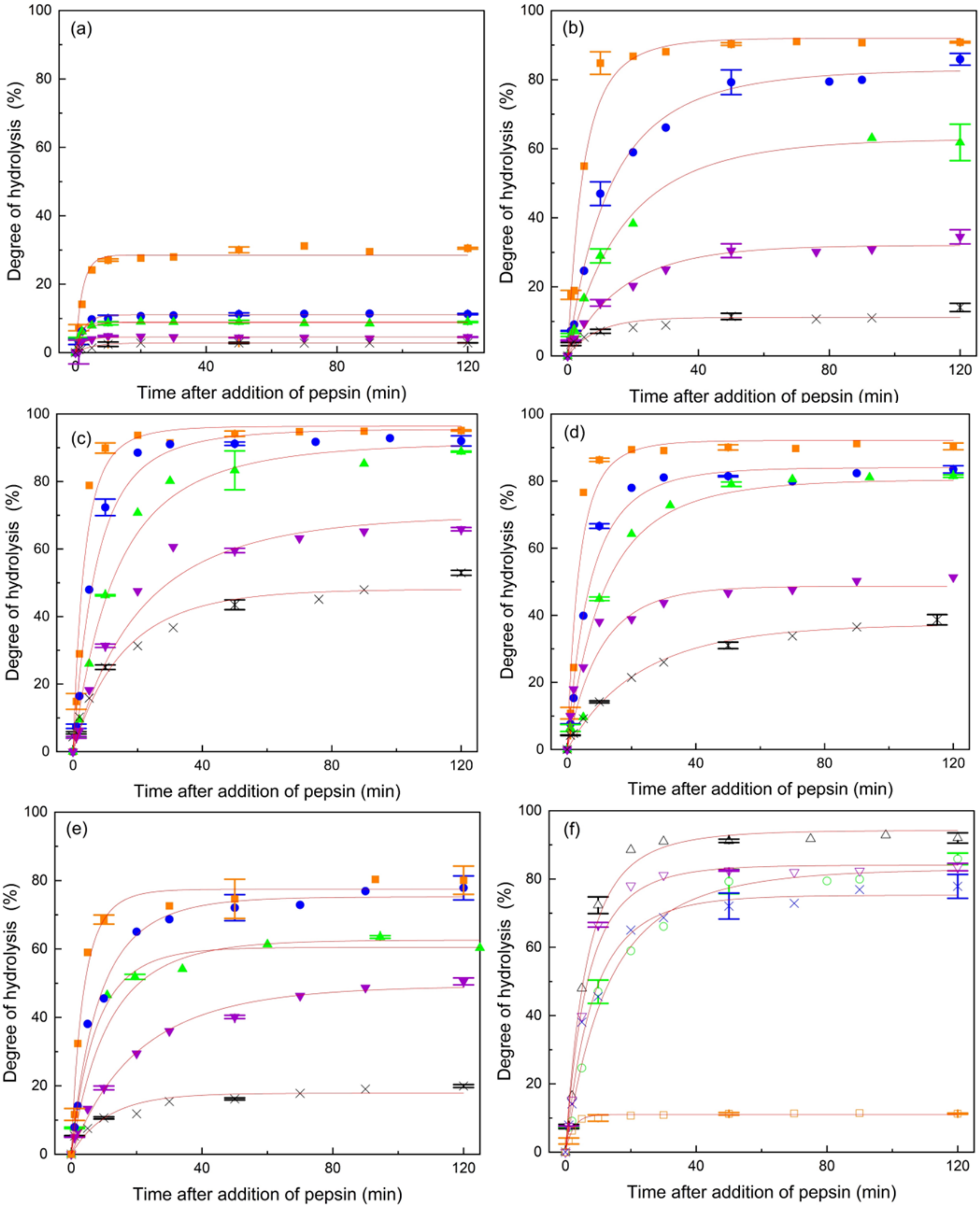

Figure 3-1 (a)–(e): Degree of hydrolysis of κ-casein in skim milk with time after the addition of pepsin (■, 2.75 U/mL; ●, 1.10 U/mL; ▲, 0.550 U/mL; ▼, 0.275 U/mL; ×, 0.110 U/mL milk) at 37°C (a) pH 6.7; (b) pH 6.3; (c) pH 6.0; (d) pH 5.7; (e) pH 5.3; (f): degree of hydrolysis of κ-casein in skim milk with time after the addition of pepsin at a concentration of 1.10 U/mL into skim milk at different pH values (□, pH 6.7; ○, pH 6.3; △, pH 6.0; ▽, pH 5.7; ×, pH 5.3). The red curves are the fits of **Equation 3-3**.

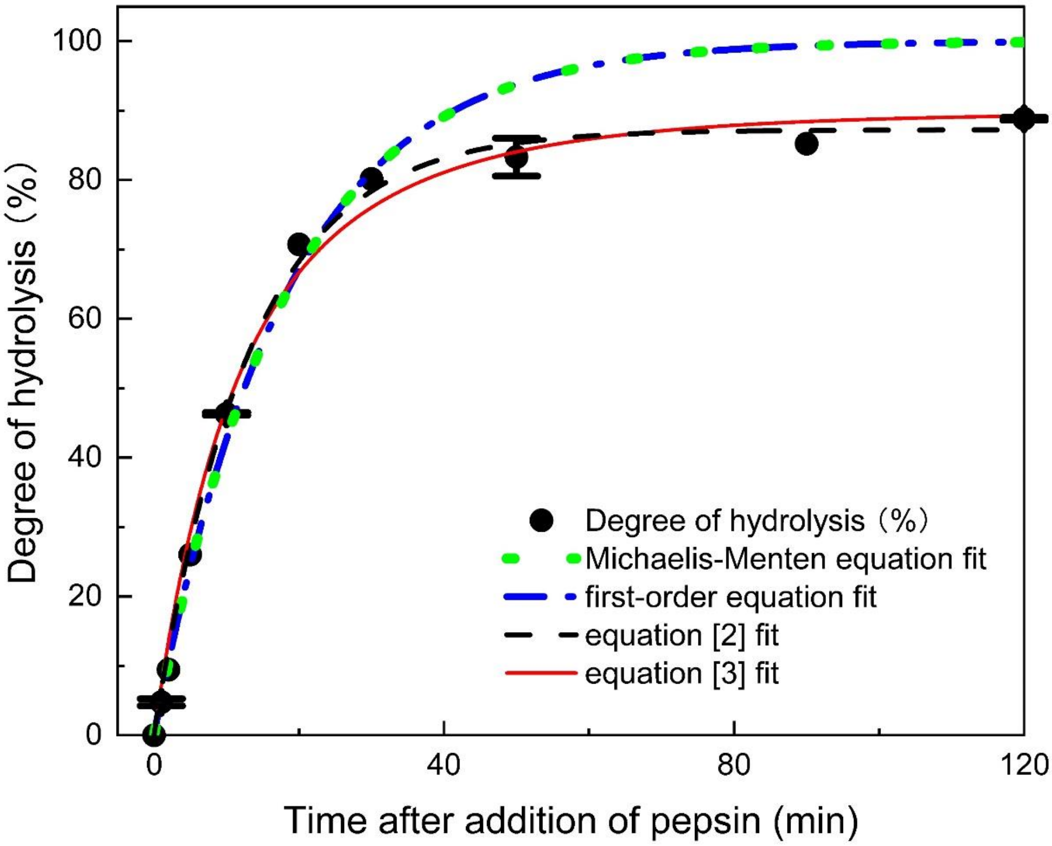

Figure 3-2 Degree of hydrolysis of κ-casein in skim milk at pH 6.0 in 120 min with a pepsin concentration of 0.550 U/mL (black dots). The green dashed curve is the Michaelis–Menten equation fit, the blue dashed curve is according to the first-order equation, the black dashed curve is according to **Equation 3-2**, and the red curve is according to **Equation 3-3**.

3.3.2. Effect of pepsin concentration on the hydrolysis of κ-casein

According to **Figure 3-3 a**, the V_i values increased at higher pepsin concentrations; this is apparent for the samples at all pH values. The enzymatic reaction rate constant K ($K_{enz} \cdot C$, min^{-1}) (**Table 3-1**) increased as pepsin concentration increased, and there was a positive linear correlation between K and the pepsin concentration (**Figure 3-4 a**), which was found at all pH values. The slope of the curve, equal to K_{enz}, is shown in **Figure 3-4 b**. By plotting the reaction constants (K) as a function of the initial velocities (V_i) (**Figure 3-4 c**), K was observed to have a proportional relationship to V_i. This indicates that, at the same pH, higher pepsin concentrations resulted in higher K, and thus higher V_i.

Table 3-1 Hydrolysis kinetics of κ-casein in bovine skim milk at different pH values and pepsin concentrations.

		According to Equation 3-2			**According to Equation 3-3**		
Pepsin concent-ration (U/mL)	**Milk pH**	**Enzymatic reaction rate constant K_2 (min^{-1})**	**Hydrolysis degree at 120 min (H_{120}) (%)**	**R^2**	**Enzymatic reaction rate constant K (K_{enz}· C) (min^{-1})**	**Denaturation rate constant (K_{den}) (min^{-1})**	**R^2**
2.75	6.7	0.292±0.028 ^{Ab}	29.32±0.28 ^{Ca}	0.989	0.104±0.032 ^{Ca}	0.230±0.194 ^{Acd}	0.964
	6.3	0.228±0.036 ^{Aab}	91.16±0.71 ^{Aa}	0.987	0.218±0.046 ^{Ba}	0.083±0.014 ^{ABa}	0.980
	6	0.239±0.017 ^{Aa}	95.29±0.63 ^{Aa}	0.982	0.258±0.017 ^{Aa}	0.074±0.013 ^{Ba}	0.973
	5.7	0.225±0.019 ^{Aa}	91.44±0.19 ^{Aa}	0.972	0.237±0.015 ^{Aa}	0.092±0.007 ^{Ba}	0.961
	5.3	0.212±0.049 ^{Aa}	78.29±3.53 ^{Ba}	0.987	0.190±0.034 ^{Ba}	0.123±0.033 ^{Ba}	0.985
1.10	6.7	0.403±0.045 ^{Aa}	11.41±0.37 ^{Db}	0.996	0.046±0.005 ^{Cb}	0.377±0.040 ^{Aab}	0.991
	6.3	0.324±0.420 ^{ABa}	82.50±1.30 ^{Bb}	0.990	0.072±0.012 ^{Cb}	0.038±0.008 ^{Ba}	0.989
	6	0.138±0.001 ^{Ba}	92.58±0.37 ^{Aa}	0.995	0.146±0.001 ^{Ab}	0.053±0.002 ^{Ba}	0.990
	5.7	0.145±0.005 ^{Ba}	82.76±0.73 ^{Ab}	0.994	0.131±0.019 ^{ABb}	0.072±0.012 ^{Ba}	0.983
	5.3	0.122±0.014 ^{Ba}	74.05±3.56 ^{Ca}	0.989	0.104±0.010 ^{Bb}	0.076±0.013 ^{Ba}	0.992
0.550	6.7	0.418±0.170 ^{Aa}	8.26±0.22 ^{Dbc}	0.993	0.029±0.005 ^{Bbc}	0.318±0.055 ^{Aabc}	0.932
	6.3	0.056±0.005 ^{Bb}	68.72±5.82 ^{Cc}	0.993	0.060±0.011 ^{ABbc}	0.037±0.003 ^{Ba}	0.995
	6	0.075±0.003 ^{Ba}	86.51±2.89 ^{Ab}	0.997	0.072±0.003 ^{Ac}	0.033±0.003 ^{Ba}	0.993
	5.7	0.079±0.004 ^{Ba}	80.64±0.56 ^{Bb}	0.976	0.059±0.019 ^{ABc}	0.054±0.023 ^{Ba}	0.967
	5.3	0.088±0.016 ^{Ba}	64.87±1.94 ^{Cb}	0.988	0.067±0.008 ^{Ac}	0.056±0.016 ^{Ba}	0.975
0.275	6.7	0.606±0.007 ^{Aa}	4.20±0.19 ^{Ecd}	0.977	0.013±0.005 ^{Abc}	0.298±0.119 ^{Abcd}	0.750
	6.3	0.060±0.002 ^{Bb}	33.89±1.67 ^{Cd}	0.982	0.020±0.001 ^{Acd}	0.051±0.001 ^{Ba}	0.985
	6	0.056±0.012 ^{Ba}	65.87±0.45 ^{Ac}	0.976	0.035±0.003 ^{Ad}	0.031±0.004 ^{Ba}	0.986
	5.7	0.049±0.014 ^{Ba}	54.30±6.71 ^{Bc}	0.963	0.022±0.003 ^{Ad}	0.026±0.007 ^{Ba}	0.968
	5.3	0.047±0.009 ^{Ba}	40.09±2.69 ^{Dc}	0.979	0.018±0.002 ^{Ad}	0.032±0.006 ^{Ba}	0.969
0.110	6.7	0.400±0.229 ^{Aa}	2.88±0.11 ^{Dcd}	0.979	0.012±0.005 ^{Ac}	0.438±0.170 ^{Aa}	0.964
	6.3	0.074±0.025 ^{Bb}	13.18±0.70 ^{Ce}	0.729	0.012±0.001 ^{Ad}	0.093±0.020 ^{Ba}	0.863
	6	0.046±0.016 ^{Ba}	45.88±2.36 ^{Ad}	0.960	0.019±0.003 ^{Ad}	0.030±0.007 ^{Ba}	0.993
	5.7	0.042±0.007 ^{Ba}	38.12±1.95 ^{Bd}	0.987	0.017±0.002 ^{Ad}	0.035±0.006 ^{Ba}	0.990
	5.3	0.092±0.011 ^{Ba}	17.98±0.54 ^{Cd}	0.897	0.017±0.002 ^{Ad}	0.086±0.011 ^{Ba}	0.901

$^{A–E}$ Mean values between samples at same pepsin concentration in the same column with different superscripts are significantly different ($P < 0.05$). $^{a–e}$ Mean values between samples at same pH in the same column with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of mean (n = 3).

The pepsin denaturation rate constants (K_{den}) (**Table 3-1**) appear to be independent of pepsin concentration at the same pH. The maximum degree of hydrolysis within the 120 min time period (H_{max}) decreased with a decrease in pepsin concentration (**Figure 3-3 b**). For the samples with lower hydrolysis reaction constants K where pepsin concentration is lower, a lower maximum degree of hydrolysis (H_{max}) in the 120 min time period was observed.

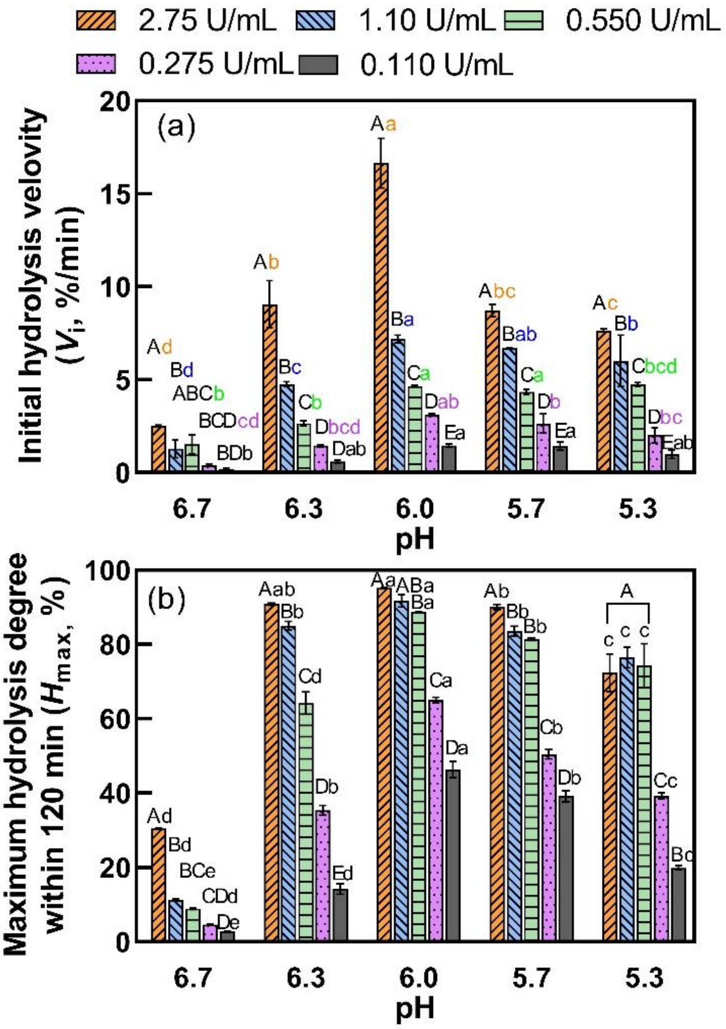

Figure 3-3 (a) Initial hydrolysis velocity (V_i, %/min) and (b) maximum hydrolysis percent within 120 min for samples at 6.7–5.3 when the concentrations of pepsin were 0.110–2.75 U/mL. $^{A–E}$ Mean values between samples at same pH with different superscripts are significantly different ($P < 0.05$). $^{a–e}$ Mean values between samples at same pepsin concentration with different superscripts are significantly different ($P < 0.05$).

3.3.3. Effect of pH on the hydrolysis of κ-casein

As shown in **Figure 3-3 b**, H_{max} values over 120 min were below 100%. According to van Hooydonk (1987), κ-casein cannot be totally hydrolyzed by chymosin because of the denaturation of this enzyme at pH > 7.0; however, $K_{\mathrm{den}} > 0$ reported in this study indicates that denaturation of pepsin occurred at all pH values (6.7–5.3)). Even though the optimum pH for milk-clotting activity (specific hydrolysis activity) of pepsin is unknown (Andrén, 2011), the milk-clotting activity of porcine pepsin is more pH dependent than chymosin. They reported that the porcine pepsin is sensitive to inactivation that denatures rapidly at the pH ~ 6.5 and temperature ~ 30 °C (when used for cheesemaking), and only 50% of its milk-clotting activity remains after 1 h (Andrén, 2011). Consequently, denaturation of pepsin could have occurred in the pH range of 6.7–5.3 in this study.

The hydrolysis processes at pH 6.7–5.3 in the presence of pepsin at a concentration of 1.10 U/mL are shown in **Figure 3-1 f**. The lowest H_{max} (11.30%) found at pH 6.7 may be related to the denaturation of pepsin (K_{den}) which was significantly higher ($P < 0.05$) for the sample at pH 6.7 compared to other pH values (**Table 3-1**). In addition, the sample at pH 6.7 had lower K values (**Table 3-1**, **Figure 3-4 a**), V_{i} values (**Figure 3-3 a**) and K_{enz} values **(Figure 3-4 b**). As both the casein micelles (pI 4.6) and pepsin (pI 2.7) are negatively charged in the pH range 6.7–5.3 (Herriott, Desreux, & Northrop, 1940; McMahon & Brown, 1984), a decrease in pH increases the ionic strength (due to the dissolution of CCP) from the casein micelles to the soluble phase) and diminishes the electrostatic repulsion between the casein micelles and pepsin, and thus promotes the binding of pepsin to casein micelles, resulting in faster enzymatic hydrolysis and higher K_{enz} (van Hooydonk, 1987). K_{enz} was highest for the sample at pH 6.0 (**Figure 3-4 b**), which indicates that the optimal pH for pepsin-induced hydrolysis of κ-casein is pH 6.0, similar to that for chymosin-induced hydrolysis (van Hooydonk, 1987). With minimum voluminosity of the casein micelles at pH near 6.0 and maximum voluminosity at pH around 5.3, van Hooydonk (1987) proposed that the accessibility of the κ-casein Phe^{105}–Met^{106} bond for chymosin might be a function of the protrusion of the caseinomacropeptide part of κ-casein into the serum, which may also be the case for pepsin accessibility. The protrusion of caseinomacropeptide is probably minimal at pH close to 6.0 due to the minimum voluminosity of casein micelles at this pH, diminishing steric hindrance and creating a higher probability for an effective interaction between pepsin and κ-casein (van Hooydonk & Berg, 1988). In the other side, pepsin diffusion probably decreases and consequently decrease in K_{enz} when casein micelles aggregate and form a gel. Since the aggregation of casein micelles occurs at lower degree of hydrolysis at lower pH, the effect of a limited diffusion of pepsin would be observed earlier at lower pH. These two opposite effects on K_{enz} could also explain an optimal pH for pepsin-induced hydrolysis of κ-casein at pH 6.0.

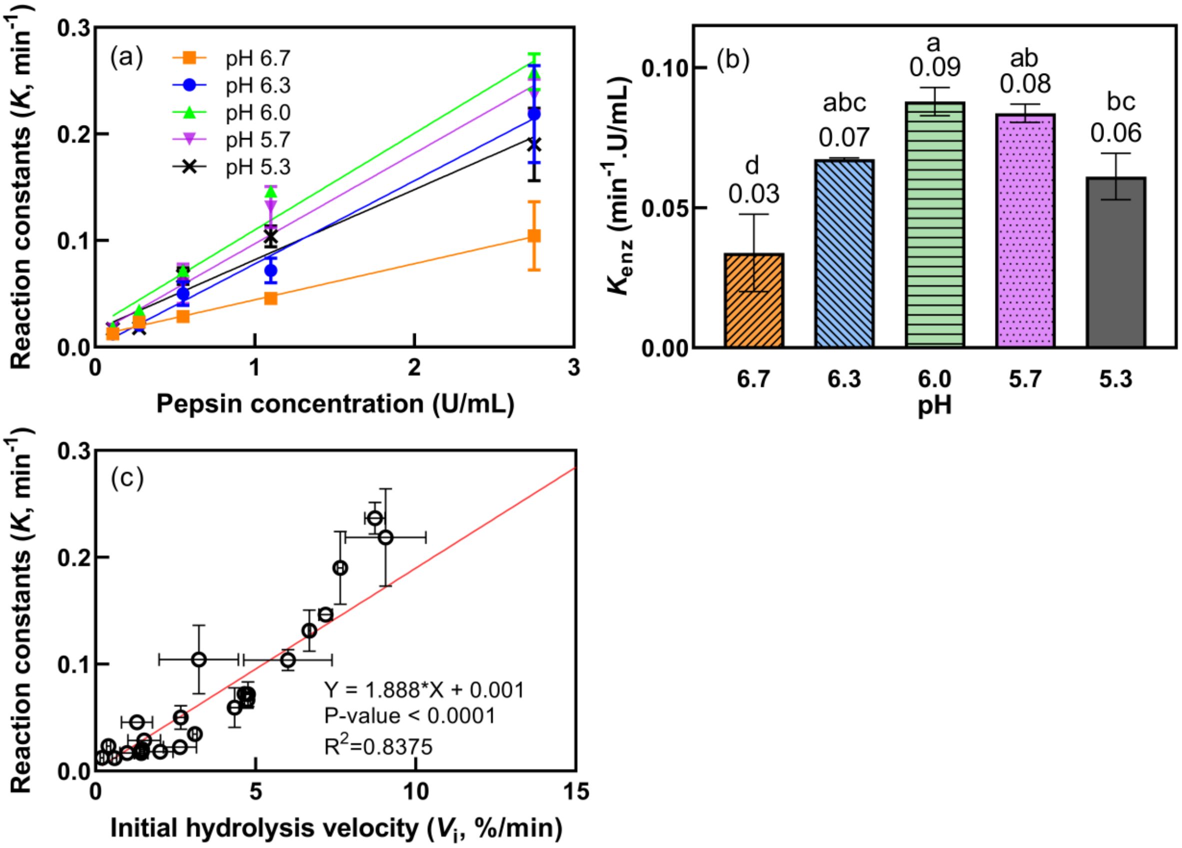

Figure 3-4 (a) Enzymatic reaction rate constant K ($K_{\text{enz}} \cdot C$, min^{-1}) as a function of pepsin concentration (0.110–2.75 U/mL) at: ■ pH 6.7; ● pH 6.3; ▲ pH 6.0; ▼ pH 5.7; × pH 5.3. (b) K_{enz} [min^{-1}/(U/mL)], calculated from the slopes of the curves in (a), at: 🟧 pH 6.7; 🟦 pH 6.3; 🟩 pH 6.0; 🟪 pH 5.7; ⬛ pH 5.3. (c) Reaction constants (K, min^{-1}) as a function of the initial hydrolysis velocity (V_i, %/min). $^{a\text{–}c}$ Mean values with different superscripts are significantly different ($P < 0.05$).

3.3.4. Pepsin-induced milk coagulation

The increase in the storage modulus (*G′*) of skim milk after the addition of pepsin is shown in **Figure 3-5 (a)–(e)**, representing the samples at pH values 6.7–5.3 (the loss modulus *G″* was also recorded, and **Figure S 3-2** shows the change in *G′* and *G″* over time for skim milk at pH 6.0 after the addition of pepsin at concentrations of 0.550 U/mL). Except samples at pH 6.7, the *G′* versus time curves showed three stages, depending upon the concentration of pepsin and the pH: (1) in the early stages of coagulation, the sample was still in liquid phase that *G′* showed a lag phase; (2) as time progressed, *G′* increased significantly with increasing hydrolysis time; (3) in the later stages, *G′* reached a plateau for the samples at pH 6.3 (**Figure 3-5 b**) or decreased for the samples at pH values 6.0–5.3 (**Figure 3-5 c–e**). For the second stage, after hydrolysis of κ-casein, steric repulsion due κ-casein is reduced and the destabilized para-casein micelles aggregate via hydrophobic interactions, leading to the formation of a casein network (Panthi et al., 2019). Calcium and hydrogen bonds also contribute to the specificity and stability of protein interactions (Lefebvre-Cases et al., 1998). According to Mellema et al. (2002) and Panthi et al. (2019), due in part to the rearrangement, movement of casein micelles or clusters by detaching at weak bonds at one junction and connecting to another junction eventually leads to increased strength of the bonds. Besides, the protein network density between para-casein micelles increases over time, which results in the observed increase in *G′* (Panthi et al., 2019). The time point at which coagulation occurs has previously been defined as the point when *G′* is equal to *G″* (Li & Dalgleish, 2006) or the point at which *G′* is greater than 1 Pa (Meletharayil, Patel, & Huppertz, 2015). Panthi et al. (2019) took the first consistent increase in *G′* as the coagulation time, which is more precise than previous methods. In this study, as shown in the inset of **Figure 3-5 d**, a linear regression can be applied to the first four points when *G′* began to increase above the data noise. The coagulation time was defined as the intercept of this regression with the time axis.

G′ increased over time, and the maximum slope of *G′* as a function of time (d*G′*/d*t*) is an indication of the curd firming rate (Guinee et al., 1997; Panthi et al., 2019).

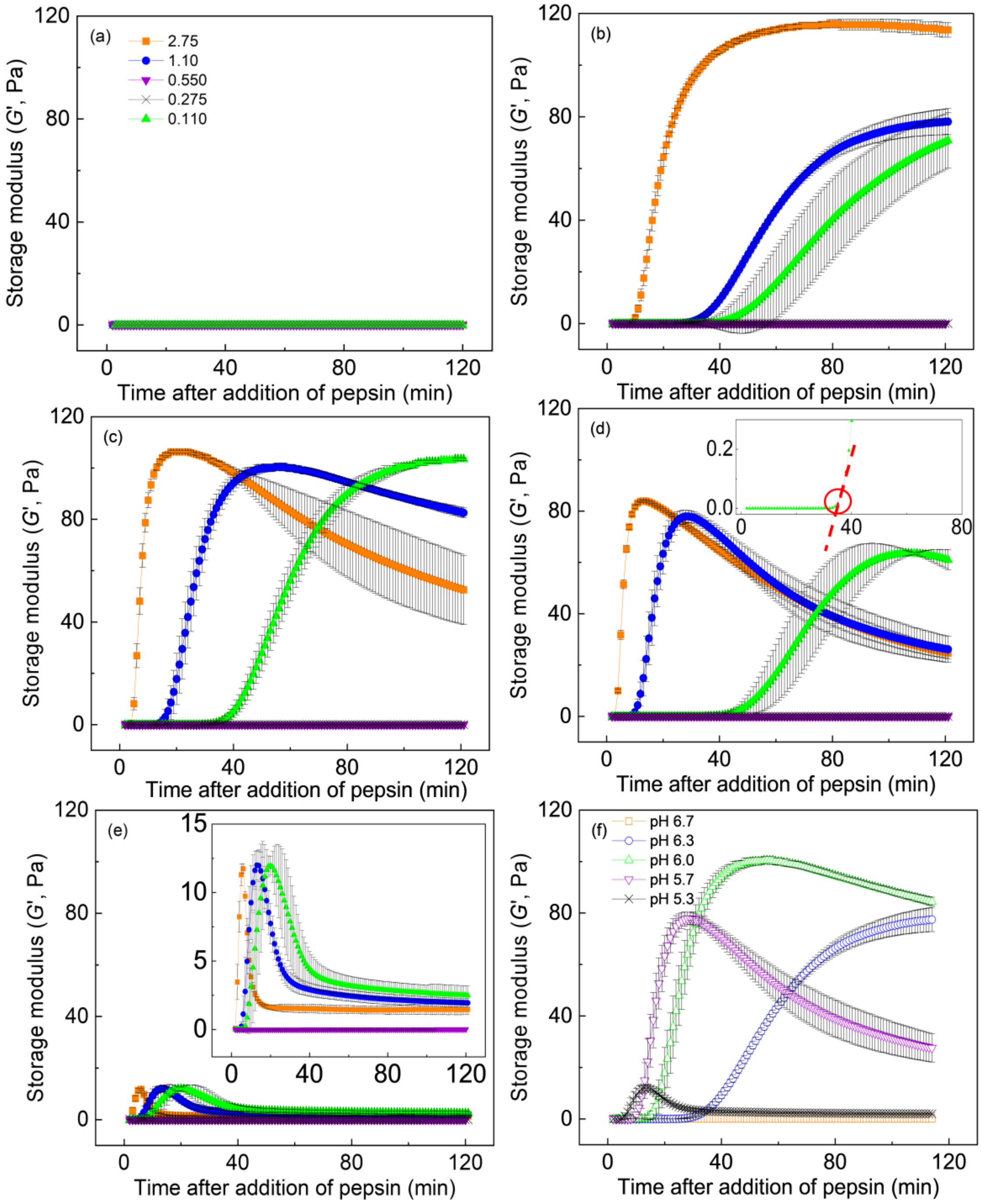

Figure 3-5 Storage modulus (*G′*) of samples as a function of pepsin concentration (■ 2.75 U/mL; ● 1.10 U/mL; ▲ 0.550 U/mL; ▼ 0.275 U/mL; × 0.110 U/mL) at 37 °C: (a) pH 6.7; (b) pH 6.3; (c) pH 6.0; (d) pH 5.7, with an inset to show coagulation time (details are described in text); (e) pH 5.3 with zoomed inset. (f) Storage modulus (*G′*) of samples over time after the addition of pepsin at a concentration of 1.10 U/mL at different pH values (□ pH 6.7; ○ pH 6.3; △ pH 6.0; ▽ pH 5.7; × pH 5.3).

3.3.5. Effect of pepsin concentration on the coagulation process

At pepsin concentrations of 0.110 and 0.275 U/mL, no coagulation occurred within 120 min. **Figure 3-5 c** illustrates the change in G' with time at pH 6.0 after the addition of pepsin at concentrations of 2.75, 1.10, and 0.550 U/mL, where the coagulation times were ~ 5, 11, and 25 min, respectively (**Figure 3-6 a**). The degree of hydrolysis of κ-casein at the coagulation time (the ‘critical’ degree of hydrolysis, H_{ct}) was calculated from H_t using equation (3). Coagulation occurred when ~ 64, 70, and 70% of the κ-casein had been hydrolyzed in the presence of pepsin concentrations of 2.75, 1.10, and 0.550 U/mL at pH 6.0, respectively (**Figure 3-6 b**). For each pH value, the differences in H_{ct} among different pepsin concentrations were not significant ($P > 0.05$). Therefore, the degree of hydrolysis of κ-casein that is required for coagulation seems to be independent of the pepsin concentration for a fixed pH. In the presence of a higher pepsin concentration (2.75 U/mL), with a higher reaction rate constant K, it took less time to reach the ‘critical’ degree of hydrolysis (~ 68% at pH 6.0), which explains the shorter coagulation time. In the presence of low pepsin concentrations (0.275 and 0.110 U/mL), as the maximum degrees of hydrolysis were found to be < 68% (65 and 46% for 0.275 and 0.110 U/mL, respectively), no coagulation occurred due to insufficient hydrolysis of κ-casein within 120 min.

As shown in **Figure 3-6 c**, higher pepsin concentrations resulted in higher firming rates (dG'/dt) at all pH values. By plotting the firming rates (dG'/dt) with the hydrolysis reaction rate constant (K), a proportional relationship was found, as shown in **Figure 3-7**. The firming rate in the aggregation phase could be considered as independent of the hydrolysis phase with respect to the different Q_{10} value (defined as the proportional rate of increase in reaction rate as temperature rises by ten degrees from 20 °C to 30 °C) of the hydrolysis phase compared to the aggregation phase (Dalgleish, 1979; van Hooydonk, 1987). However, according to (Bringe & Kinsella, 1986), the curd firming rate is determined not only by para-casein micelle aggregation but also by the initial rate of hydrolysis reaction because of the influence of the enzymatic reaction on aggregation, e.g., for the samples at pH 6.0, coagulation occurred when 68% of the para-κ-casein had been released (H_{ct} was ~ 68%), and further increase in G' requires additional unstable para-casein micelles (the limiting step of dG'/dt could shift to the further hydrolysis of κ-casein). The higher hydrolysis reaction constant (K) at higher pepsin concentrations led to more unstable para-casein micelles over time, and this is shown by the increase in the firming rate (Carlson et al., 1987b).

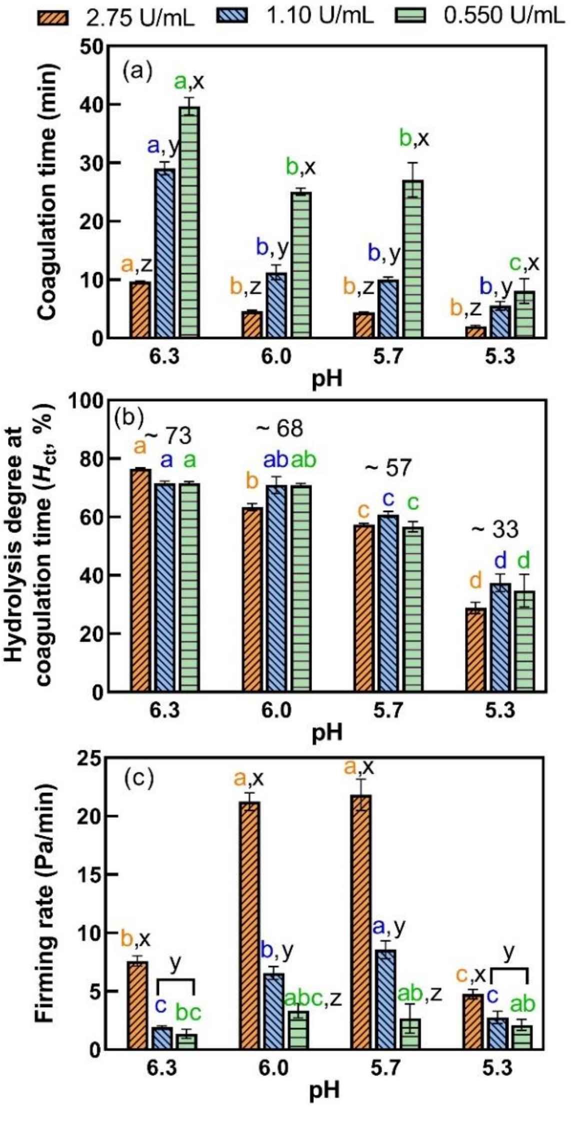

Figure 3-6 (a) Coagulation time (min), (b) hydrolysis degree at coagulation time (H_{ct}, %), and (c) firming rate (dG'/dt, Pa/min) as a function of pepsin concentration (0.110–2.75 U/mL) at pH values 6.7–5.3. $^{X-Z}$ Mean values between samples at same pH with different superscripts are significantly different ($P < 0.05$). $^{a-e}$ Mean values between samples at same pepsin concentration with different superscripts are significantly different ($P < 0.05$).

3.3.6. Effect of pH on the coagulation process

When the pepsin concentration was same at 1.10 U/mL, the coagulation time and *G'* of skim milk differed with change in pH (6.7–5.3) (**Figure 3-5 f**). This is also the case for samples at other pepsin concentrations that the effect of pH on coagulation time and firming rate were compared in **Figure 3-6**. As shown in **Figure 3-6 a**, the coagulation time decreased as pH decreased. When the coagulation time was substituted into **Equation 3-3**, the average H_{ct} values were ~ 73, 68, 57, and 33% for the samples at pH 6.3, 6.0, 5.7, and 5.3, respectively (**Figure 3-6 b**), noting that H_{ct} was found to be independent of the pepsin concentration at 0.550 U/mL and higher. The degree of hydrolysis required for coagulation to occur decreased markedly with a decrease in pH, which was also observed in chymosin-induced milk coagulation (Carlson et al., 1987b, 1987d; van Hooydonk, 1987); this was due to partial charge neutralization of the negatively charged para-casein micelles. The decrease in the magnitude of repulsion forces and increased hydrophobic interactions allowed a closer approach of the casein micelles, leading to faster onset of aggregation (van Hooydonk, 1987; Zoon, 1988). In addition, as measured by Choi et al. (2007) the CCP content of the casein micelles decreases significantly when the pH decreases from 6.7 to 5.4, which results in an increase in the concentration and activity of calcium ions in the milk serum. As calcium ions are critical for aggregation of the casein micelles (Lucey & Fox, 1993), the onset of casein aggregation (as measured by coagulation time) occurred more rapidly, even when the degree of hydrolysis of κ-casein was low. The coagulation time was therefore shorter for milk samples at lower pH.

As shown in **Figure 3-6 c**, the firming rates (dG'/dt) for samples at pH 6.0 and 5.7 were higher than at pH 6.3 and 5.3 at the two highest pepsin concentrations. As previously mentioned, the firming rate is positively correlated to the reaction rate constant (K) (**Figure**

3-7); therefore, higher firming rates were found for the samples at pH 6.0 and 5.7, which had higher *K* values (**Table 3-1**).

G′ reached a plateau for the sample at pH 6.3 (**Figure 3-5 b**) and decreased for other the samples at pH < 6.3 (**Figure 3-5 c–e**). Since pepsin hydrolysis is much less specific than chymosin, there is a possibility that other peptide bond cleavages may occur during the experiments. However, as the pH in this study did not change during 120 min (results not shown) and according to the RP-HPLC profile, this hypothesis was excluded. The decrease in *G′* was probably caused by microsyneresis (Roefs, de Groot-Mostert, & van Vliet, 1990) – a shrinkage of the casein curd over time – due to small oscillations of the sample (Zoon, 1988), thus eliminating the required adhesion of the coagulum to the rheometer surface during measurements. Therefore, the value of *G′* likely does not accurately reflect the coagulum strength at low pH at longer coagulation times. Further research using nondestructive structural characterization techniques, such as small angle neutron scattering, may provide more information (Gilbert, 2019).

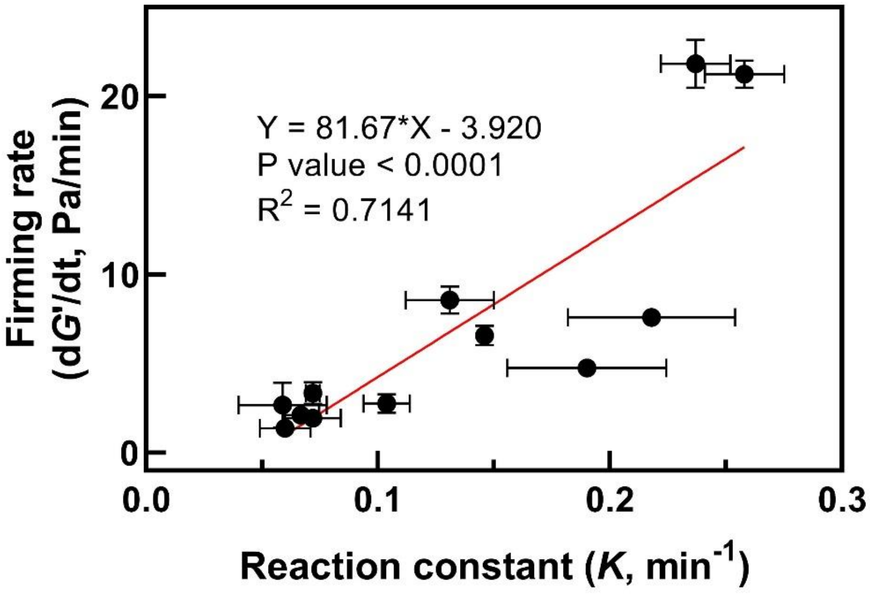

Figure 3-7 Firming rate (d*G′*/d*t*, Pa/min) of samples as a function of reaction rate constant *K* (min^{-1}).

3.3.7. Microstructure of pepsin-induced milk coagulum

Confocal micrographs of the coagulum formed from skim milk with a pepsin concentration of 0.550 U/mL at pH 6.0 are shown in **Figure 3-8 a**; the proteins appear as green and the associated pores in the aqueous phase appear as black. In the initial stage of coagulation (~ 16 min), the proteins were evenly distributed and homogeneous, indicating that the casein micelles had not yet coagulated. A protein network with some voids and large aggregated structures was observed from ~ 20 min. The pores in the matrix became larger (diameter > 20 μm) and the network became closely knitted with an increase in reaction time.

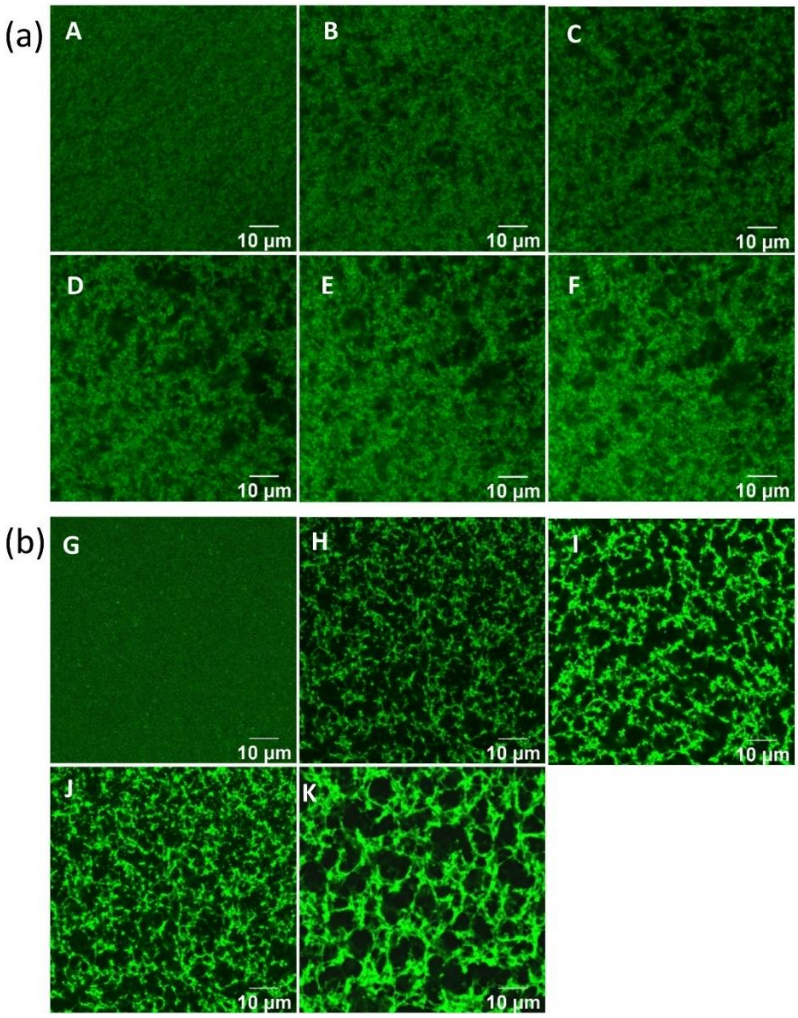

Figure 3-8 (a) Confocal micrographs at 37°C for the pepsin-induced coagulation of milk at: A, 16 min; B, 20 min; C, 30 min; D, 40 min; E, 60 min; F, 80 min (skim milk pH 6.0 with the addition of pepsin at a concentration of 0.550 U/mL); the micrographs were observed at the same position. (b) Microstructures of the coagulum after 120 min with the addition of pepsin at a concentration of 1.10 U/mL and at: G, pH 6.7; H, pH 6.3; I, pH 6.0; J, pH 5.7; K, pH 5.3. The scale bars are 10 μm in length for all micrographs.

The microstructures of the coagulum after a prolonged incubation of 120 min at 37°C are shown in **Figure 3-8 b**. As coagulation did not occur for the sample at pH 6.7, no protein network was observed at 120 min. The observed network structure and the pore size within samples at pH values of 6.3, 6.0, and 5.7 were similar, whereas the pores observed within the sample at pH 5.3 were larger than in the samples at other pH values. This observation is consistent with observations of rennet curd at different pH by Ong et al. (2013a). As previously mentioned, G' for the sample at pH 5.3 decreased in the later stages because of curd syneresis. The rearrangement of the casein micelle network at pH 5.3 appears more extensive and syneresis would likely be more pronounced (Mellema et al., 2002).

3.4. Conclusion

The pepsin-induced hydrolysis of κ-casein followed a combined kinetic model of first-order hydrolysis and pepsin denaturation, depending on both pepsin concentration and pH. The rate of hydrolysis increased with the pepsin concentration and was pH dependent, reaching a maximum velocity at pH 6.0. Coagulation of the casein micelles occurred when a critical amount of κ-casein had been hydrolyzed. At a given pH, the critical degree of hydrolysis was independent of the pepsin concentration. The degree of κ-casein hydrolysis required for coagulation decreased markedly from ~ 73 to ~ 33% when the pH decreased from 6.3 to 5.3, likely due to the lower pH promoting aggregation of casein micelles through neutralization of surface charges and releasing Ca^{2+} by colloidal calcium phosphate dissolution. The coagulation time decreased with an increase in pepsin concentration and a decrease in pH. These results suggest that changes in pH and pepsin concentration markedly affect the hydrolysis of κ-casein, the milk coagulation behavior, and the coagulum properties. The knowledge obtained from this study provides further understanding the observation on the coagulation of milk occurring at the initial stage of gastric digestion in previous *in vitro* and *in vivo* studies, which is high pH and low pepsin concentration.

3.5. Acknowledgements

This study was funded by New Zealand Milks Mean More (NZ3M) program and Riddet Institute Centre of Research Excellence, Tertiary Education Commission, New Zealand. The author Mengxiao Yang thanks the Australian Institute of Nuclear Science and Engineering (AINSE) for a Post Graduate Research Award. We would like to acknowledge the support of Michael Agnew (AgResearch Limited, New Zealand) for training in reverse-phase HPLC, Michelle Tamehana (Massey University, New Zealand) for training in rheometric studies and Matthew Savoian (Manawatu Microscopy and Imaging Centre, New Zealand) for training in confocal laser scanning microscopy. We would like to thank Claire Woodhall (Havelock North, New Zealand) for proofreading the manuscript.

3.6. Supplementary material

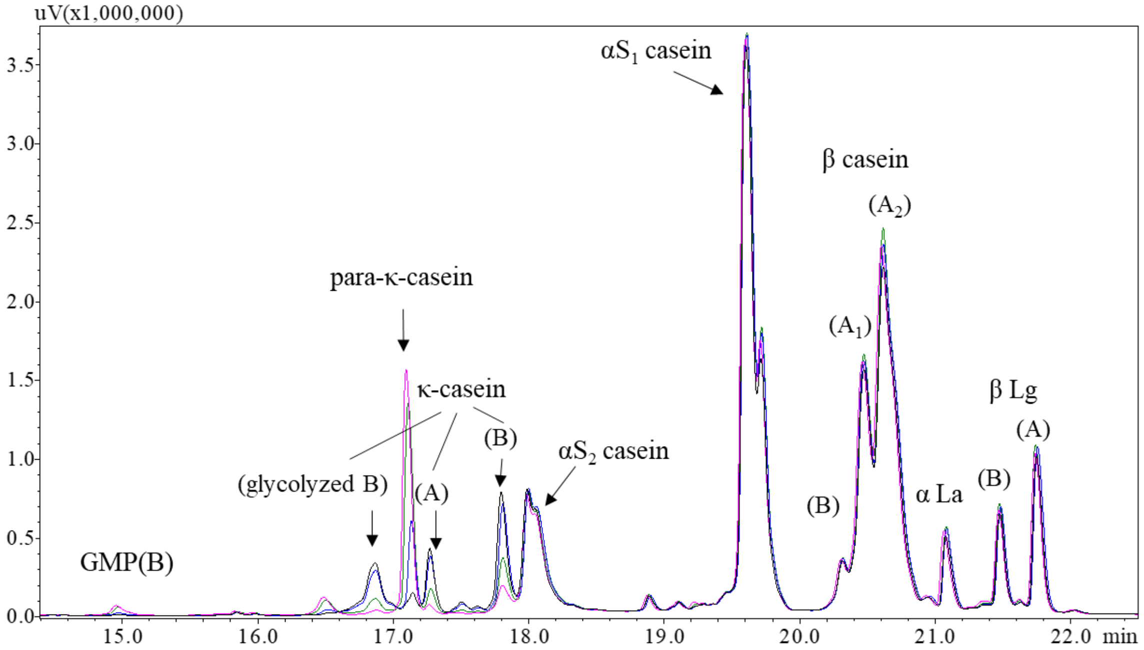

Figure S 3-1 Overlay of UV chromatograms analyzing protein profiles before (−) and after addition of pepsin at 5 min (−), 20 min (−), 8 h (−). All major milk proteins were identified.

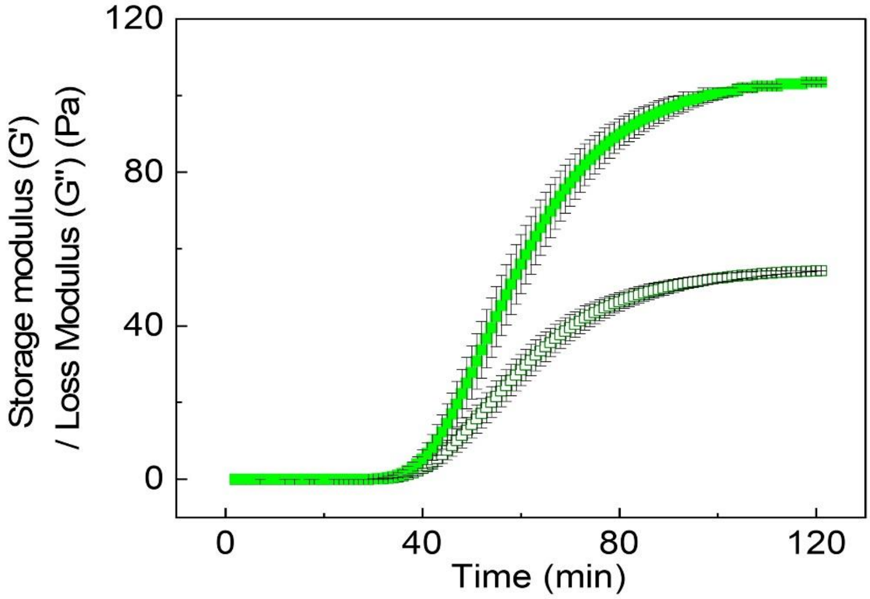

Figure S 3-2 The storage modulus (G') and loss modulus (G'') of a sample with time after the addition of pepsin at a concentration of 0.550 U/mL at pH 6.0 (■ G'; □ G'').

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Mengxiao Yang
Name and title of main supervisor:	Professor Aiqian Ye
In which chapter is the manuscript/published work?	Chapter 4

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Mengxiao Yang: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft.
Aiqian Ye: Conceptualization, Funding acquisition, Supervision, Resources, Writing – review & editing. Zhi Yang: Supervision, Writing – review & editing. David W. Everett: Supervision, Writing – review & editing. Elliot Paul Gilbert: Supervision, Writing – review & editing. Harjinder Singh: Supervision, Resources, Writing – review & editing.

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Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Effect of ingestion temperature on the pepsin-induced coagulation and the in vitro gastric digestion behavior of milk. Food Hydrocolloids. https://doi.org/10.1016/j.foodhyd.2023.108550

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Chapter 4 Effect of ingestion temperature on the pepsin-induced coagulation and the *in vitro* gastric digestion behavior of milk[3]

Abstract

Pepsin-induced protein coagulation occurs in the gastric environment when the milk pH is above the isoelectric point of casein proteins. In this study, the effect of milk temperature (4–48 °C) on the hydrolysis of κ-casein by pepsin and the consequent protein coagulation was studied at pH 6.0 for 120 min. Quantitative determination of the released para-κ-casein showed that both the κ-casein hydrolysis reaction rate constant and the pepsin denaturation rate constant increased with an increase in temperature. The temperature coefficient (Q_{10}) of the specific hydrolysis of κ-casein was calculated to be ~ 1.95. The coagulation process was investigated by the evolution of the storage modulus (G'). At higher temperature, the milk coagulated faster but had a lower firming rate and $G'_{\max}$ with larger aggregates and voids were observed. The digestion behavior of the milk ingested at 4 °C, 37 °C, or 50 °C was investigated for 240 min in a human gastric simulator, in which the milk temperature increased or decreased to 37 °C (body temperature) over ~ 60 min. The coagulation of the 4 °C milk was slower than for the 37 °C and 50 °C milk. The curd obtained from the 4 °C milk had a looser and softer structure with a significantly higher moisture content at the initial stage of digestion (20 min) which, in turn, facilitated the breakdown and hydrolysis of the caseins by pepsin. During the digestion, the curd structure became more cohesive, along with a decrease in moisture content. The knowledge gained from this study provides insight into the effect of temperature on the kinetics of pepsin-induced milk coagulation and the consequent digestion behavior.

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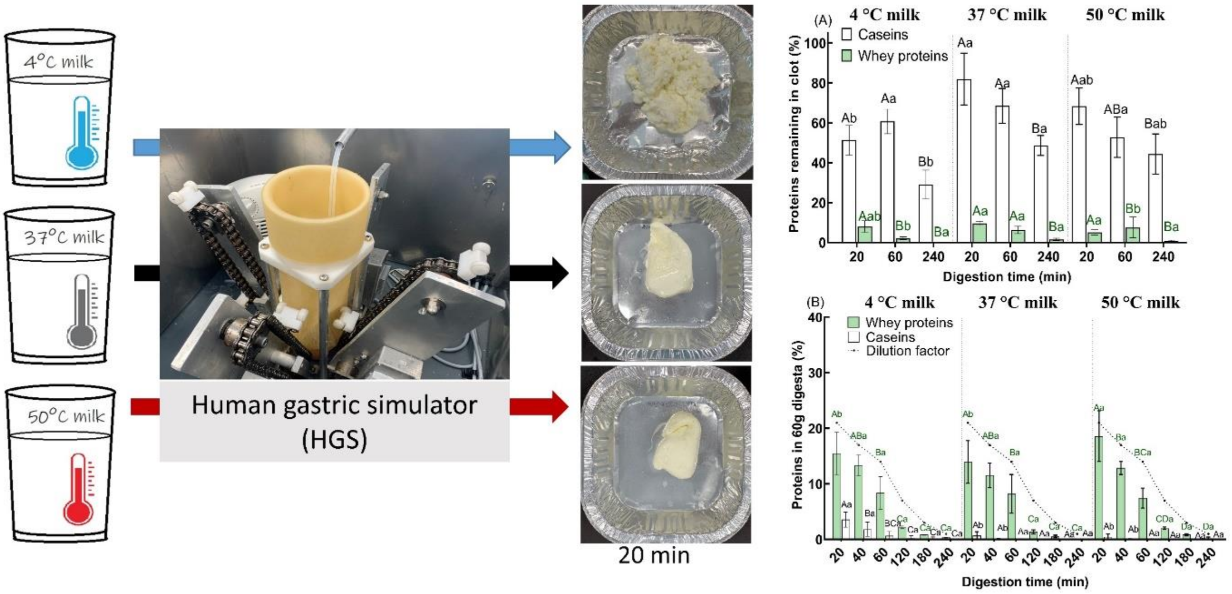
[3] This chapter has been published as Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Effect of ingestion temperature on the pepsin-induced coagulation and the in vitro gastric digestion behavior of milk. Food Hydrocolloids, 139, 108550.

Graphical abstract



4.1. Introduction

Milk protein coagulation, including the specific hydrolysis of κ-casein and protein aggregation, is the initial step during gastric digestion of milk. Pepsin-induced hydrolysis and the coagulation behavior of the casein micelles in cow skim milk at 37 °C have been extensively investigated (Huppertz & Chia, 2021; Yang et al., 2022a; Ye et al., 2016b, 2017; Ye et al., 2019b) . Depending on their drinking habits, some people like to drink warm milk, whereas some prefer to drink cold milk. As temperature is known to influence pepsin activity (Zhao et al., 2011) and enzyme-induced coagulation behavior (Dalgleish, 1983; Horne & Lucey, 2014; Nájera et al., 2003), the temperature of the milk could influence the structure of the curd/clot that is induced by pepsin. The emptying of the stomach contents and release of nutrients would be expected to depend on the structural properties of the milk curd.

Pepsin hydrolyzes κ-casein specifically at the Phe^{105}–Met^{106} bond at pH > 5 with an optimum at pH ~ 6.0, which is similar to the action of chymosin on κ-casein (Yang et al., 2022a). The reaction rate constant for the chymosin-induced hydrolysis of κ-casein increases with an increase in temperature from 7 to 37 °C (Turhan & Mutlu, 1998). The temperature coefficient (Q_{10}), defined as the rate ratio of a given process taking place at temperatures differing by 10 units (°C or K), was used as a measure of the temperature sensitivity of chymosin-induced hydrolysis of κ-casein by van Hooydonk (1987). These authors reported that Q_{10} varied between 1.3 and 2.0 and that hydrolysis occurred even at 0 °C. However, the effect of temperature on pepsin-induced hydrolysis of κ-casein has not been fully explored.

The aggregation phase of para-casein micelles occurs due to hydrophobic association and a decrease in electrostatic repulsion after a certain level of (glyco)macropeptide from κ-casein has been removed. The effect of temperature on the chymosin-induced aggregation phase has been investigated (Dalgleish, 1983; Gunasekaran & Ay, 1996; Horne & Lucey, 2014; Nájera et al., 2003)The effect of temperature on the chymosin-induced aggregation phase has been investigated (Dalgleish, 1983; Gunasekaran & Ay, 1996; Horne & Lucey, 2014; Nájera et al., 2003). A greater effect of temperature has been found on the aggregation phase, with a Q_{10} of ~ 16 (Fox et al., 2017a). Aggregation occurs very slowly, or not at all, at temperatures < 15 °C (Fox et al., 2017a); the velocity of curd formation increases progressively from 20 to 42 °C but slows down at much higher temperatures because of denaturation of chymosin (Nájera et al., 2003). Mellema et al. (2002) demonstrated that rearrangement of the curd network structure was facilitated at higher temperature, resulting in soft curds at 35–36 °C, whereas hard or semihard curds formed at 31–32 °C. Panthi et al. (2019) and Ong, Dagastine, Kentish, & Gras (2011b) reported that set temperature had a significant effect on the formation of curd microstructure; the casein micelles appeared to be smaller and less crosslinked in curds coagulated at a lower temperature (28 °C) than at a higher temperature (36 °C). In addition, after coagulation, syneresis was reported to occur more extensively at the higher temperature (Panthi et al., 2019).

According to Sun et al. (1988), temperature, *i.e.,* 4 °C , 37 °C, or 50 °C, impacts the rate of gastric emptying of a radiolabeled isosmotic drink of orange juice in humans. The intragastric temperature returned to body temperature within 20–30 min of ingestion of the 4 °C and 50 °C drinks, which appeared to empty from the stomach more slowly than the 37 °C drink. However, Webber, Nouri, & Bell (1980) found that cold meals increased the rate of gastric emptying in a study on calves, which was associated with increased acid and pepsin secretion. Despite the importance of temperature on the coagulation of milk proteins, no reports on the effect of temperature of milk on its digestion behavior have been published; therefore, an understanding of the interactive effects of milk temperature on the hydrolysis of κ-casein, the consequent coagulation, the curd structure, and the subsequent digestion behavior is desirable.

The objective of this study was to investigate the pepsin-induced protein hydrolysis and aggregation characteristics of skim milk (pH ~ 6.0, which is the optimum pH for the hydrolysis of κ-casein) at different temperatures. In addition, the different digestion behavior of the milk ingested at 4 °C, 37 °C, or 50 °C was investigated using a dynamic *in vitro* gastric model, the human gastric simulator (HGS) (Kong & Singh, 2010). The knowledge gained provides better understanding of the effect of drinking temperature on the coagulation and digestion of milk in a gastric environment.

4.2. Materials and methods

4.2.1. Materials

Fresh bovine milk was obtained from Dairy Farm 4 (Massey University, Palmerston North, New Zealand) and was skimmed by centrifugation with a swing bucket rotor (Thermo Fisher Scientific Multifuge Heraeus 3SR+ centrifuge, Thermo Electron LED GmbH, Langenselbold, Germany) at 3,000 *g* and 4 °C for 15 min. The skimmed milk contained 0.1% fat, 4.3% protein and 4.7% lactose as determined by a MilkoScan FT120 (Foss Electric, Hillerød, Denmark). The pH of the skim milk samples was measured to be ~ 6.7 at room temperature. Sodium azide was added at a concentration of 0.02% (wt/vol) to act as a bacteriostatic agent and stored for up to five days at 4 °C before experiments.

Pepsin (EC 3.4.23.1) from porcine gastric mucosa with an enzymatic activity of 550 U (mg protein)$^{-1}$ was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals (analytical grade) were also obtained from Sigma-Aldrich unless otherwise specified. Simulated salivary fluid (SSF, pH 7.0) and simulated gastric fluid (a 1.25 × concentrated electrolyte SGF, pH 1.5) were prepared according to Brodkorb et al. (2019).

4.2.2. Measurement of pepsin-induced milk hydrolysis and coagulation

The pH of skim milk samples was adjusted to 6.0 at 25 °C, by the gradual addition of SGF (1 × concentrate, pH 1.5) under vigorous stirring conditions (Yang et al., 2023b). Different temperature profiles were applied by using a cold room, a water bath, an oven or a rheometer cup: (1) the temperatures were maintained at 4, 15, 25, 37, 40, 43, and 48 °C for 120 min; (2) increased from 4 to 37 °C or decreased from 50 to 37 °C in 30 min and kept at 37 °C for 90 min (these last two samples were designated as C4 °C and W50 °C milk, respectively, to distinguish them from the samples held at a constant temperature of 4 °C). The pH of samples at each temperature did not change more than 0.1 unit. Porcine pepsin (2 mg) was dissolved in Milli-Q water (10 mL; 110 U mL^{-1}). The diluted pepsin solutions were added to the skim milk samples at a ratio of 10 µL per 1 mL of milk and resulted in a final pepsin concentration of 1.10 U $(mL\ milk)^{-1}$. Hydrolysis and aggregation occurred within 120 min at this pepsin concentration.

4.2.2.1. Measurement of κ-casein hydrolysis

RP-HPLC was used to quantify the release of para-κ-casein (Yang et al., 2022a). A 20 µL aliquot of diluted pepsin solution was added to the skim milk sample (2 mL), which was then immediately transferred into nine different test tubes, with 0.2 mL per tube, in a cold room or a water bath at each desired temperature. HPLC buffer solution (0.8 mL: 6 *M* guanidinium hydrochloride, 0.1 *M* bis-Tris, 19.5 m*M* dl-dithiothreitol, and 5.37 m*M* sodium citrate, pH 7) was subsequently added into each tube at different time points (1, 2, 5, 10, 20, 30, 60, 90, and 120 min) to stop the pepsin reaction. Each sample was shaken for 30 s, incubated for 1 h at room temperature and centrifuged before HPLC injection.

RP-HPLC analysis was carried out using a Nexera-X2 ultra-HPLC instrument equipped with an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). Separation was carried out using a Phenomenex Aeris WIDEPORE XB-C18 column (100 mm × 4.6 mm, 3.6 µm particles). The column temperature was maintained at 45 °C and the detection wavelength was 214 nm. Chromatographic runs were carried out with an injection volume of 10 μL at a flow rate of 0.8 mL min^{-1}, with (A) 0.1% (vol/vol) trifluoroacetic acid and (B) acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid as solvents. The following solvent gradients were then applied: 0–2.5 min, isocratic conditions, 10% B; 2.5–22 min, 10–49% B; 22–23 min, 49–10% B; 23–30 min, isocratic conditions, 10% B. The amount of para-κ-casein was quantified using LabSolutions Main (Shimadzu) software, based on the changes in the peak areas.

4.2.2.2. Measurement of protein coagulation

The coagulation process was carried out in a stress-controlled rheometer (MCR301 Anton Paar, Graz, Austria) equipped with a Couette geometry (Anton Paar; CC27, with a 28.93-mm cup diameter and a 26.64-mm bob diameter). Milk samples (20 mL) were equilibrated at each desired temperature for 15 min. After the addition of pepsin solution (200 μL), the samples were stirred for 30 s and loaded into the rheometer, which was set to the desired temperature. The C4 °C and W50 °C samples progressed through a temperature setting protocol. For the C4 °C sample, the temperature was gradually raised from 4–30 °C over the first 8 minutes, to 37 °C over 8 to 22 min and remaining at 37 °C to 120 min. For the W50 °C sample, the temperature was gradually decreased from 50–40 °C over the first 8 minutes, to 37 °C over 8 to 30 min and remaining at 37 °C to 120 min. A time sweep measurement was carried out at a constant frequency of 0.1 Hz with a strain of 1% (within the linear viscoelastic region as determined by strain sweep measurements). The storage modulus (G′) and the loss modulus (G″) were recorded every minute over 120 min.

4.2.2.3. Water-holding capacity of the milk curd

The water-holding capacity (WHC) of the samples was measured gravimetrically using a procedure from Yang et al. (2023b). Milk (10 g) was placed in a 15-mL centrifuge tube and treated with the same concentration of pepsin (100 µL) at 25, 31, 37, or 43 °C. The samples were then centrifuged at 500 *g* for 2 min at room temperature. The expelled whey was carefully removed and weighed. WHC was calculated as the weight of the pellet expressed as a percentage of the weight of the total sample.

4.2.2.4. Microstructural characterization of the curd

The microstructure of the pepsin-induced curd at different temperatures was examined using CLSM. Fast Green fluorescent dye (3 µL; 1% wt/vol, in distilled water) was added to the skim milk (100 µL) and stirred for 30 s. After prewarming to 25, 31, 37, or 43 °C, pepsin was added to the milk, reaching a final pepsin concentration of 1.10 U (mL milk)$^{-1}$. The milk–pepsin mixture was then transferred to the cavity of a glass microscope slide and covered with a glass cover slip. The glass slides were incubated in an oven at each desired temperature for 120 min. The samples were then examined using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 63× magnification lens at room temperature.

4.2.3. Dynamic gastric digestion

Dynamic gastric digestion was carried out using an HGS (Kong & Singh, 2010). The method described in Li et al. (2022c) was used in this study. Before the start of the gastric digestion, 20 mL of SGF (1 × concentrate, including pepsin) was added to the latex HGS chamber as the basal level in the fasted state. The oral phase of digestion was performed by mixing 20 g of SSF (prewarmed to 37 °C) into 200 g of milk (prewarmed to 4, 37, or 50 °C), equal to the solids content of the milk (Mulet-Cabero et al., 2020a). The temperature of 4 °C and 50 °C samples after mixing was recorded. To simulate gastric secretion, a 1.25 × concentrated electrolyte SGF (pH 1.5) and the pepsin solution (pepsin and $CaCl_2$) were pumped gradually into the latex gastric chamber separately at flow rates of 2.0 and 0.5 mL min^{-1}, respectively. The HGS simulated the peristaltic movement in the stomach chamber at a frequency of three times per minute. The digestion was carried out for 240 min at a set temperature of 37 °C. The temperature of the gastric chyme was measured by a digital thermometer and recorded every 5 min. The digesta were emptied every 20 min (3 mL min^{-1}) through a 1-mm sieve to mimic gastric sieving and stored at −20 °C for further compositional analysis. The pH profiles during the digestion were determined every 20 min by measuring the freshly emptied digesta at 25 °C. The dilution curves of whey proteins in 60 g of digesta were calculated during the period of SGF secretion (including pepsin) and egress of the digesta.

The curds were sampled at 20, 60, and 240 min of digestion in triplicate for further analysis. Solid curds were collected by passing the contents of the gastric chamber through a 1-mm sieve. The wet weight of the fresh curds was determined immediately after sampling. A sub-sample of each curd sample was freeze dried and ground into a powder for further compositional analysis, and another sub-sample dried at 105 °C for 24 h. The dry weight and moisture content was measured by gravimetric analysis, based on the wet weight ratio between the whole curd and the portion taken for oven drying (Li et al., 2022b).

4.2.3.1. Macro- and microstructures of the digesta and the curds

To compare the macrostructures of the curds and the digesta, photographs of the fresh curds and the digesta samples were taken. CLSM imaging of the fresh curds was carried out as described in Ye et al. (2017). CLSM imaging of the fresh curds was carried out as described in Ye et al. (2017).

4.2.3.2. Rheological analysis of the curds

The rheological properties of the fresh curds were determined according to Li et al. (2022b). The complex moduli (G^*) of the milk curds at 20, 60, and 240 min were recorded after a 10-min time sweep with a shear strain of 0.5 and a frequency of 1 Hz at 37 °C, using a rheometer (AR-G2; TA Instruments, Crawley, West Sussex, UK) with a parallel plate geometry (40-mm diameter) and a 2 mm gap.

4.2.3.3. Texture analysis of the curds

The curd texture was measured as described by Roy et al. (2021) using a TA-XT Plus texture analyzer (Stable Micro Systems, Surrey, UK), fitted with a 5.0-kg load cell and a 12.7-mm diameter cylinder probe. The curd was cut into 1.0 cm^3 cubes with a wire cutter immediately after sampling. Exponent software (version 6.1.15.0, Stable Micro Systems) was used for the analysis of results. The experiments were run, and the sample penetrated to a depth of 5.0 mm at a constant speed of 2.0 mm s^{-1}. The hardness value of the curds was calculated and expressed in Newtons.

4.2.3.4. Protein hydrolysis

The changes in the protein compositions of the curds and the emptied digesta were determined using RP-HPLC as described in **Section 4.2.2.1**. Sample buffer (0.8 mL) was added to 0.2 mL of digesta (immediately collected at 10, 20, 40, 60, 120, 180, and 240 min) or 8 mg of curd powder (collected at 20, 60, and 240 min and freeze dried). The protein content was determined by comparing the peak area of the proteins in the curds or digesta with that of the skim milk at each digestion time point.

4.2.4. Statistical analysis

Samples were prepared in triplicate from two different batches of milk, and the measurements of each sample were carried out in triplicate; values (or data points) are expressed as mean ± standard deviation. Experimental data were analyzed by running analysis of variance (ANOVA) tests using Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The hydrolysis kinetics and coagulation results were analyzed using one-way ANOVA with a Tukey post-hoc test. The changes in pH, wet and dry weights, moisture content, curd consistency, and protein hydrolysis were analyzed using two-way ANOVA, with the temperature of the ingested milk, the digestion time, and interaction (ingestion milk temperature × time) as fixed effects. If significant interaction effects were found, further analysis was carried out with Tukey post-hoc tests. Differences were considered significant at $P < 0.05$.

4.3. Results and discussion

4.3.1. Effect of temperature on the pepsin-induced hydrolysis of κ-casein and the coagulation of milk

4.3.1.1. Hydrolysis of κ-casein

Yang et al. (2022a) reported that the pepsin-induced hydrolysis of κ-casein is pH dependent, and that the hydrolysis rate reaches a maximum at pH ∼ 6.0 and 37 °C. **Figure 4-1 A** shows the degree of κ-casein hydrolyzed to para-κ-casein (%, written as degree of hydrolysis in the rest of the manuscript) at pH 6.0 after the addition of pepsin at different temperatures as a function of the reaction time. The experimental data were fitted to the equation:

$$ln\left(1-\frac{H_t}{100}\right)=\frac{K_{\mathrm{enz}}\cdot C}{K_{\mathrm{den}}}\cdot\left[exp(-K_{\mathrm{den}}\cdot t)-1\right] \qquad \textbf{(Equation 4-1)}$$

The reaction rate constants K ($K_{\mathrm{enz}} \cdot \mathrm{C}$, min^{-1}) and K_{den} (min^{-1}) are shown in **Table 4-1**. Both K and K_{den} were significantly affected by temperature ($P < 0.05$). This indicates that the hydrolysis of κ-casein, as induced by pepsin, was markedly temperature-dependent. The Arrhenius plot (**Figure 4-1 B**) shows the effect of temperature on the rate of the hydrolysis reaction. The Q_{10} for κ-casein hydrolysis induced by pepsin was calculated to be ~ 1.95, which is in the range of Q_{10} values for κ-casein hydrolysis by chymosin (1.3–2.0) (van Hooydonk, 1987). Relatively high K values and low K_{den} values were found at 37−40 °C, indicating that the optimum temperature for pepsin to specifically hydrolyze κ-casein was around 37−40 °C. The highest K_{den} value was found at 48 °C, indicating that extensive pepsin denaturation occurred at this temperature. As a result, after 20 min, the degree of hydrolysis of κ-casein reached a plateau at around 60% (**Figure 4-1 A**), which was lower than for the samples at 31−43 °C.

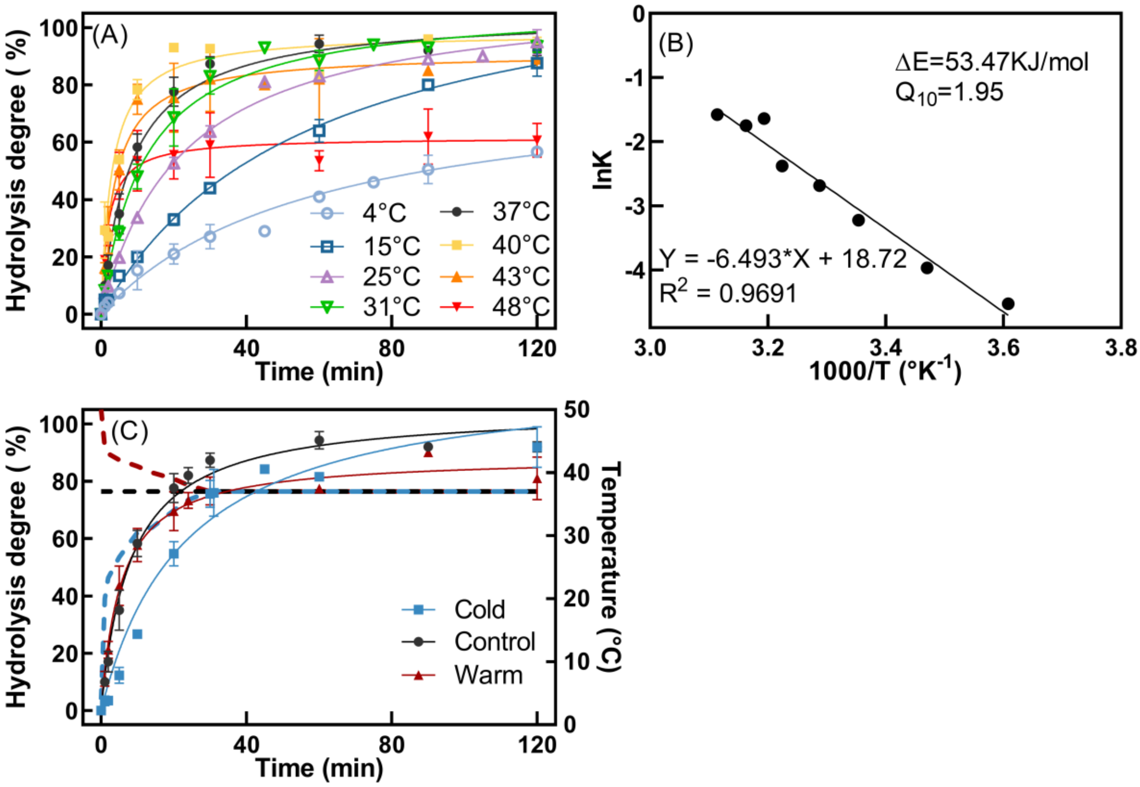


Figure 4-1 (A) Degree of hydrolysis of κ-casein in skim milk as a function of time after the addition of 1.10 U pepsin mL^{-1} at different temperatures (○ 4°C; □ 15°C; △ 25°C; ▽ 31°C; ● 37°C; ■ 40°C; ▲ 43°C; ▼48°C). (B) Arrhenius plot of the reaction rate constant together with a least-squares linear fit through the experiment points. (C) Temperature profile of the C4 °C (---), 37 °C (---), and W50 °C (---) milk samples during the measurement and the degree of hydrolysis of κ-casein in C4 °C (■), 37 °C (●), and W50 °C (▲) milk. Error bars represent standard deviations from triplicate measurements.

The hydrolysis of the C4 °C and W50 °C milks was compared with that of the 37 °C milk (**Figure 4-1 C**). The dashed curves show the change in temperature of the C4 °C, 37 °C, and W50 °C milks, which increased or decreased to 37 °C over a period of 30 min. The W50 °C milk had significantly higher ($P < 0.05$) K and K_{den} values than the 37 °C and C4 °C milks. Therefore, the initial hydrolysis reaction rate between pepsin and κ-casein was faster in the

W50 °C milk than in the 37 °C and C4 °C milks. Due to the more extensive denaturation of pepsin, the final degree of hydrolysis at 120 min was relatively lower for the W50 °C milk, at ~ 80%.

Table 4-1 Hydrolysis kinetics of κ-casein in skim milk at pH 6.0 with the addition of 1.1 U pepsin mL^{-1}.

	Hydrolysis kinetics according to Eq. (1)	
Sample	**K (min^{-1})**	**K_{den} (min^{-1})**
4 °C	0.0119 ± 0.0041^{e}	0.011 ± 0.009^{cd}
15 °C	0.0208 ± 0.0005^{e}	0.004 ± 0.004^{cd}
22 °C	0.0439 ± 0.0038^{de}	0.013 ± 0.005^{cd}
31 °C	0.0750 ± 0.0127^{cde}	0.023 ± 0.003^{cd}
37 °C	0.1021 ± 0.0187^{cd}	0.035 ± 0.008^{cd}
40 °C	0.2137 ± 0.0275^{ab}	0.081 ± 0.012^{bc}
43 °C	0.1910 ± 0.0384^{ab}	0.105 ± 0.007^{b}
48 °C	0.2272 ± 0.0533^{a}	0.267 ± 0.072^{a}
C4 °C	0.0432 ± 0.0013^{de}	0.007 ± 0.006^{d}
W50 °C	0.1257 ± 0.0101^{bc}	0.076 ± 0.022^{bc}

K, Enzymatic reaction rate constant ($K_{enz} \cdot C$); K_{den}, Denaturation rate constant.

$^{a–e}$ Mean values between samples in the same column with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of the mean ($n = 3$).

4.3.1.2. Rheological properties.

The evolution of the storage modulus (G') as a function of time in the milk samples at various set temperatures (25, 31, 37, and 43 °C) is shown in **Figure 4-2 A**. Values for the coagulation time, the firming rate (dG'/dt), and G'_{max} are summarized according to Yang et al. (2022a) in **Table 4-2**; all values were significantly affected by temperature ($P < 0.001$). At 25 °C, there was no significant increase in G' within 120 min (although it may have increased with a longer incubation time). The coagulation time decreased from ~ 39 to 13 min when the temperature was increased from 31 to 43 °C, in agreement with work of Panthi et al. (2019) on chymosin-induced coagulation. This probably occurred because of an increase in hydrophobic associations and enhanced enzymatic activity, which led to the formation of aggregates earlier in the coagulation process (Mishra et al., 2005).

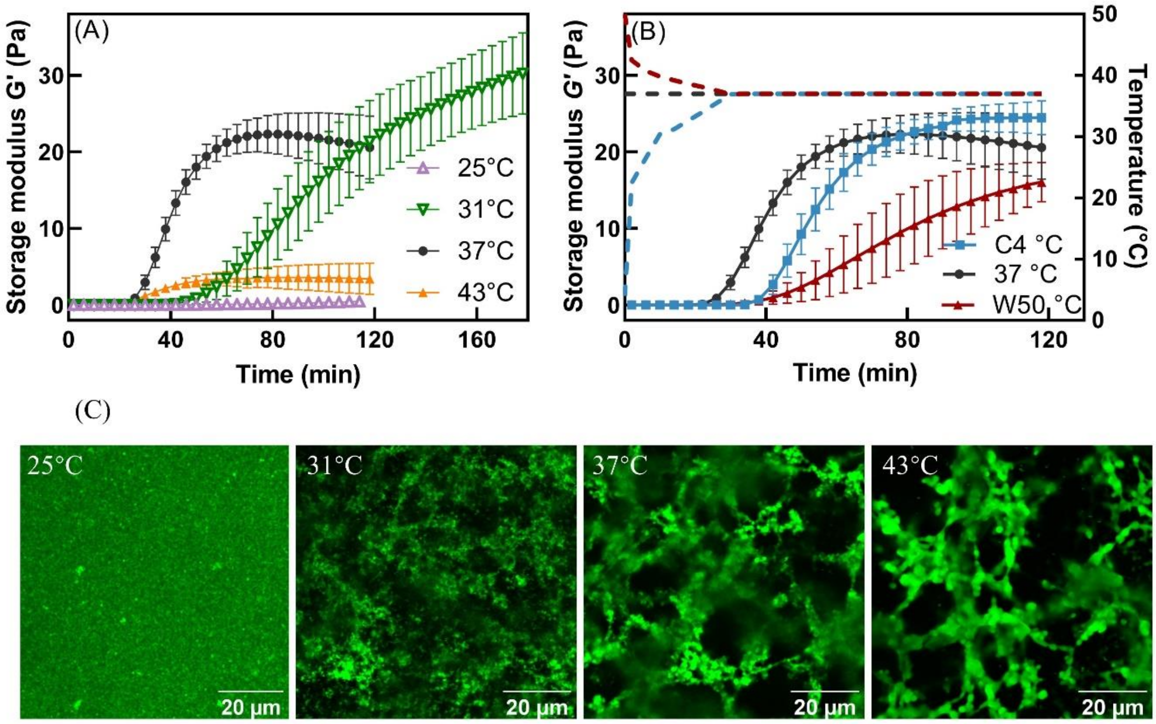


Figure 4-2 (A) Storage modulus (*G*′) of the pepsin-induced coagulum in samples at different constant set temperatures (△ 25 °C; ▽ 31 °C; ● 37 °C; ▲ 43 °C). (B) *G*′ of the pepsin-induced coagulum in C4 °C (■), 37 °C (●), and W50 °C (▲) milk at pH 6.0 and temperature profile of the C4 °C (---), 37 °C (---), and W50 °C (---) milk samples during the associated measurements. Error bars represent standard deviations from triplicate measurements. (C) Microstructures of the pepsin-induced coagulum at 120 min at different constant set temperatures (25, 31, 37, 43 °C) as analysed by confocal laser scanning microscopy. The scale bars are 20 μm in length for all micrographs.

The degree of hydrolysis at the coagulation time (H_{ct}) was calculated by substituting the coagulation time into the hydrolysis equation (**Equation 4-1)**, and the results are shown in **Table 4-2**. Coagulation began before the end of the primary hydrolysis phase, when the degree of hydrolysis was ~ 83, 75, and 71% at 31, 37, and 43 °C, respectively. This indicates that relatively less degree of hydrolysis of κ-casein was required for coagulation to occur at higher temperatures, in agreement with Carlson, Hill Jr, & Olson (1987c) and Garg & Johri

(1994). Greater hydrophobic association and diffusion (due to Brownian motion) between casein molecules is expected at higher temperatures (Dalgleish, 1983; Horne & Lucey, 2014; O'meara & Munro, 1982). Since the increasing number of hydrophobic domains in casein, generated from the hydrolysis process, seeks to reduce contact with water molecules, a lowering of energy favors self-association; an increased frequency of collisions between the para-casein micelles would facilitate coagulation at a lower degree of κ-casein hydrolysis.

Table 4-2 Effect of temperature on the coagulation properties of skim milk at pH 6.0 with the addition of 1.1 U pepsin mL^{-1}.

	25 °C	31 °C	37 °C	43 °C	C4 °C	W50 °C
Coagulation time (min)	> 120	39.48 ± 5.17^{b}	20.80 ± 1.08^{c}	13.05 ± 2.14^{d}	33.43 ± 2.64^{b}	25.26 ± 2.96^{c}
Firming rate (Pa min^{-1})	–	0.41 ± 0.06^{b}	0.95 ± 0.06^{a}	0.13 ± 0.03^{d}	0.91 ± 0.12^{c}	0.34 ± 0.05^{b}
G'_{max} (Pa)	–	28.60 ± 1.91^{a}	22.57 ± 2.75^{b}	4.04 ± 1.09^{d}	23.93 ± 1.81^{ab}	16.33 ± 2.27^{c}
H_{ct} (%)	–	83 ± 2^{b}	75 ± 1^{c}	71 ± 3^{cd}	69 ± 3^{d}	74 ± 2^{cd}
WHC (%)	–	58.46 ± 9.46^{a}	19.64 ± 2.25^{b}	12.11 ± 1.28^{c}	–	–

G'_{max}, maximum storage modulus value during the measuring time; H_{ct}, degree of hydrolysis of κ-casein at the coagulation time; WHC, water-holding capacity.
$^{a–d}$ Mean values between samples in the same row with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of the mean ($n = 3$).

The maximum slope of the G' curve (dG'/dt) is defined as the firming rate and was significantly higher ($P < 0.05$) at 37 °C than at 25, 31, and 43 °C (**Table 4-2**). **Figure 4-2 A** shows that the G' of the sample at 37 °C was higher than that of the sample at 31 °C until ~ 115 min. However, beyond 115 min, the G' of the sample at 31 °C increased continually whereas that of the sample at 37 °C reached a plateau (decreasing slightly); thus, a higher G' was found in the later stages at 31 °C. Therefore, as shown in **Table 4-2**, G'_{max} decreased with increasing temperature. This is consistent with Mellema et al. (2002), who reported that a higher G' of chymosin-induced curd was found at a higher temperature (25 and 30 °C were compared), as long as the curd was not fully developed (*i.e.*, in the early stage of coagulation). As mentioned above, stronger hydrophobic association and increased frequency of collisions between the para-casein micelles perhaps resulted in more rapid intra-/interparticle protein rearrangements (types A and B according to Mellema et al. (2002)). This led to the formation of a stronger protein network at higher temperatures up to 37 °C, as indicated by the faster G' development (Sandra, Cooper, Alexander, & Corredig, 2011). In the later stages of coagulation, the lower G' at 37 °C could have been due to the more extensive inter-cluster rearrangements of the curd at 37 °C, resulting in syneresis, as indicated by a decrease in G' after 80 min. For the sample at 43 °C, G' was lower than that for the sample at 37 °C. There is little published information on the rheological properties of enzyme-induced coagulated milk at temperatures > 40 °C. However, as the fusion of para-casein micelles is expected to increase rapidly at higher temperatures because of greater hydrophobic association, faster syneresis would occur concurrently; the evolution of G' could therefore be related to the fact that the increase in firmness of the coagulum was masked by syneresis (Lagoueyte, Lagaude, & De La Fuente, 1995). In addition, according to Zoon, van Vliet, & Walstra (1988), G' was lower at 40 °C than at 35 °C, probably because of the inactivation of chymosin at the higher temperature. In the present study, the rate of hydrolysis at 43 °C was rapid during the first 10 min (**Figure 4-1 A**); however, the degree of hydrolysis reached a plateau at ~ 80% due to denaturation of pepsin. Fewer exposed para-casein surfaces in the curd network could also attribute the lower firming rate and lower G'_{max}.

The evolution of G' for the C4 °C and W50 °C milks was compared with that of the 37 °C milk in **Figure 4-2 B** and the rheological parameters are summarized in **Table 4-2**. The C4 °C and W50 °C milks coagulated at ~ 33 and 25 min, respectively, when ~ 70% of the caseins had been hydrolyzed. The coagulation time was longer for the C4 °C milk than for the 37 °C and W50 °C milks. Dissociation of β-casein has been reported in 4 °C milk, which could generate more space internally in the casein micelle (Zhang et al., 2018), and resulted in the longer coagulation time. On the other hand, it may have been related to the hydrolysis kinetics, in that, during the first 40 min, the degree of hydrolysis of κ-casein in the C4 °C milk was relatively lower than that in the other two milks (**Figure 4-1 C**). As the aggregation was markedly dependent on the temperature, the lower temperature resulted in slower aggregation. The firming rate and $G'_{\max}$ of C4 °C milk were ~ 0.91 Pa $\min^{-1}$ and 23.93 Pa, respectively, which were similar to those of the 37 °C milk. However, the firming rate and $G'_{\max}$ for the W50 °C milk were 0.34 Pa $\min^{-1}$ and 16.33 Pa, respectively, i.e., lower than for the other two milks. As the hydrolysis reaction rate constant K_{enz} and the denaturation rate constant K_{den} were similar for the C4 °C and the 37 °C milk ($P > 0.05$), the developments of G' were similar. The relatively lower firming rate and $G'_{\max}$ for the W50 °C milk can be attributed to the hydrolysis kinetics. Even though the temperature decreased to 37 °C after around 30 min, the denaturation of the pepsin was relatively high for the W50 °C milk. The degree of hydrolysis of the casein micelles was maintained at ~ 80% from 40 to 120 min, lower than for the C4 °C and 37 °C milk samples (**Figure 4-1 C**). As a consequence, fewer exposed para-casein surfaces were involved in curd formation, resulting in the lower $G'_{\max}$.

4.3.1.3. Water-holding capacity and microstructure of the curd

As shown in **Table 4-2**, the WHCs of the curds were significantly influenced by temperature, *i.e.*, highest at 31 °C, followed by 37 and 43 °C. In agreement with Teo, Munro, Singh, & Hudson (1996), the lower WHC could be largely attributed to increased hydrophobic association between the casein molecules with an increase in temperature. In addition, the difference in WHCs of the curds could also be related to the network structures formed during coagulation. As shown in **Figure 4-2 (C)**, temperature had a significant effect on the curd microstructure. Except for the sample at 25 °C, which did not show a network structure the casein micelles assembled into strands and were interconnected by large numbers of crosslinks in the samples at 31, 37, and 43 °C. A lower level of crosslinks with larger strands was observed in the samples at higher temperature. This is consistent with the findings of Ong et al. (2011a), who observed that chymosin-induced curds formed a finer network when the milk was treated at 27 °C compared with 36 °C. At 43 °C, the casein aggregates and the pores in the network were large. This may have been due to the rapid formation of larger aggregates at temperatures > 40 °C (Muñoz, Torres, Guerrero, & Talavera, 2017) and extensive inter-cluster rearrangements (Mellema et al., 2002). Under an external centrifugal force (500 *g*), the larger pores in the samples at higher temperatures may have been more easily destroyed, with water (liquid) being expelled, resulting in a lower WHC.

4.3.2. *In vitro* digestion behavior of 4 °C, 37 °C and 50 °C milks

4.3.2.1. Temperature and pH changes during digestion.

The temperature of the HGS was set at 37 °C (body temperature). The temperature profiles of the milks during digestion are shown in **Figure 4-3 A**. The 4 °C milk at 4 °C gradually increased to 37 °C over 60 min; the 50 °C milk at 50 °C decreased to 37 °C over 60 min. The digesta were collected every 20 min and the pH was measured at 25 °C. The pH values decreased from ~ 6.7 to ~ 2.0 over 240 min (**Figure 4-3 A**) and were significantly affected by the digestion time and the temperature of the ingested milk ($P < 0.001$). The pH of the 4 °C milk decreased more slowly than those of the 37 °C and 50 °C milks during the first 40 min (0.2–0.3 units higher), indicating a slightly higher buffering capacity of the milk at the lower temperature. From 40 to 240 min, the pH profile became similar for all three samples.

4.3.2.2. Macro-/microstructures of the curd and digesta during digestion

Gastric coagulation of all milk samples occurred at pH > 6.0, in agreement with Mulet-Cabero et al. (2019) and Ye et al. (2017). During the experiments, protein coagulation was visible immediately after the addition of 37 °C and 50 °C milk into HGS, whereas visible coagulation occurred at a later time for the 4 °C milk. The late coagulation of the 4 °C milk was consistent with the observation that C4 °C milk had a longer coagulation time than the

37 °C and 50 °C milks at pH 6.0 (**Table 4-2**). The appearances of the digesta are shown in **Figure 4-3 B**. At 20 min, the digesta of 4 °C milk was more turbid than the 37 °C and W50 °C milks. This explains the relatively higher pH of the 4 °C milk during the first 40 min which arises from the buffering capacity of CCP and milk proteins in the liquid phase. In general, the digesta became clearer with an increase in the digestion time, and lower turbidity could be observed after 240 min of digestion.

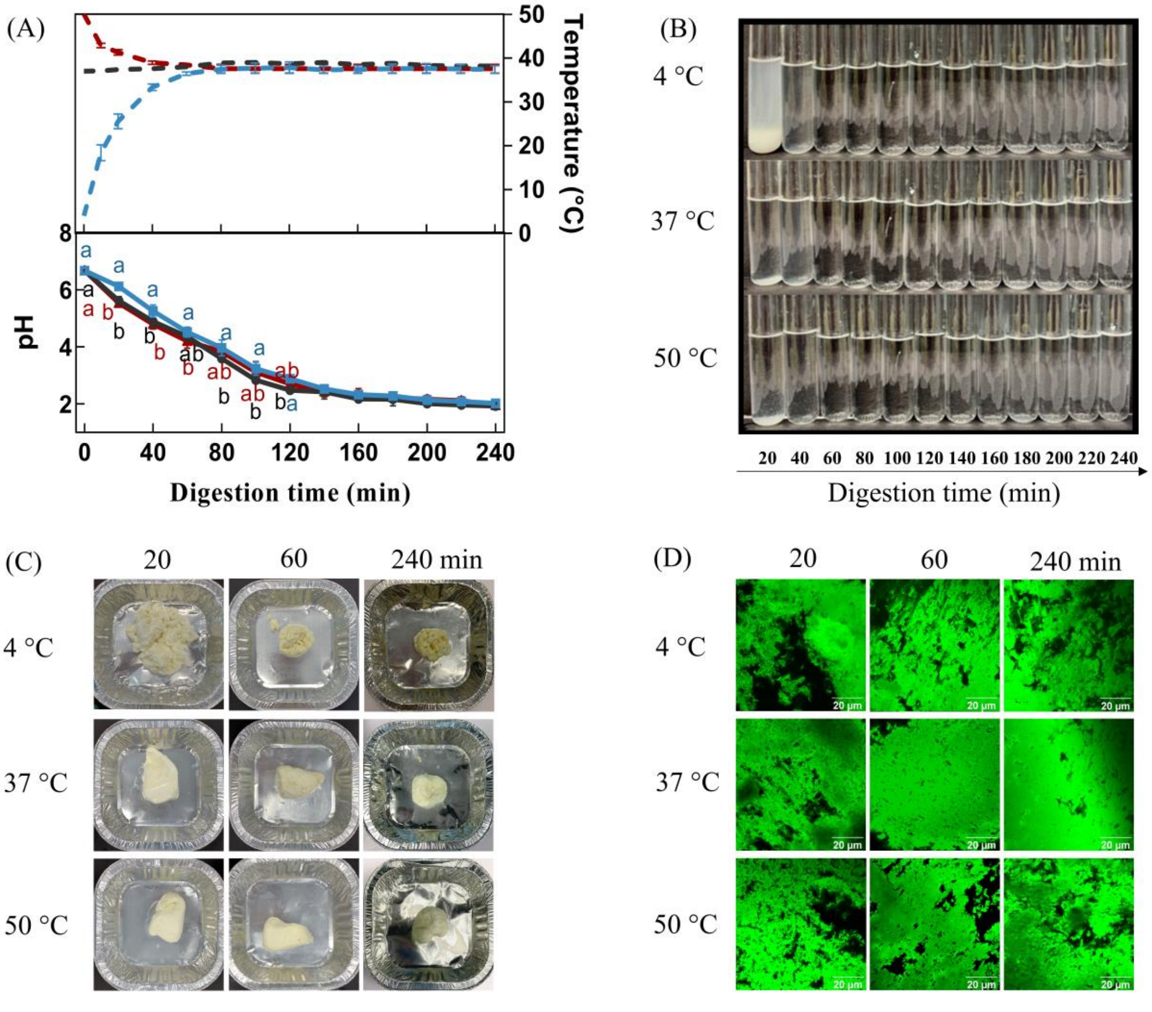


Figure 4-3 (A) Measured pH (solid curves with symbols) and temperature (dashed curves) profiles of the gastric digesta during 240 min of digestion. Blue color: 4 °C milk; black color: 37 °C milk; Red color: 50 °C milk. $^{a–c}$ Mean values between samples with different superscripts are significantly different ($P < 0.05$). Error bars represent standard deviations from triplicate measurements. (B) Photographs of emptied digesta from 20 to 240 min. (C) Photographs (macrostructure) and (D) confocal micrographs (microstructure) of the coagulum formed following 20, 60, and 240 min of gastric digestion in the human gastric simulator.

Photographs of the curds formed during gastric digestion of the 4 °C, 37 °C and 50 °C milks at 20, 60 and 240 min are presented in **Figure 4-3 C**. After 20 min of digestion, the curds formed from the 37 °C and 50 °C milks were intact with fairly smooth surfaces, whereas the curd formed from the 4 °C milk was crumbly and fragmented with small granules. After further digestion, at 60 min, the 4 °C milk curd was firmer with a smooth surface. At this stage, the serum became clear indicating that most of the casein was incorporated into the curd. At 240 min, the entire block of the curd was smaller but remained integrated. These results indicate that the initial temperature of the milk could lead to the formation of differently structured curds during the early stage of digestion.

CLSM images of the curd samples at 20, 60, and 240 min are given in Fig. 3D and reveal the protein microstructure of the curds. These images are consistent with curd formation in the HGS that was observed visually (macrostructure, **Figure 4-3 C**). At 20 min, the curd network consisted of a loose, irregular protein matrix with tiny pores. After 240 min of digestion, there were fewer pores in all samples, which may have been due to contraction and exchange of water between the curd and the gastric fluid during digestion. Liquid in the pores was expelled with mechanical deformation of the curd. The protein matrix for the 4 °C curd sample appeared to be loose, with an open and porous structure at all times during digestion. The microstructure of the 37 °C curd sample was denser than those of the other two samples at all time points.

4.3.2.3. Curd weights and moisture

The dry matter content, water content, and wet weight (total content) of the curds at 20, 60, and 240 min are presented in **Figure 4-4 A**. The wet weight and the water content were significantly affected by the digestion time and by the interaction between the digestion time and the temperature of ingested milk ($P < 0.001$). The wet weight of the curds followed the order 4 °C > 37 °C ≈ 50 °C at the beginning of the digestion (20 min) and the order 4 °C <

37 °C ≈ 50 °C after 60 min of digestion. The wet weights of all curds decreased significantly over the period of digestion. This occurred because more proteins in the curd were hydrolyzed into peptides or amino acids during digestion (discussed in **Section 4.3.2.5**); thus, the dry matter content decreased with an increase in digestion time. In addition, water was expelled with mechanical deformation of the curds by peristaltic movement in the HGS. At each digestion time, the wet weights of the curds formed from the 37 °C and 50 °C milks did not vary significantly ($P > 0.05$), whereas the wet weight of the curd formed from the 4 °C milk was relatively higher ($P < 0.05$) at 20 min. This can be explained by the structure of the protein matrix during the early stages of digestion. It is expected that fragmented curds would have larger surface areas which would bind more water (Li et al., 2022b). As mentioned previously, the curd structure for the 4 °C milk was relatively looser and a greater number of small particles passed into the liquid phase, resulting in the highest water content and the lowest dry matter content at 20 min. In the later stages (60 and 240 min), the water content in the 4 °C milk curd decreased significantly and became similar to those of the other two curds. The water contents of the curds formed from the 37 °C and 50 °C milks were similar and remained constant throughout the whole digestion process. In summary, the curd weights of the 37 °C and 50 °C milks were similar at each digestion time, whereas a lower total weight was found in the curd formed from the 4 °C milk after 240 min of digestion.

4.3.2.4. Rheological and texture analysis of the curds

Figure 4-4 B presents the G^* values for the curds formed by the 4 °C, 37 °C and 50 °C milks at different digestion times. The G^* value can be used as an indicator of curd consistency (Li et al., 2022b; Mulet-Cabero et al., 2019). The G^* values of the curds formed from the 37 °C and 50 °C milks did not change markedly during digestion ($P > 0.05$), at around 2,000 Pa. However, the G^* value of the curd formed from the 4 °C milk was ~ 84 Pa in the early stages of digestion (20 min), which was significantly lower ($P < 0.05$) than those of the curds formed from the 37 °C and 50 °C milks. The *G** value of the curd formed from the 4 °C milk increased significantly and became similar to those of the curds formed from the 37 °C and 50 °C milks with an increase in digestion time (at 60 and 240 min).

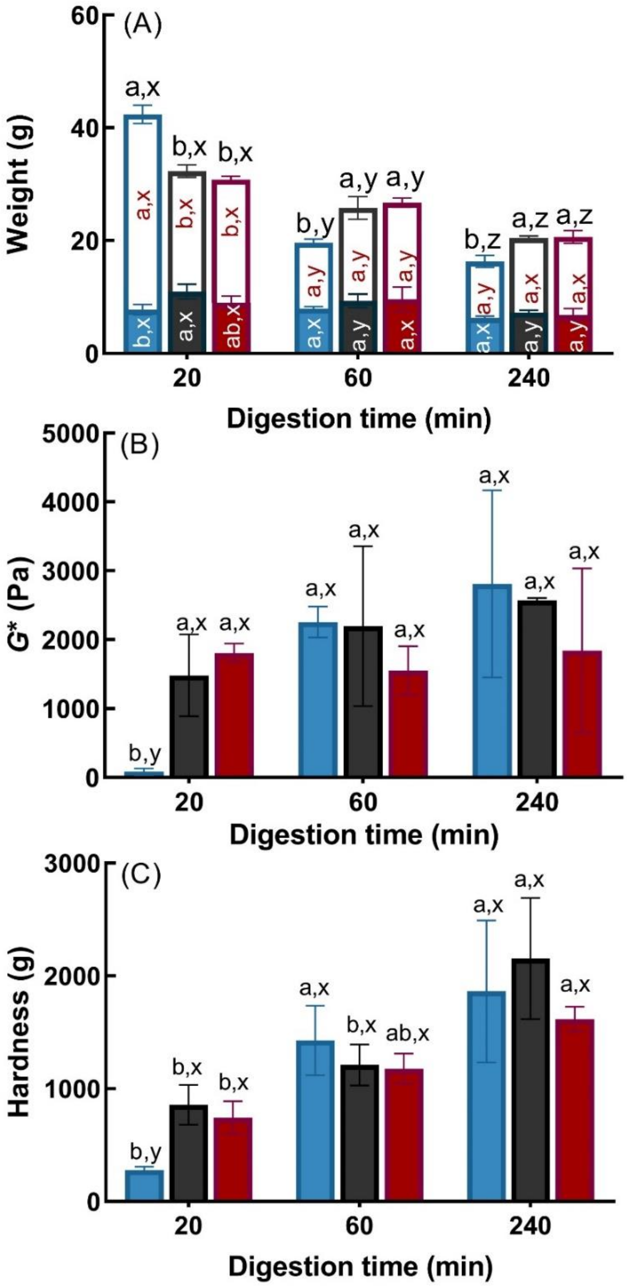


Figure 4-4 (A) Dry matter weight (bottom) and water weight (top) of fresh clots formed during the gastric digestion at 20, 60, and 240 min. (B) *G** values of the coagulum formed during the gastric digestion at 20, 60, and 240 min. (C) Hardness of the coagulum formed during the gastric digestion at 20, 60, and 240 min. Blue color: 4 °C milk; black color: 37 °C milk; Red color: 50 °C milk. $^{x–z}$ Mean values for the same sample at different digestion times with different superscripts are significantly different ($P < 0.05$). $^{a–b}$ Mean values between samples at the same digestion time with different superscripts are significantly different ($P < 0.05$). Error bars represent standard deviations from triplicate measurements.

According to Roy et al. (2021), texture analysis gives an indication of the heterogeneous and dynamic nature of the curds during gastric digestion. The hardness of the curds formed by the 4 °C, 37 °C and 50 °C milks at different digestion times are shown in **Figure 4-4 C**. A significantly lower hardness ($P < 0.05$) was found in the curd formed from the 4 °C milk at 20 min. However, no significant differences ($P > 0.05$) were detectable for hardness at 240 min for all samples.

During digestion, a structural rearrangement of the protein network may have taken place due to mechanical peristaltic movement and more water may have been expelled from the curd over time. According to Li et al. (2022b) and Roy et al. (2021), the consistency and the texture of the curd are negatively correlated with moisture content. Therefore, this contributed to the formation of a stronger curd (higher values of G^* and hardness) in the HGS during the later stages of dynamic gastric digestion. The lowest consistency and hardness were found in the 4 °C milk curd at 20 min. This could be attributed to the more open structure (**Figure 4-5 B** and **C**) and the higher moisture content (**Figure 4-4 A**) that were observed in the 4 °C milk curd at 20 min.

4.3.2.5. Protein content in the curds and digesta during digestion

The changes in specific protein components in the curds and the digesta during digestion, as determined by RP-HPLC, are shown in **Figure S 4-1** and **Figure S 4-2**, respectively. Based on the peak area, the amount of each major protein (αs_1-casein, αs_2-casein, β-casein, κ-casein, whey proteins) was calculated. The percentage of each major protein remaining in the curd or digesta was calculated as the ratio of each protein remaining in the curd or digesta to each protein in the 200 mL of milk that was ingested in the HGS (wt/wt). **Figure 4-5 A** shows the percentages of casein and whey proteins remaining in the curd, and **Figure 4-5 B** shows the percentages remaining in the digesta. The hydrolysis of casein proteins in the curds and digesta was significantly affected by the digestion time ($P < 0.05$) and by the temperature of the ingested milk ($P < 0.05$). The hydrolysis of the whey proteins in the curds and digesta was significantly affected by the digestion time ($P < 0.05$) but was not significantly affected by the temperature of the ingested milk ($P > 0.05$). In agreement with previous research, most of the whey proteins in unheated samples are soluble and present in the liquid digesta (Ye et al., 2016b). The small amount of whey proteins observed in the curds may have been caused by entrapment during the formation of the curds; these could have been expelled from the curds and gradually hydrolyzed as digestion progressed.

The percentages of each individual casein protein remaining in the curd during digestion are shown in **Figure 4-5 A1–A4**. At 20 min, little of the intact κ-casein (12, 8, and 10% for the 4 °C, 37 °C, and 50 °C milks, respectively) remained in the curds of all milk samples. Because of the rapid specific hydrolysis at pH > 6, κ-casein was expected to be hydrolyzed to para-κ-casein within the first several minutes. A decrease in intact proteins and an increase in peptides was observed after 240 min of digestion for all samples (**Figure S 4-1** and **Figure S 4-2**). Due to the slower coagulation and looser structure (**Figure 4-3 C**) of the 4 °C milk sample, some of the casein proteins and/or some small crumbly particles (containing mostly caseins) were still in the liquid phase (**Figure 4-5 B**). From 20 to 60 min of digestion, these casein proteins gradually assembled into the curds, which resulted in the relatively higher average value of caseins (especially αs_1-casein, **Figure 4-5 A1**) for the 4 °C sample in **Figure 4-5 A**. After 240 min of digestion, the lowest percentages of caseins were found in the 4 °C milk curd, whereas there was no significant difference ($P > 0.05$) between the 37 °C and 50 °C milk curds (**Figure 4-5 A**). This indicated that 4 °C milk led to a faster release rate of the casein proteins during gastric digestion. Faster degradation of proteins has been shown to be caused by the looser and crumbly structure of curds at the early stage of digestion, which allows pepsin to diffuse into the curds rapidly and to hydrolyze the proteins (Ye et al., 2017). There was no significant difference between the amount of each individual protein as a fraction of total protein in the curds (results not shown) during the whole period of digestion, indicating that the hydrolysis rate of each protein type by pepsin was similar in each curd.

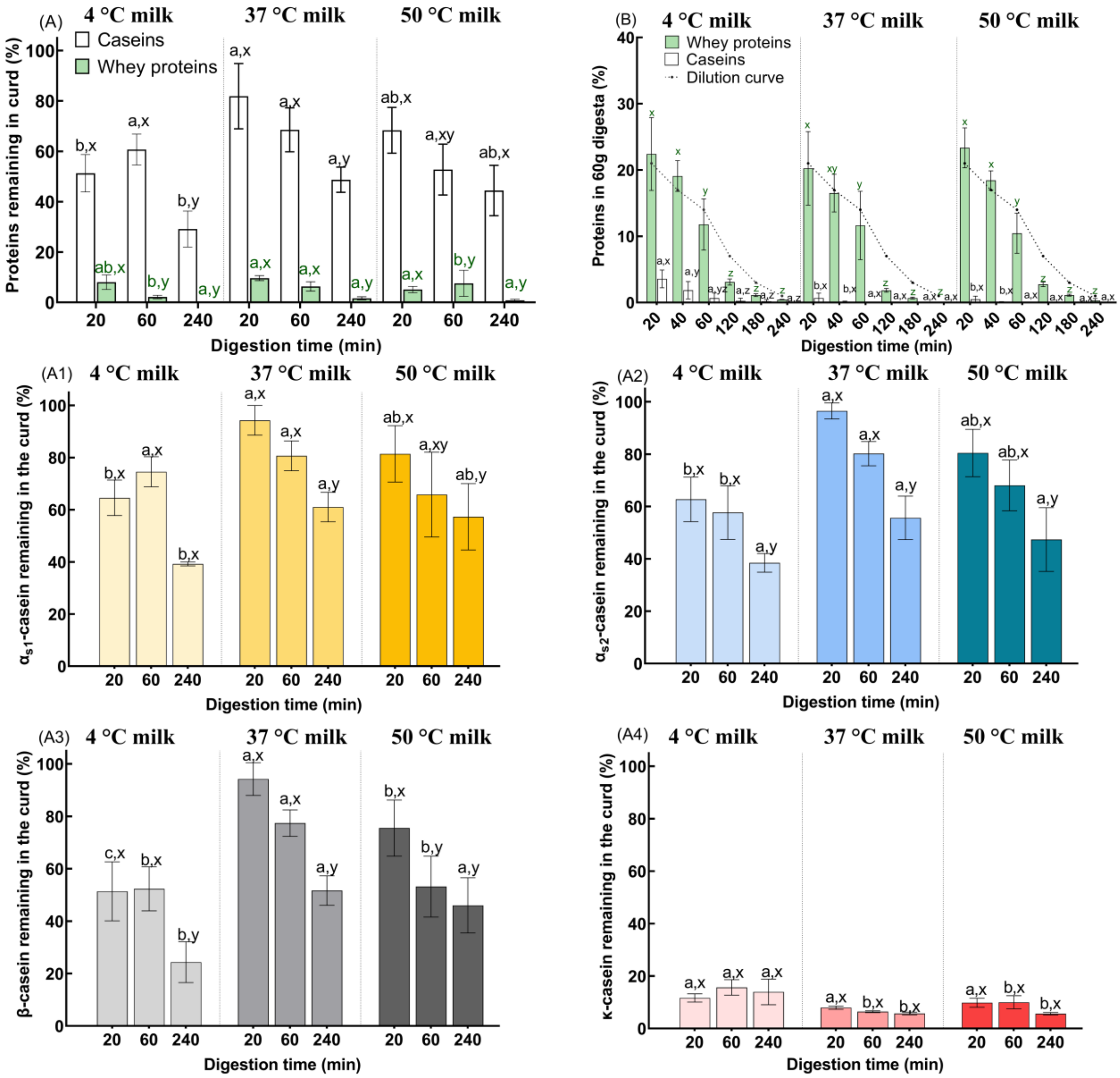


Figure 4-5 (A) Percentage of caseins (□) and whey proteins (🟩) remaining in the clot at 20, 60, and 240 min. (A1)–(A4) Percentage of α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein remaining in the clot at 20, 60, and 240 min. (B) Percentage of caseins (□) and whey proteins (🟩) detected in the 60 g of emptied digesta at 20, 40, 60, 120, 180, and 240 min. The dashed curves are the expected trends of the whey protein content resulting from the dilution caused by the SGF addition. $^{\mathrm{x–z}}$ Mean values for the same sample at different digestion times with different superscripts are significantly different ($P < 0.05$). $^{\mathrm{a–b}}$ Mean values between samples at the same digestion time with different superscripts are significantly different ($P < 0.05$). Error bars represent standard deviations from triplicate measurements.

A small amount of casein protein was present at 20 min in the digesta for all samples (**Figure 4-5 B**), likely due to some caseins not coagulated and incorporated into the curd, and were therefore emptied from the stomach into the digesta. As discussed above, because of the looser structure of the curd in the 4 °C milk sample, a greater amount of casein proteins ($P < 0.05$) was found in the 4 °C milk sample digesta at 20 min. The values shown as grey dashed lines in **Figure 4-5 B** are expected trends of whey proteins based on dilution due to the continual addition of SGF. After 120 min, the amount of whey proteins decreased to below the dilution (dashed line) caused by the continuous addition of SGF. This may have been because of the hydrolysis of α-lactalbumin by pepsin at pH < 4 (Wang et al., 2018).

These results suggested that the dry matter in the curds was mainly casein proteins for all milk samples. The protein emptied from the stomach was composed mainly of whey proteins in the early stages of digestion and was digested to peptides by pepsin at longer digestion times. After 240 min of digestion, fewer intact proteins were found in the 4 °C milk curd, indicating a faster rate of hydrolysis.

4.4. Conclusions

The present study demonstrates the impact of temperature on pepsin-induced hydrolysis and coagulation of milk proteins. The temperature coefficient of the hydrolysis of κ-casein was ~ 1.95. The optimum temperature for pepsin to specifically hydrolyze κ-casein was found to be around 37−40 °C. The coagulation behavior and the structure of the curd depended markedly on the set temperature. Different coagulation and digestion behaviors were found for the milks with different initial temperatures during dynamic *in vitro* gastric digestion. It took longer to coagulate under the gastric conditions for the 4 °C milk than for the 50 °C milk or the milk at body temperature. Protein digestion was relatively faster for the 4 °C milk than for the other two milk samples, which was attributed to the looser and softer structure during the early stages of digestion. After 240 min of digestion, the total curd weights of the 37 °C and 50 °C milk samples were similar but higher than that of the 4 °C milk sample. The findings of the present study provide insight into gastric coagulation and digestion of milk at different initial temperatures.

4.5. Acknowledgments

This study was funded by the New Zealand Milks Mean More (NZ3M) program and the Riddet Institute Centre of Research Excellence, Tertiary Education Commission, New Zealand. The author Mengxiao Yang thanks the Australian Institute of Nuclear Science and Engineering (AINSE) for a Post Graduate Research Award. We would like to acknowledge the support of Michael Agnew (AgResearch Limited, New Zealand) for helping with the RP-HPLC and Claire Woodhall (Havelock North, New Zealand) for proofreading the manuscript.

4.6. Supplementary material

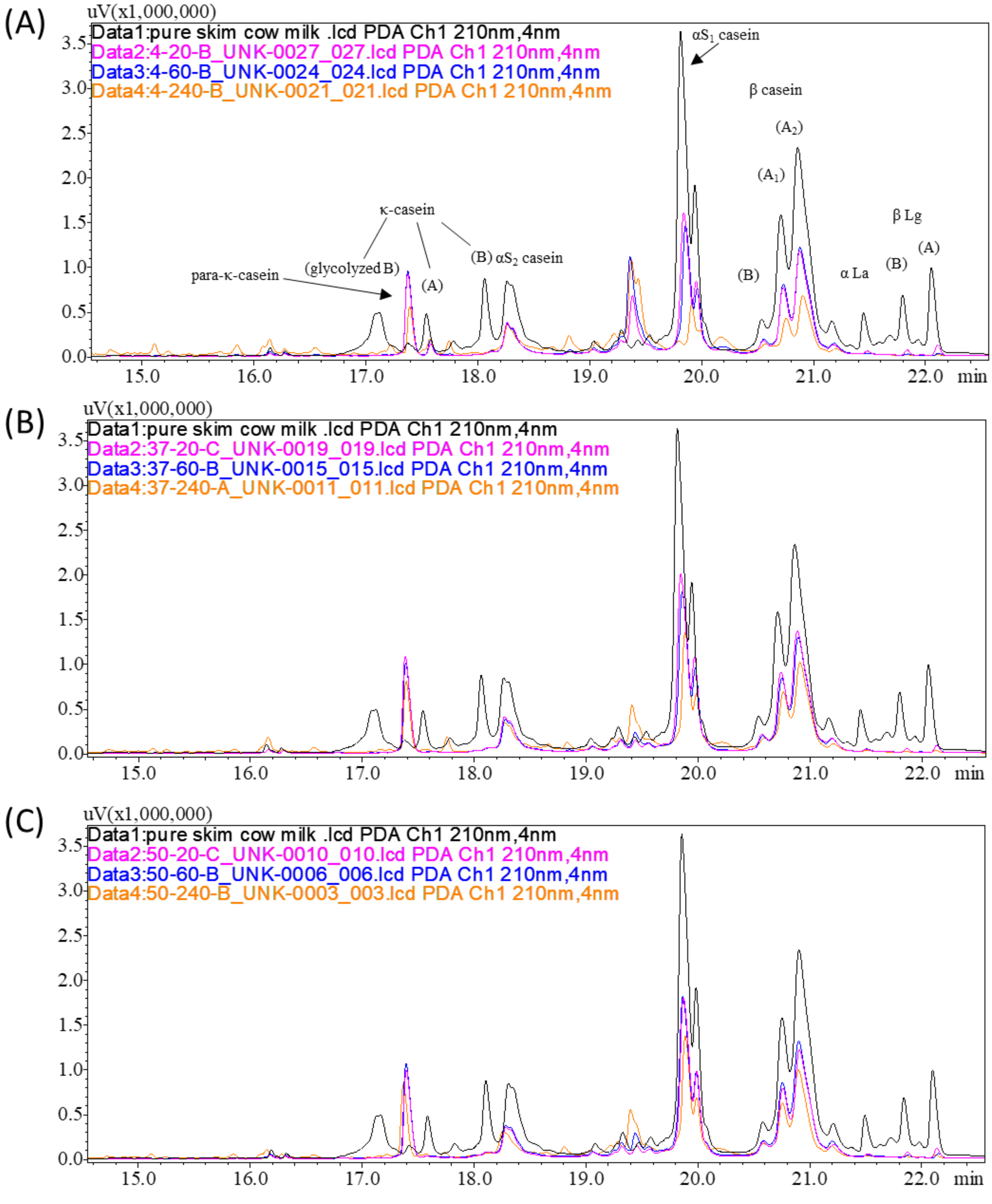


Figure S 4-1 Overlay of UV chromatograms analyzing the protein profiles of skim milk (−) and the coagulum that formed after 20 min (−), 60 min (−), and 240 min (−) of digestion. All major milk proteins were identified: (A) cold milk; (B) control milk; (C) warm milk.

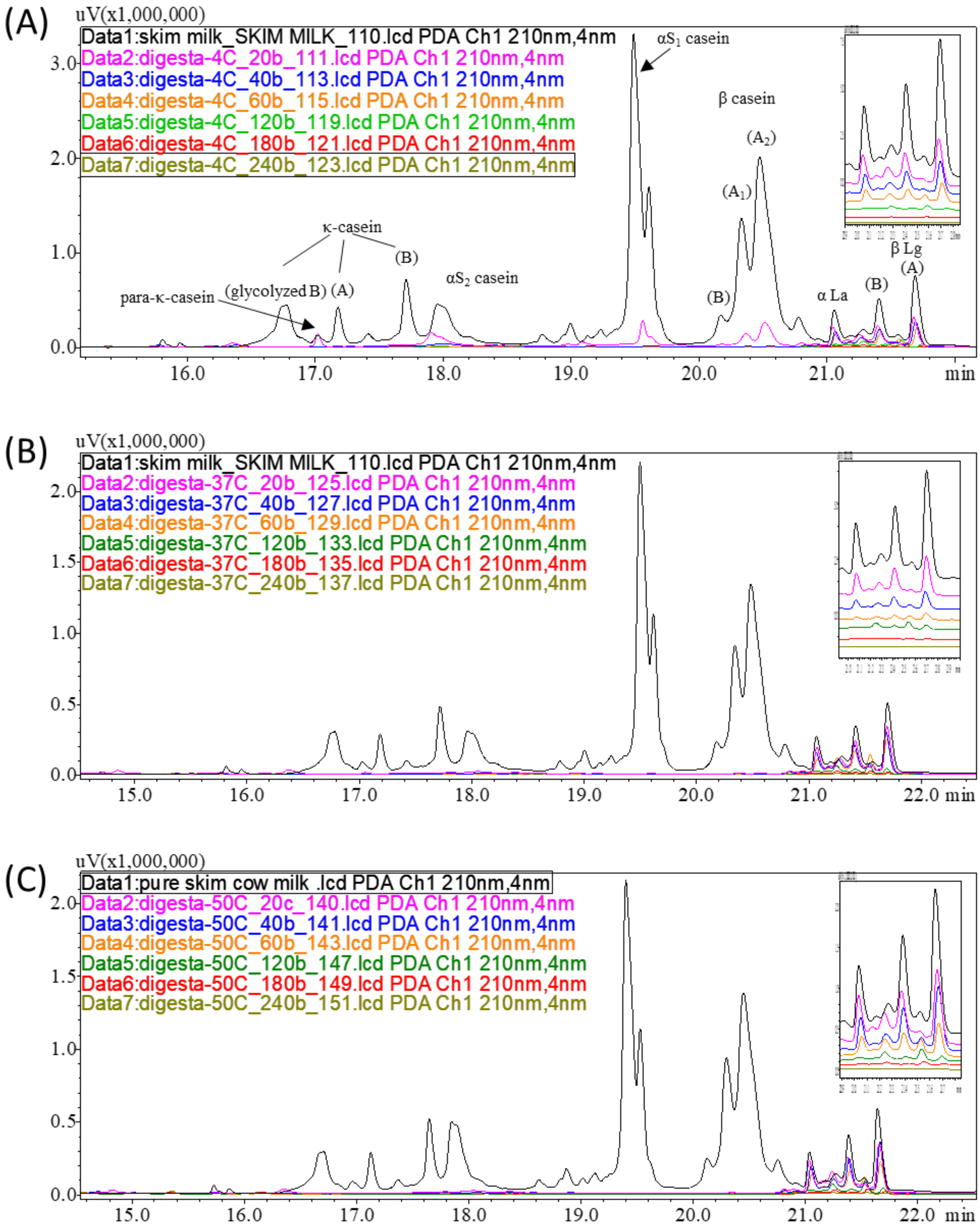


Figure S 4-2 Overlay of UV chromatograms analyzing the protein profiles of skim milk (−) and the emptied digesta: 20 min (−), 40 min (−), 60 min (−), 120 min (−), 180 min (−), 240 min (−). All major milk proteins were identified: (A) 4 °C milk; (B) 37 °C milk; (C) 50 °C milk.

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Student name:	Mengxiao Yang
Name and title of main supervisor:	Professor Aiqian Ye
In which chapter is the manuscript/published work?	Chapter 5

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Mengxiao Yang: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft.
Aiqian Ye: Conceptualization, Funding acquisition, Supervision, Resources, Writing – review & editing. Zhi Yang: Supervision, Writing – review & editing. David W. Everett: Supervision, Writing – review & editing. Elliot Paul Gilbert: Supervision, Writing – review & editing. Harjinder Singh: Supervision, Resources, Writing – review & editing.

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Chapter 5 Pepsin-induced coagulation of casein micelles: Effect of whey proteins and heat treatment[4]

Abstract

The effect of whey proteins and heat treatment (90 °C, 5 min) on pepsin-induced hydrolysis of κ-casein, and subsequent coagulation of casein micelles, was investigated at pH 6.3 and 6.0 using reverse phase high performance liquid chromatography (RP-HPLC), oscillatory rheology, and confocal laser scanning microscopy. Whey proteins did not affect the hydrolysis of κ-casein but retarded the coagulation process. Heat treatment did not affect the hydrolysis kinetics in whey protein (WP)-free samples, but slightly impaired the hydrolysis rate in WP-containing samples. The coagulation process of WP-free samples was little affected by heat-treatment. However, compared with unheated WP-contained sample at the same pH, the coagulation process of the heated sample was retarded at pH 6.3 but enhanced at pH 6.0. The curd in heated samples with smaller pores had higher water holding capacity. This knowledge provides further understanding on the role of whey proteins and heat treatment on the coagulation mechanisms of milk under gastric conditions.

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[4] This chapter has been published as Yang, M., Ye, A., Yang, Z., Everett, DW., Gilbert, EP., & Singh, H. (2023). Pepsin-induced coagulation of casein micelles: Effect of whey proteins and heat treatment. Food Chemistry, 402, 134214.

5.1. Introduction

Caseins and whey proteins are the main proteins in cow milk, comprising ~ 80% and ~ 20%, respectively, of total milk protein. Casein micelles are large colloidal aggregates, comprising calcium phosphate nanoclusters and casein proteins that are stabilized through electrostatic and steric repulsion (Walstra, 1999a) where κ-casein is located at the surface. It has been reported that the action of pepsin on casein micelles in skim milk at pH > 5 resembles that of chymosin by selective hydrolysis of the Phe^{105}-Met^{106} bond of κ-casein, yielding the C-terminal glycosylated caseinomacropeptide and para-κ-casein. This follows an equation combining first order hydrolysis and enzyme deactivation (Yang et al., 2022a). When a minimum amount of the caseinomacropeptide has been removed (arising following a ‘critical’ degree of hydrolysis), aggregation of the destabilized para-casein micelles occurs, resulting in the formation of a protein coagulum/curd (Dalgleish, 1993b). When milk transits into the gastric environment, milk coagulation occurs at pH > 6 which is above the isoelectric point of casein proteins in milk (pI 4.6) (Ye et al., 2017). Whey proteins, primarily β-lactoglobulin and α-lactalbumin, have globular structures, and are heat-sensitive (Anema & Li, 2003). Over the years, few studies have reported on the effect of whey proteins on chymosin-induced milk curd (cheese) properties. According to de Koning et al. (1981) and van den Berg (1979), undenatured whey proteins appear to act largely as an inert filler and disturb the formation of the milk coagulum. Gamlath et al. (2018) stated that whey proteins present a physical barrier to para-casein aggregation, resulting in an increased coagulation time and decreased firming rate. These authors reported the inhibition by whey proteins of the enzymatic action of chymosin by comparing a sample with a casein:whey (CN:WP) ratio of 1:4 to a sample CN:WP ratio of 1:0.03 (with fixed casein concentrations). Although the overall mechanisms of pepsin-induced casein coagulation are similar to chymosin-induced coagulation, to date, there have been no studies investigating the effect of whey proteins on pepsin-induced κ-casein hydrolysis and the subsequent coagulation process. However, more extensive coagulation of the caseins was observed at a higher CN:WP ratio from a study on the digestibility of infant formula powder using an in vitro digestion model (Phosanam et al., 2021; Ye et al., 2019a). Therefore, the ratio of CN:WP is important to protein digestion of dairy products (Mulet-Cabero et al., 2020b; Phosanam et al., 2021).

Heat treatment is an essential step in the processing of milk. When comparing the digestion behavior of heated or ultra-high-temperature (UHT) milk with fresh milk, Ye et al. (2017) found more fragmented and crumbly structures of the curd formed from heated and UHT milk after digestion in a human gastric simulator. These authors hypothesized that the aggregation of the casein micelles was sterically hindered by the binding of β-lactoglobulin to κ-casein after heat treatment. However, the effect of heat treatment on the hydrolysis of κ-casein induced by pepsin and subsequent coagulation has not been explored. It was reported that heat treatment induces changes in the physicochemical properties of milk, including the pH, buffering capacity, and calcium distribution, depending upon the heating temperature and duration (Lewis, 1994). According to Anema et al. (2007), about 80% of the whey proteins denature after 5 min heating at 90 °C. Through hydrophobic bonding and thiol–disulfide exchanges, the denatured whey proteins eventually aggregate with themselves and/or with κ-casein at the micelle surface (whey protein/κ-casein complexes) (Anema & Li, 2003). Previous reports suggested that the whey protein/κ-casein complexes inhibit the enzyme action of chymosin on κ-casein (Guyomarc'h et al., 2003; Jang & Swaisgood, 1990; Singh & Creamer, 1991), whereas according to Vasbinder et al. (2003) and Anema et al. (2007), heat treatment of milk has little effect on the hydrolysis reaction, regardless of whether the whey proteins are associated with the casein micelles or in the serum as non-sedimentable aggregates. Vasbinder et al. (2003) explained that these different results were due to different methods of isolating the glycosylated caseinomacropeptide. It is generally accepted that the aggregation phase of skim milk is retarded by heat treatment (Dalgleish, 1993b; Lucey, 1995; Marshall, 1986; Singh et al., 1988; van Hooydonk, 1987; Vasbinder et al., 2003), due to calcium precipitation and/or the steric hindrance of aggregable para-casein micelles by whey protein/κ-casein complexes. The pH of heated milk before and during enzymatic treatment is important to the coagulation process. By either decreasing the pH to about 6.2 or pH adjustment of acidified milk (acidifying milk to below 5.5 followed by adjustment to 6.6), the whey protein/κ-casein complexes were reported to facilitate the formation of a coagulum complex (Reddy & Kinsella, 1990) or could overcome the retardation of coagulation (Singh et al., 1988). This could be due to the changed structure and electrostatic repulsion of casein micelles at lower pH (< 6.2) (Zhao & Corredig, 2020).

As the pepsin-induced protein curd plays an important role in further hydrolysis, establishing the role of whey proteins and the effect of heat treatment on the hydrolysis kinetics and coagulation properties in a gastric environment will provide new insights into milk protein digestion. In this study, samples were prepared with micellar casein and whey protein isolate (WPI) ratios of 4:0, 4:0.6, 4:1 and 4:2 (at a constant casein concentration) and were either unheated or heated at 90 °C for 5 min. According to Yang et al. (2022), the pepsin-induced hydrolysis of κ-casein and the coagulation of proteins highly depends upon the pH (5.3–6.7) of milk, in which the hydrolysis of κ-casein is very low at pH > 6.3 and significantly decreases at pH < 6. Moreover, the association of whey proteins with casein micelles after heat treatment is largely influenced by pH (Lucey, Gorry, & Fox, 1994; Marshall, 1986; Singh et al., 1988). We hypothesize that the coagulation process of heated milk may differ according to pH, especially in the case where whey proteins are present in the samples. Therefore, in this study, the pH of all samples was adjusted to be constant at 6.3 or 6.0 by slow addition of simulated gastric fluid (SGF). The hydrolysis kinetics and coagulation behavior were compared over a period of 120 min after the addition of pepsin. The microstructure and water holding capacity of the curd were compared at 120 min.

5.2. Materials and methods

5.2.1. Materials

Micellar casein powder (84.2% protein, 2.1% fat, 6.9% ash, 3.5% moisture, wt/wt) was provided by FrieslandCampina (Amersfoort, The Netherlands). Whey protein isolate (WPI) (93.9% protein, 0.3% fat, 1.5% carbohydrate, 4.8% moisture, 1.7% ash, wt/wt) was supplied by Fonterra Co-operative Group (Auckland, New Zealand). All other chemicals (analytical grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Porcine pepsin (EC 3.4.23.1; Sigma-Aldrich), with stated activity of 541 U/mg protein was dissolved in Milli-Q water (5 mg per 5 mL; activity 541 U/mL) and then added to the micellar casein samples at a ratio of 10 µL per 1 mL with a final pepsin concentration of 5.4 U/mL at pH 6.3. According to previous results (Yang et al., 2022a), coagulation occurs much faster when pH decreases from 6.3 to 6.0. Therefore, to better observe the reaction within 120 min, an 8× diluted pepsin solution was used for samples at pH 6.0 with a final pepsin concentration of 0.68 U/mL.

5.2.2. Methods

5.2.2.1. Simulated milk ultrafiltrate and micellar casein solutions

The formulation of simulated milk ultrafiltrate (SMUF) follows the methods of Dumpler (2017). Micellar casein systems were prepared by dissolving the micellar casein and WPI powder into SMUF to achieve casein:whey protein (CN:WP) ratios of 4:0, 4:0.6, 4:1 (the ratio in natural bovine milk), 4:2 wt/vol while keeping the casein concentration constant at 0.032 g/mL to match the casein content in bovine skim milk. These samples, at pH 6.6 ± 0.1, were designated as U4:0, U4:0.6, U4:1, U4:2 and were dissolved at room temperature for at least 10 h to allow complete hydration before use.

Half volumes of the samples were filled into glass tubes (8 mL, diameter 1 cm) for heat treatment. The glass tubes were placed in a water bath until the center temperature of the sample reached 90 °C, and was then held for 5 min. The heated systems were designated as H4:0, H4:0.6, H4:1, and H4:2. All samples were prepared in triplicate and stored at 4 °C after the addition of sodium azide (0.02%, wt/vol).

5.2.2.2. Simulated gastric fluid

Simulated gastric fluid (SGF) was prepared by slight modification of a published method (Minekus et al., 2014). A fresh solution of a mixture of KCl (6.9 m*M*), KH_2PO_4 (0.9 m*M*), NaCl (72.2 m*M*), $MgCl_2(H_2O)_6$ (0.1 m*M*), $(NH_4)_2CO_3$ (0.5 m*M*) and $CaCl_2$ (0.15 m*M*) was prepared in Milli-Q water with stirring for 30 min. The pH of SGF was adjusted to 1.5 using HCl, and no pepsin was added into the SGF solution. By addition of SGF, the pH of samples was adjusted to 6.3 or 6.0 and equilibrated for approximately 3 h at room temperature. The samples were stored at 4 °C and small pH adjustments were made before further experiments, if required, to maintain pH at either 6.3 or 6.0.

5.2.2.3. Ionic calcium concentration

The ionic calcium concentration [Ca^{2+}] in the samples was determined using a calcium-selective electrode (Orion 9720BNWP; Thermo Scientific, Waltham, MA, USA). The electrode was calibrated with a series of aqueous calcium chloride standards ranging from 1 to 10 m*M*. Ionic strength of each standard was adjusted to ~ 80 m*M* by KCl addition (Chandrapala, McKinnon, Augustin, & Udabage, 2010). The [Ca^{2+}] of all samples were measured before and after pH adjustment at room temperature.

5.2.2.4. Particle size measurement

The particle size was measured by dynamic light scattering using a Malvern Zetasizer (Nano ZS; Worcestershire, UK) with disposable cells (DTS0012) at 25 °C. The samples were diluted 50× with calcium-imidazole buffer solution (20 m*M*-imidazole, 5 m*M*-$CaCl_2$, 30 m*M*-NaCl, pH 7.0) and analyzed under the 'milk protein size analysis' option (Anema & Li, 2003).

5.2.2.5. Measurement of κ-casein hydrolysis

The action of pepsin on κ-casein was tracked by measuring the changes in the amount of para-κ-casein using RP-HPLC. An aliquot of diluted pepsin solution (20 μL) was added to 2 mL samples, which was then immediately divided into 10 different test tubes (0.2 mL per tube) and incubated in a water bath at 37 °C. The reaction was stopped at different times (1, 2, 5, 10, 20, 30, 60, 90, and 120 min) with HPLC buffer solution (0.8 mL, 6 *M* GdnHCl, 0.1 *M* Bis-Tris, 19.5 m*M* DL-dithiothreitol and 5.37 m*M* sodium citrate, pH 7). Four time points (1, 10, 50, and 120 min) were examined by HPLC in triplicate. Each sample was shaken for 10 s and left at room temperature for 1 h before HPLC injection.

RP-HPLC analysis was carried out using a Nexera-X2 ultra-HPLC instrument equipped with an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan) by an established method described previously (Yang et al., 2022a). The amount of para-κ-casein was quantified using LabSolutions Main (Shimadzu) software based on the peak areas. The degree of hydrolysis of κ-casein at each time point was thus determined (Yang et al., 2022a), calculated as the ratio between the obtained chromatographic peak area of para-κ-casein at a given time and the chromatographic peak area of para-κ-casein after complete hydrolysis.

5.2.2.6. Determination of coagulation process

The coagulation process was determined by measuring the storage modulus (G') and loss modulus (G'') as a function of time using a stress-controlled rheometer (MCR301 Anton Paar, Graz, Austria) equipped with a Couette geometry (Anton Paar; CC27, with 28.93 mm cup diameter and 26.64 mm bob diameter). An aliquot of diluted pepsin solution (200 µL) was added into 20 mL samples and stirred for 30 s before loading. By performing a time sweep measurement with a strain of 1% and a constant frequency 0.1 Hz at 37°C for 120 min, G' was recorded every three minutes. The measurement of rheological properties was carried out in triplicate. The ratio of G'' to G' gives the value of loss tangent (tan δ). The onset of coagulation (coagulation time) was defined as the intercept of a linear regression of the first four G' points that consistently increase above the time axis (Yang et al., 2022a). The firming rate was defined as the maximum slope of G' as a function of time (dG'/dt) (Yang et al., 2022a).

5.2.2.7. Microstructural characterization of the coagulum

The microstructure of the pepsin-induced coagulum was examined by confocal laser scanning microscopy (Yang et al., 2022a). Fast Green fluorescent dye (1% wt/vol) was used to stain proteins (3 µL/100 µL). An aliquot of diluted pepsin solution was added into the pre-stained sample, reaching a final pepsin concentration of either 5.4 or 0.68 U/mL, depending on the pH. The sample–pepsin mixture was transferred to the cavity of a microscope glass slide, covered with a glass cover slip and incubated at 37 °C for 120 min. The fluorescence images were observed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 63× magnification lens at room temperature.

5.2.2.8. Water holding capacity of the coagulum

A pepsin solution (100 µL) was added to a 10 mL sample in centrifuge tubes. After 120 min incubation in a 37 °C water bath, the samples were centrifuged at 500×g for 2 min. The supernatants were drained, and the remaining pellets were weighed. Water holding capacity (WHC) was calculated as:

$$\text{WHC}\ (\%) = \frac{pellets\ weight}{sample\ weight} \times 100 \qquad \textbf{(Equation 5-1)}$$

5.2.2.9. Statistical analysis

Statistical analysis was carried out using Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Two-way ANOVA with Tukey post-hoc test was used to determine significant differences between samples grouped by two factors: CN:WP ratio and heat treatment. All analysis was done at the 95% confidence level ($P < 0.05$). The data was represented as the mean ± standard deviation.

5.3. Results and discussion

Experimental data from the multifactorial design were analyzed by two-way ANOVA. The results of the ANOVA (**Table S 5-1**and **Table S 5-2**) showed that changes in the casein micelle diameters were significantly affected by pH ($P < 0.001$), the milk systems (i.e., a combination of heat treatment and CN:WP ratio, $P < 0.001$), and their interaction ($P < 0.001$). The [Ca^{2+}] was significantly affected by pH ($P < 0.001$), the milk system (i.e., a combination of heat treatment $P < 0.05$), but was not significantly affected by their interaction ($P > 0.05$). At either pH 6.3 or 6.0, the changes in the reaction constants (K_{enz}), coagulation time (H_{ct}), dG'/dt, G'_{120} and WHC were significantly affected by the CN:WP ratio ($P < 0.001$), the treatment of milk (i.e., unheated or heated milk, $P < 0.001$), and their interaction ($P < 0.001$).

5.3.1. Particle size and Ca^{2+} concentration

As shown in **Figure 5-1 (a)**, the casein micelle diameters for the unheated samples U4:0, U4:0.6, U4:1 and U4:2 were similar, at ~180 nm, independent of the concentration of whey proteins. After heat treatment, the diameters of H4:0, H4:0.6, H4:1 and H4:2 were 178.6 ± 0.6, 188.6 ± 1.6, 207.5 ± 2.0 and 351.6 ± 3.0 nm, respectively. The diameter of H4:0 was similar to U4:0, and larger diameters were found in samples with higher whey protein concentration after heat treatment due to the association of the denatured whey proteins with the casein micelles (Anema & Li, 2003). It has previously been reported that lowering the pH from 6.7 to 6.0 results in smaller casein micelle size (Sinaga, Bansal, & Bhandari, 2017), however, the differences between pH 6.6, 6.3 and 6.0 in this study were not significant.

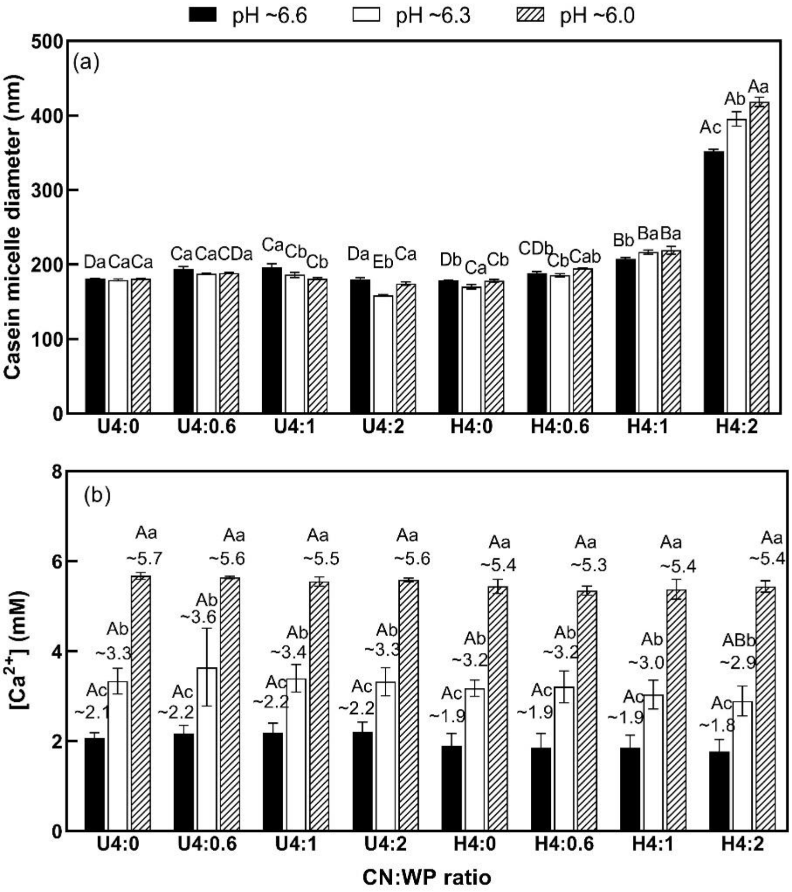

Figure 5-1 Changes in the casein micelle diameters (a) and [Ca^{2+}] (b) of samples at different CN:WP ratio after pH adjustment. Mean values between samples at same pH with different uppercase letters (A–D) are significantly different ($P < 0.05$). Mean values between samples with different lowercase letters (a–c) are significantly different ($P < 0.05$). U4:0, U4:0.6, U4:1, U4:2, unheated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol. H4:0, H4:0.6, H4:1, H4:2, heated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol. Error bars represent SD from triplicate.

According to **Figure 5-1 b**, the [Ca^{2+}] was ~2.2 m*M* for unheated samples at pH 6.6 and independent of the whey protein concentration. When the sample pH was adjusted to 6.3 or 6.0, the [Ca^{2+}] increased to ~3.4 and ~5.6 m*M*, respectively, due to solubilization of colloidal calcium phosphate. After heat treatment and before pH adjustment, the [Ca^{2+}] was similar to unheated samples ($P > 0.05$). It has been reported that heating at 100 °C for 10 min precipitates up to 30% of calcium phosphate (Schreiber, 2001), and this heat-precipitated calcium phosphate can be resolubilized after cooling (Chandrapala et al., 2010). With the decrease in pH, the [Ca^{2+}] for heated samples increased and reached a similar value as to that observed in unheated samples ($P > 0.05$) at the same pH (either 6.3 or 6.0).

5.3.2. Effect of whey protein and heat treatment on κ-casein hydrolysis

The major protein patterns for unheated samples with different CN:WP ratios are shown as black curves in **Figure 5-2 a**. After the addition of pepsin, a decrease in the peak area of κ-casein and an increase in the para-κ-casein peak area were observed over the reaction time. The peak areas of other major proteins remained the same, indicating that hydrolysis of other proteins did not occur, possibly because of the high pH (> 6) where general hydrolysis of other caseins and whey protein by pepsin is very limited. To investigate whether the presence of whey proteins affected the hydrolysis of κ-casein, the degree of hydrolysis for samples U4:0, U4:0.6, U4:1 and U4:2 was plotted as a function of reaction time at pH 6.3 and 6.0 at different pepsin concentrations, shown in **Figure 5-2 b** and **c**, respectively. The data were fitted to the equation used by (Yang et al., 2022a):

$$ln\left(1-\frac{H_t}{100}\right)=\frac{K_{enz}\cdot C}{K_{den}}\cdot[exp(-K_{den}\cdot t)-1] \qquad \textbf{(Equation 5-2)}$$

According to **Table 5-1**, the differences in reaction constants (K_{enz}) and denaturation constants (K_{den}) between U4:0, U4:0.6, U4:1 and U4:2 were not significant ($P > 0.05$) at pH 6.3. Except for the K_{enz} of U4:1 being significantly lower, no significant differences for K_{enz} and K_{den} were found between U4:0, U4:0.6, and U4:2 at pH 6.0. This indicates that the hydrolysis of κ-casein was generally not affected by the presence of whey proteins up to a CN:WP ratio of 4:2. Although a slightly decreased hydrolysis rate of κ-casein was observed by Gamlath et al. (2018) when rennet (chymosin produced via fermentation) was added to milk systems (pH ~ 6.7) with a CN:WP ratio of 4:16, these data were not fitted to a hydrolysis kinetics model. As the ratio of CN:WP in our study was much higher, the inhibition of hydrolysis was not significant. Besides, when the pH was adjusted to 6.3 or 6.0 prior to pepsin addition, the electrostatic repulsions between the casein micelles and pepsin would diminish, thus promoting the binding of pepsin to casein micelles (Yang et al., 2022a). The enzymatic inhibitory role of whey proteins, as proposed by Gamlath et al. (2018), to compete with κ-casein for the chymosin active site, or for binding either to the enzyme-substrate (chymosin-κ-casein) complex or substrate (κ-casein), may not be relevant at the lower pH used in our study. In addition, these authors used chymosin rather than pepsin, as used in our study.

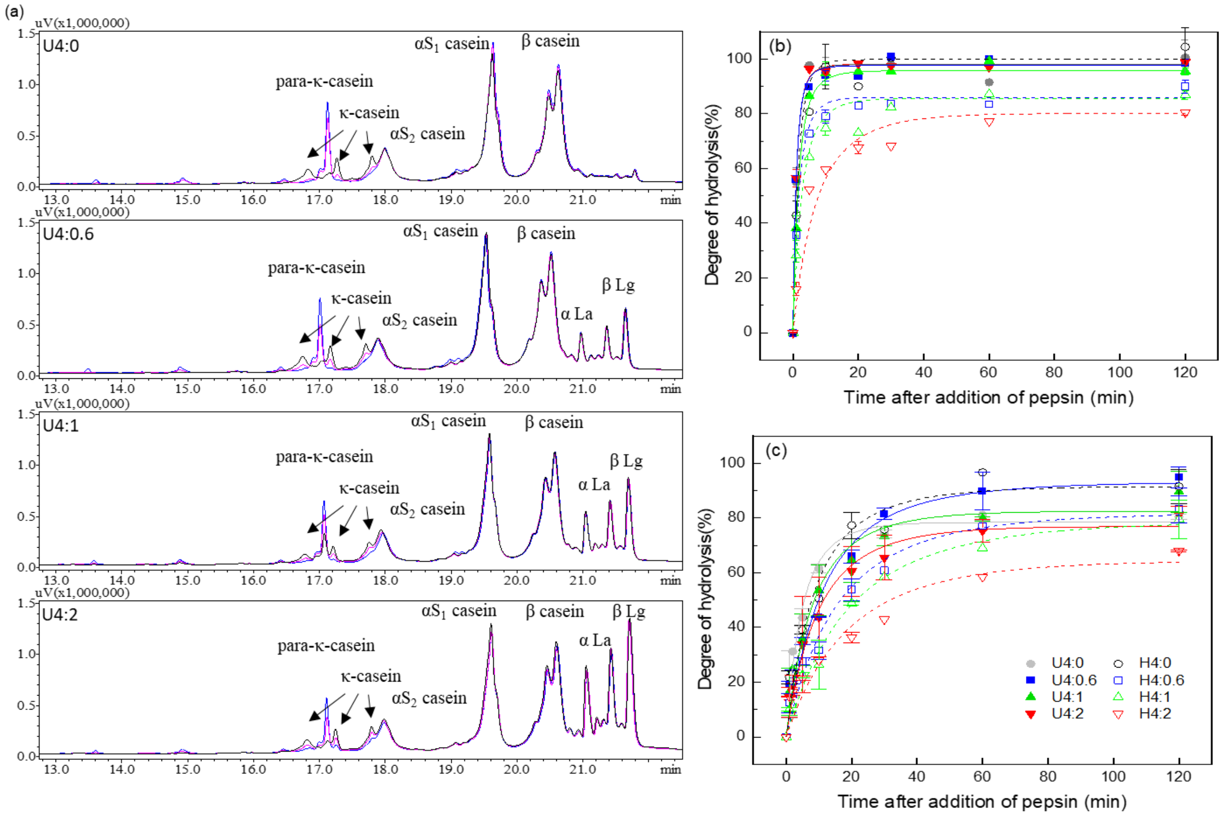

Figure 5-2 (a) Representative example of overlay of UV chromatograms analyzing protein profiles before (−) and after addition of pepsin in to sample U4:0, U4:0.6, U4:1, U4:2 at pH 6.3, (−) 60 min, (−) 120 min. (b) Degree of hydrolysis of κ-casein over time after the addition of pepsin at 37°C, pH 6.3 with addition of 5.4 U/mL pepsin. (c) Degree of hydrolysis of κ-casein over time after the addition of pepsin at 37°C, pH 6.0 with addition of 0.68 U/mL pepsin. The curves are fits to **Equation 5-2**, solid curves for unheated samples and dashed curves for heated samples. U4:0, U4:0.6, U4:1, U4:2, unheated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol. H4:0, H4:0.6, H4:1, H4:2, heated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol. Error bars represent SD from triplicate.

The degree of hydrolysis in the heated samples at pH 6.3 and 6.0 are shown in **Figure 5-2 b** and **c**, respectively. By fitting the experimental data to the hydrolysis **Equation 5-2**, the reaction constants K_{enz} and denaturation constants K_{den} for U4:0 and H4:0 (WP-free samples) were found to be similar ($P > 0.05$), whereas K_{enz} for the heated WP-containing samples were lower ($P < 0.05$) than the unheated samples at the same CN:WP ratio (**Table 5-1**). This suggests that the applied heat treatment (90°C, 5 min) did not affect the hydrolysis reaction for WP-free samples but impaired the WP-containing samples. Therefore, heat treatment had no significant effect on enzymatic hydrolysis in the absence of whey proteins. This is consistent with the result that heating at 90°C for 10 min did not affect the hydrolysis of κ-casein induced by chymosin in WP-free milk (Vasbinder et al., 2003).

Table 5-1 The hydrolysis kinetics of κ-casein in different systems after the addition of pepsin.

	Hydrolysis kinetics of κ-casein in samples at pH 6.3, 5.4 U/mL pepsin		**Hydrolysis kinetics of κ-casein in samples at pH 6.0, 0.68 U/mL pepsin**	
Sample	**K_{enz} (min^{-1})**	**K_{den}(min^{-1})**	**K_{enz} (min^{-1})**	**K_{den}(min^{-1})**
U4:0	0.165±0.020 $^{A, a}$	0.228±0.088 $^{A, a}$	0.087±0.006 $^{ABC, a}$	0.053±0.005 $^{AB, a}$
U4:0.6	0.168±0.011 $^{A, a}$	0.268±0.035 $^{A, a}$	0.093±0.009 $^{A, a}$	0.043±0.008 $^{AB, a}$
U4:1	0.164±0.018 $^{A, a}$	0.280±0.015 $^{A, a}$	0.071±0.004 $^{C, a}$	0.037±0.005 $^{AB, a}$
U4:2	0.172±0.015 $^{A, a}$	0.246±0.034 $^{A, a}$	0.091±0.009 $^{AB, a}$	0.060±0.005 $^{A, a}$
H4:0	0.139±0.011 $^{A, a}$	0.143±0.126 $^{A, a}$	0.083±0.009 $^{A, a}$	0.050±0.006 $^{A, a}$
H4:0.6	0.086±0.007 $^{B, b}$	0.241±0.041 $^{A, a}$	0.053±0.012 $^{B, b}$	0.033±0.012 $^{AB, a}$
H4:1	0.060±0.006 $^{B, b}$	0.183±0.017 $^{A, a}$	0.041±0.008 $^{BC, b}$	0.029±0.013 $^{B, a}$
H4:2	0.026±0.005 $^{C, b}$	0.098±0.026 $^{B, b}$	0.034±0.002 $^{C, b}$	0.034±0.002 $^{AB, b}$

$^{A–E}$ Mean values within the subset of heated or unheated samples at the same pH in the same column with different superscripts are significantly different ($P < 0.05$).

$^{a–e}$ Mean values between heated and unheated samples at same CN:WP ratio in the same column with different superscripts are significantly different ($P < 0.05$).

U4:0, U4:0.6, U4:1, U4:2: unheated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol.
H4:0, H4:0.6, H4:1, H4:2: heated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol.

Results are expressed as the mean ± SD of the mean (n = 3).

The observation that, in the presence of whey proteins, the K_{enz} for heated samples was lower than unheated samples at pH 6.3 and 6.0 (**Table 5-1**), suggests that the hydrolysis rate was impaired by heat treatment. It seems likely that the small reduction in enzymatic hydrolysis after heat treatment was caused by the denaturation of whey proteins and subsequent interactions with casein micelles, which is the general consensus for chymosin-induced hydrolysis (Dalgleish, 1990; Vasbinder et al., 2003; Walstra & Jenness, 1984b). However, according to Anema et al. (2007), heat-induced denaturation of whey proteins has no significant effect on the enzymatic reaction of chymosin on κ-casein in skim milk (CN:WP ~ 4:1) at pH 6.67; the rate of formation of para-κ-casein in all heated samples were similar to those in the unheated samples. As reported by Vasbinder et al. (2003), different methods for quantifying the degree of hydrolysis could be a reason for the variation. The inconsistent results could also be related to the different enzymes and pH used for the hydrolysis reaction. On the other hand, the casein micelle size of H4:2 was higher than the U4:2 (**Figure 5-1**), due to the interaction of denatured whey proteins with the casein micelles and/or the heat-induced whey protein aggregation. This might affect the diffusivity of the enzyme sufficiently to induce an appreciable change in the rate of κ-casein hydrolysis. Therefore, the pepsin diffusivity of H4:2 would be slower than U4:2, a possible explanation, in part, as to why K_{enz} of H4:2 is much lower than for U4:2.

5.3.3. Effect of whey proteins and heat treatment on the coagulation process

In the presence of pepsin, the changes in G' and loss tangent (tan δ) over time for unheated samples at pH 6.3 and 6.0 are shown in **Figure 5-3** and **Figure S 5-1**, respectively. In general, G' showed a lag phase immediately after pepsin addition, followed by an increase. For some of the samples, G' reached a plateau within 120 min. The coagulation properties, including the coagulation time, degree of hydrolysis at coagulation time (H_{ct}), firming rate ($\mathrm{d}G'/\mathrm{dt}$) and G' at 120 min (G'_{120}) are summarized in **Figure 5-4**. The increase in whey protein concentration, for the unheated samples, resulted in lower firming rate ($\mathrm{d}G'/\mathrm{dt}$) (**Figure 5-4 e**) at pH 6.3; and resulted in longer coagulation times (**Figure 5-4 b**), lower firming rate ($\mathrm{d}G'/\mathrm{dt}$) (**Figure 5-4 f**) and lower final G' (G'_{120}) (**Figure 5-4 h**) at pH 6.0. This is similar to the effect of whey proteins on milk coagulation induced by chymosin (Gamlath et al., 2018; Lelievre, Creamer, & Tate, 1990; Vasbinder et al., 2003) where the coagulation of WP-free milk was reported to occur earlier than for the WP-containing milk. The coagulation time of each sample was substituted into **Equation 5-2**, and the degree of hydrolysis at coagulation time (H_{ct}) was calculated and reported in **Figure 5-4 (c) and (d)** for samples at pH 6.3 and 6.0, respectively. The H_{ct} for samples at pH 6.0 were lower than samples with same CN:WP ratio at pH 6.3, consistent with a previous report that a lower degree of hydrolysis was required for coagulation at lower pH (Yang et al., 2022a). At both pH values, the required degree of hydrolysis of U4:2 is higher than U4:0 ($P < 0.05$). At pH 6.0, although the hydrolysis degree at 120 min reached 81% for sample U4:2 (**Figure 5-2 c**), there is no obvious increase in G'. Since the whey proteins did not affect the kinetics of hydrolysis (similar values for K_{enz} and K_{den}, as shown above), the increased concentration of whey proteins delayed the onset of coagulation by requiring more hydrolysis of κ-casein before coagulation could occur.

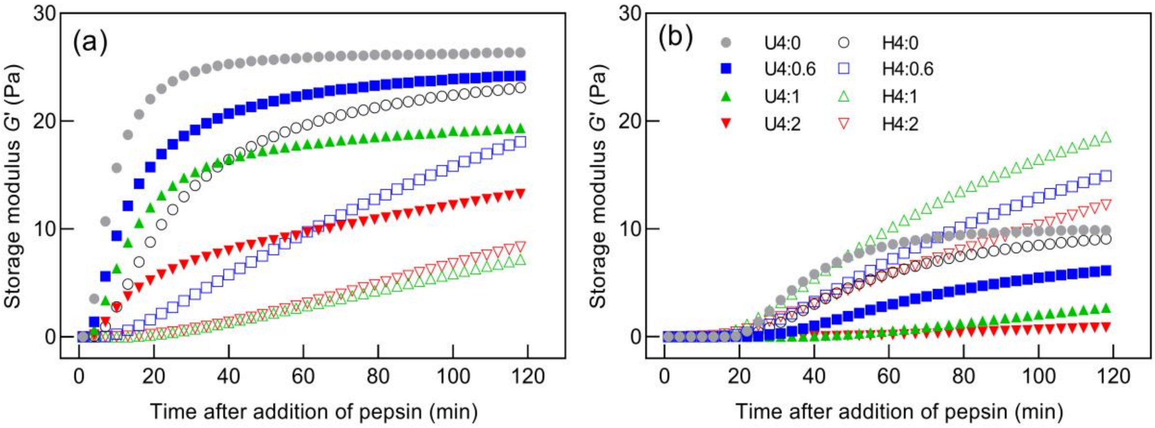

Figure 5-3 Storage modulus (G') of samples with time after the addition of pepsin at 37°C: (a) pH 6.3 with addition of 5.4 U/mL pepsin, (b) pH 6.0 with addition of 0.68 U/mL. U4:0, U4:0.6, U4:1, U4:2, unheated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol. H4:0, H4:0.6, H4:1, H4:2, heated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol.

It is well known that aggregation of para-casein micelles requires overcoming the energy barrier presented by electrostatic and steric repulsion (Gamlath et al., 2018; Payens, 1979).

The electrostatic repulsion is predominantly affected by pH, calcium ion activity and ionic strength. As mentioned above, the pH was controlled in these experiments; the [Ca^{2+}] of U4:0, U4:0.6, U4:1 and U4:2 samples were similar ($P > 0.05$) at either 6.3 or 6.0 (**Figure 5-1 b**), and the effect of up to 1.6 % WPI addition on the ionic strength can be ignored. Consequently, in agreement with Gamlath et al. (2018), none of the above factors could explain the observed impairment in coagulation and can only be explained by physical or steric hindrance. When κ-casein is hydrolyzed on the micelle surface, the whey proteins are sufficiently small to occupy gaps between the para-casein micelles (Gamlath et al. 2018). In this way, the whey proteins can provide a physical barrier and shield collisions between para-casein micelles. It is plausible then, that since whey proteins may partially reduce the extent of aggregation arising from a reduction in steric repulsion caused by removal of the κ-casein caseinomacropeptide layer, more extensive hydrolysis is required for coagulation to occur. However, this attachment of whey proteins to para-casein micelles may not be permanent and therefore not incorporated into the coagulum, resulting in the observed lower G'_{120} at the higher whey protein concentration.

Similar rheological properties were found in unheated and heated WP-free samples at either pH 6.3 or 6.0 (**Figure 5-3**), indicating that heat treatment (90 °C for 5 min) had little effect on the coagulation time and G'_{120} of WP-free samples. This is consistent with Vasbinder et al. (2003) who found the coagulation process was not affected when heating (90 °C for 10 min) WP-free milk when analyzed using diffusing wave spectroscopy. In the presence of whey proteins, the differences between unheated and heated samples for coagulation time, firming rate, and G'_{120} were highly dependent on pH. Compared to the unheated samples at the same CN:WP ratio and at pH 6.3, the heat-treated samples showed a longer coagulation time and a slower firming rate. This result is consistent with reports on chymosin-induced milk coagulation at pH ~ 6.5 where heat treatment delayed the coagulation process (Anema et al., 2007). According to Anema et al. (2007), 90 °C heating for 5 min at pH 6.6 resulted in 80% whey protein denaturation and ~ 50% of whey proteins associated with the κ-casein on the micellar surface; the latter partially stabilized the micelles and led to a much slower aggregation process, i.e. longer coagulation time and lower final G' at pH 6.5 (Anema et al., 2007; Dalgleish, 1990; Raynal & Remeuf, 1998; Singh & Waungana, 2001; Vasbinder et al., 2003).

At pH 6.0, heating the samples with high concentrations of whey proteins enhanced the coagulation process with shorter coagulation times (**Figure 5-4 b**), lower H_{ct} (**Figure 5-4 d**), and higher dG'/dt (**Figure 5-4 f**) and G'_{120} (**Figure 5-4 h**). These observations demonstrate that even though the enzymatic hydrolysis was retarded (lower K_{enz}, $P < 0.05$) by the heat treatment, the coagulation process was improved by heat treatment when the pH was adjusted to 6.0. This result for coagulation time, firming rate, and G'_{120} are the opposite to samples at pH 6.3, indicating that the pH of milk before pepsin treatment is an important factor for the coagulation behavior. Compared to the unheated samples, the coagulation behavior of heated samples was impaired at the higher pH 6.3 and enhanced at the lower pH 6.0.

According to Marshall (1986), Singh et al. (1988), and Lucey et al. (1994), an acidification step can overcome the adverse effects of heat treatment on rennet coagulation and that will improve the cheese yield of heated milk. These authors explained that the acidification of heated samples results in a greater increase in [Ca^{2+}] compared to the unacidified unheated control sample. However, in our study, the unheated sample was also acidified to pH 6.0 such that the difference in [Ca^{2+}] between heated and unheated samples was not significant ($P > 0.05$, **Figure 5-1 b**). The mechanism to explain the enhanced impact of acidification on heated samples compared to unheated samples at pH 6.0 is not fully understood. Denatured β-Lg was reported to increase curd tension (resistance to penetration) and G' values of acidified milk gels (Lucey et al., 1997). When the sample pH decreased to

6.0, which is closer to the isoelectric pH of whey proteins [pI ~ 4.2–5.5 (Marshall, 1982)], the net repulsive charge on the proteins is reduced with a decrease in pH towards the isoelectric point. Therefore, the denatured whey proteins that are associated with the casein micelles become more susceptible to aggregation and the protein–protein interactions between denatured whey proteins would be enhanced. This is consistent with the results in **Figure 5-4 d** where the degree of hydrolysis required to induce coagulation for H4:0 at pH 6.0 was ~ 68%, whereas for H4:1 and H4:2 the degrees of hydrolysis were ~ 42% and ~ 33%, respectively.

According to Singh et al. (1988), ~ 45% additional whey protein nitrogen (N) is incorporated into the rennet coagulum in acidified heated milk (pH 6.0) compared to pH 6.5. The strength of protein-protein bonds, the number of bonds per cross-section of the strand, and the orientation of the strands in the matrix all contribute to the yield stress of gels (Lee & Lucey, 2003; van Vliet, van Dijk, Zoon, & Walstra, 1991); consequently, the increased protein-protein interactions (or contact area) may have contributed to the increase in the G' values of heated samples at higher whey protein concentrations (**Figure 5-4 h**). Although the denatured whey protein may reduce casein-casein interactions and alter the basic framework of the coagulum, the concentration of coagulated protein would increase because of active participation of denatured whey proteins in the gel structure (Marshall, 1986). This suggests that whey protein interaction impacts more upon aggregation process than the retardation of hydrolysis (lower K_{enz}).

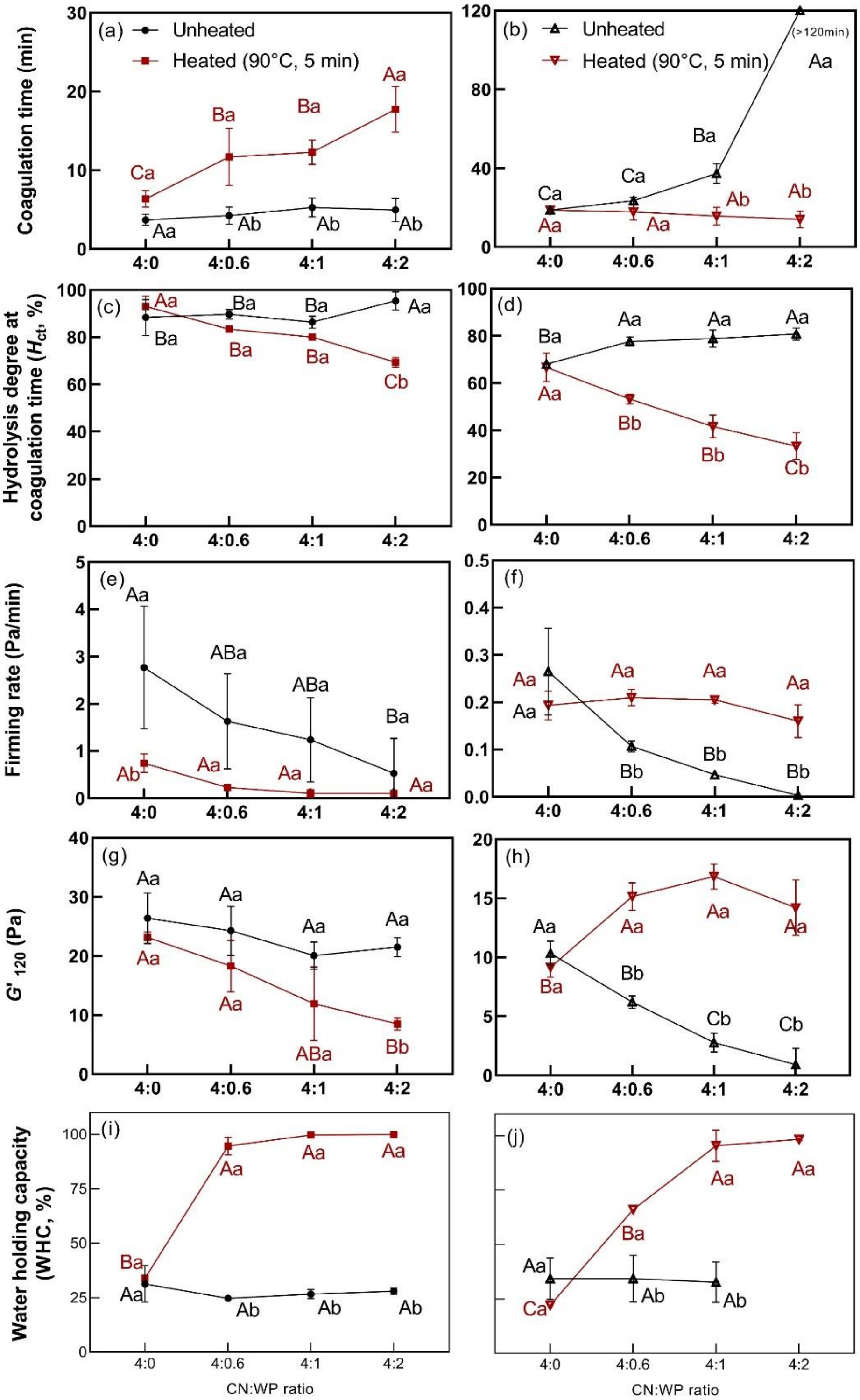

Figure 5-4 Coagulation time (min), degree of hydrolysis at coagulation time (*H*ct,%), firming rate (d*G′*/dt, Pa/min), G'_{120} (Pa) and water holding capacity (WHC, %) of gels 120 min after the addition of pepsin as a function of the casein to whey (CN:WP) ratio for samples at pH 6.3 after the addition of 5.4 U/mL pepsin (a, c, e, g, i) and for samples at pH 6.0 after the addition of 0.68 U/mL pepsin (b, d, f, h, j). Mean values between heated or unheated samples with different uppercase letters (A–C) are significantly different ($P < 0.05$). Mean values between samples at same CN:WP ratio with different lowercase letters (a–b) are significantly different ($P < 0.05$). Error bars represent SD from triplicate.

5.3.4. Effect of whey proteins and heat treatment on water holding capacity and microstructure of coagulum

After the addition of pepsin for 120 min, the water holding capacity (WHC) was measured (**Figure 5-4 i, j**) by subjecting the samples to 500×g centrifugal force for 2 min, whereupon water was expelled, and the curd shrank (note: WHC was not measured for uncoagulated samples). The presence of whey proteins did not induce changes in WHC at either pH 6.3 or 6.0. After heat treatment, the WHC of WP-free samples remained similar ($P > 0.05$). However, WHC of samples in the presence of whey proteins was higher for heated samples than for unheated samples at the same WP:CN ratio for both pH 6.3 and 6.0. This is in agreement with the observation of denatured whey proteins increasing WHC in heated milk and impairing syneresis of a coagulum (Kethireddipalli & Hill, 2015).

The microstructure of pepsin-induced coagula from different samples was observed at 120 min by confocal laser scanning microscopy, allowing a visualization of the protein networks (appearing as green) and other structural properties. The unheated samples at pH 6.3 and 6.0 are shown in **Figure 5-5 a-c** and **g-i**, respectively. The casein micelles assembled into strands and underwent crosslinking for U4:0, resulting in a more compact network. In the presence of whey proteins, the microstructure became more heterogenous and less crosslinked. Sparse and weak structures were observed in sample U4:2 at pH 6.3 (**Figure 5-5 c**) and U4:1, U4:2 at pH 6.0 (**Figure 5-5 h, i**, respectively). This is supported by the rheological data, which shows that U4:0 had a higher G'_{120} (**Figure 5-3 a**) than U4:1 and U4:2 (**Figure 5-3 b**) at both pH 6.3 and 6.0.

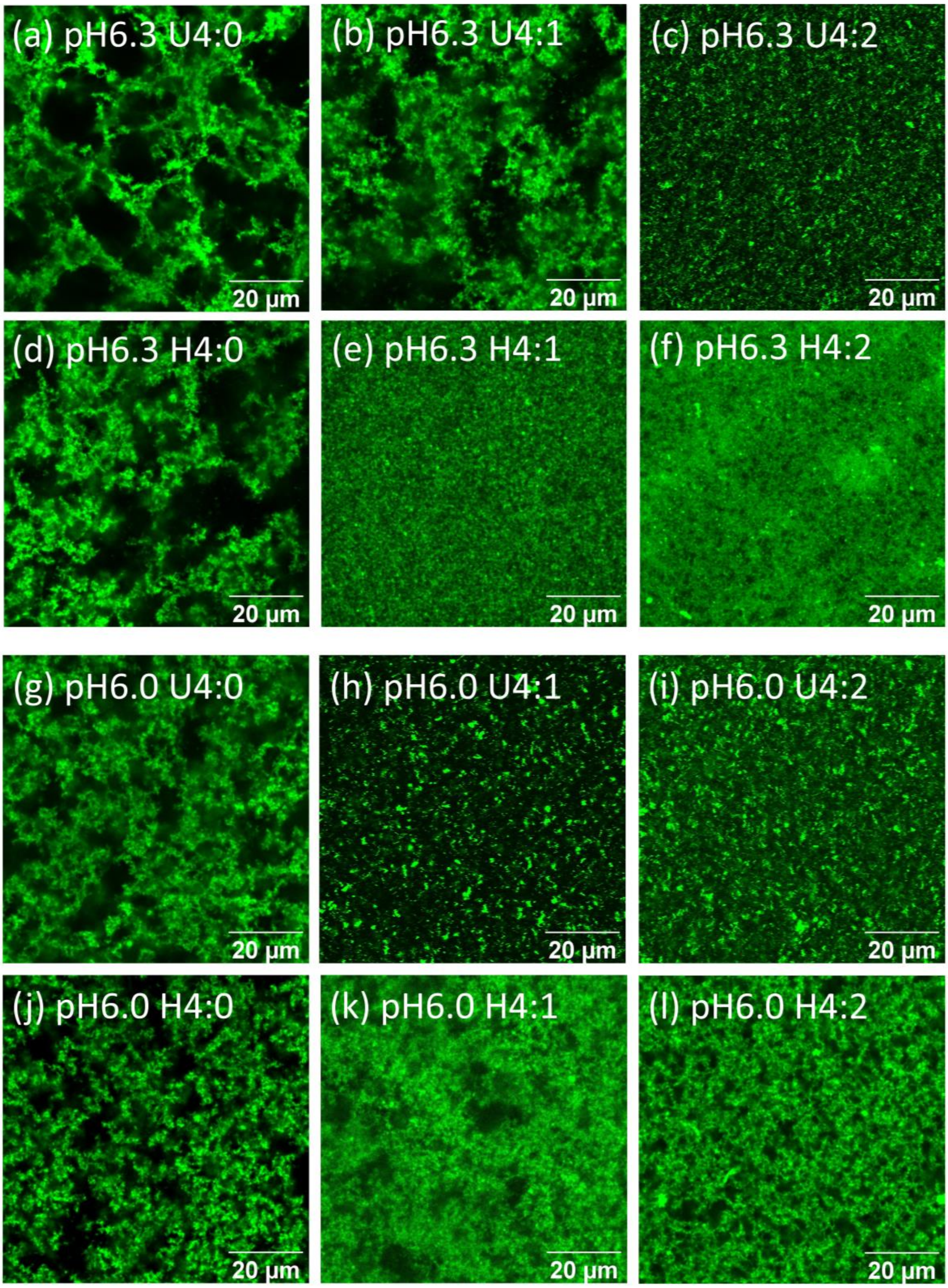

Figure 5-5 Confocal micrographs of gel samples 120 minutes after the addition of 5.4 U/mL pepsin at pH 6.3 (a-f) and confocal micrographs of gel samples 120 minutes after the addition of 0.68 U/mL pepsin at pH 6.0 (g-l). Sample abbreviations are given in **Section 2.2.1**. Scale bars are 20 μm.

The microstructures of heated samples are shown in **Figure 5-5 Figure 5-3d-f** and **j-l**, respectively. The protein networks between U4:0 and H4:0 was similar, but remarkable differences were observed between the heated and unheated samples in the presence of whey proteins. At pH 6.3, the observed particles of protein for H4:1 (**Figure 5-5 e**) and H4:2 (**Figure 5-5 f**) were smaller than for U4:1 (**Figure 5-5 b**) and U4:2 (**Figure 5-5 c**), respectively. As the firming rate for H4:1 and H4:2 was slower than for U4:1 and U4:2, respectively (**Figure 5-4 e**), the presence of small particles may have arisen from the slower rate of aggregation of the proteins. At pH 6.0, a homogenous and compact structure with small porous spaces was observed in H4:1 and H4:2 (**Figure 5-5 k** and **l**) compared to unheated samples, likely due to heat-denatured whey proteins associating with casein micelles that act as bridging material by interacting with other adsorbed denatured whey proteins (Lucey et al., 1997). When comparing the heated samples at different CN:WP ratios, a more interrupted structure with larger pores was observed for H4:0 (**Figure 5-5 j**). The crosslinked network became denser and finer, and the pores became smaller with more denatured whey proteins for H4:1, H4:2, as shown in **Figure 5-5 k**, **l**, respectively. H4:2 (**Figure 5-5 l**) appeared the most homogeneous and fine-stranded for the heated samples at pH 6.0, with the smallest and most evenly distributed pores. The homogenous and compact structure of H4:2 led to a firmer coagulum with higher storage modulus G' than U4:2 at pH 6.0 (**Figure 5-4 h**). Higher WHC was observed from samples with small network pores formed by heating in the presence of whey proteins because casein micelles are likely to trap water more effectively due to the high capillary force of small pores (Chantrapornchai & McClements, 2002).

5.4. Conclusions

The current study demonstrates that whey proteins have little effect on κ-casein hydrolysis by pepsin but significantly impair the rate of aggregation of the para-casein micelles over a wide range of CN:WP ratios. Heat treatment has little effect on the hydrolysis and coagulation process in the WP-free micellar casein system. In the presence of whey proteins, heat treatment impairs the hydrolysis rate, whereas the effect on coagulation behavior depends upon the pH of samples before addition of pepsin. Denatured whey proteins retard aggregation of casein micelles at the higher pH (6.3); however, when the pH decreases to 6.0 (closer to the average electric point of whey proteins), aggregation was enhanced, mainly due to the incorporation of whey proteins into the network. At both pH 6.3 and 6.0, heated samples, with greater association of denatured whey proteins, were found to form a network with smaller pores, which resulted in higher water holding capacity.

Milk coagulation occurs in a gastric environment as a consequence of the secretion of acidic gastric fluid and pepsin. The knowledge obtained from this study provides further understanding of the role of whey proteins and heat treatment on the mechanisms of coagulation of milk during the initial gastric stage of digestion. The results of the present study also explain previous findings on the different digestion behaviors of infant formula with varying casein-to-whey ratios, as well as the different digestive behavior of fresh milk and pasteurized or UHT milk.

5.5. Acknowledgements

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5.6. Supplementary material

Table S 5-1 Significance levels (*P*) of the two-way ANOVA for the effects of pH and sample system.

	pH	Sample system	pH×Sample system
Casein micelle diameters	***	***	***
[Ca^{2+}]	***	*	NS

NS: not significant; * P <0.050; ** P < 0.010; *** P < 0.001.

Table S 5-2 Significance levels (*P*) of the two-way ANOVA for the effects of heat-treatment and CN:WP ratio.

		Heat-treatment	CN:WP ratio	Heat-treatment × CN:WP ratio
pH 6.3	K_{enz}	***	***	***
	K_{den}	***	NS	NS
	Coagulation time	***	***	***
	H_{ct}	***	*	NS
	dG'/dt	***	***	***
	G'_{120}	***	***	***
	WHC	***	***	***
pH 6.0	K_{enz}	***	***	***
	K_{den}	***	**	NS
	Coagulation time	***	**	***
	H_{ct}	***	***	NS
	dG'/dt	***	**	***
	G'_{120}	***	**	***
	WHC	***	***	***

K_{enz}, reaction constants; K_{den}, denaturation constants; H_{ct}: degree of hydrolysis at coagulation time; dG'/dt: firming rate; G'_{120}: G' at 120min; WHC: water holding capacity; NS: not significant; * P <0.050; ** P < 0.010; *** P < 0.001.

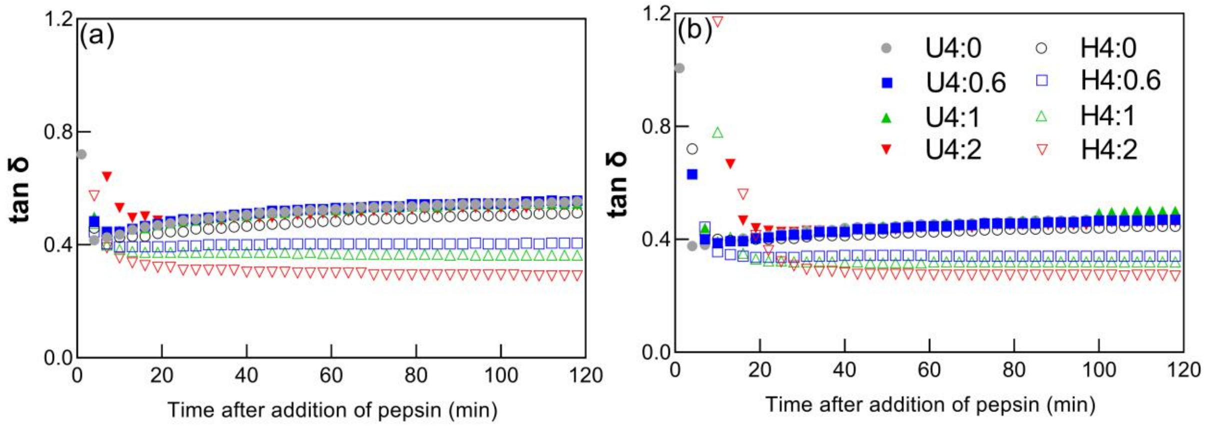

Figure S 5-1 Loss tangent (tan δ) of samples with time after the addition of pepsin at 37°C: (a) pH 6.3 with addition of 5.4 U/mL pepsin, (b) pH 6.0 with addition of 0.68 U/mL. U4:0, U4:0.6, U4:1, U4:2, unheated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol. H4:0, H4:0.6, H4:1, H4:2, heated samples with CN:WP ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol.

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Chapter 6 Role of Ca^{2+} in the pepsin-induced coagulation and in the dynamic *in vitro* gastric digestion behavior of casein micelles[5]

Abstract

The effect of Ca^{2+} on pepsin-induced hydrolysis of κ-casein and subsequent coagulation of casein micelles was studied in a micellar casein (MC) solution at pH ~ 6.0 at 37 °C without stirring. An NaCl-supplemented MC solution was used as a positive control to assess the effect of increased ionic strength after $CaCl_2$ addition. Quantitative determination of the released para-κ-casein during the reaction using reverse-phase high-performance liquid chromatography showed that specific hydrolysis of κ-casein by pepsin was little affected by the addition of either $CaCl_2$ or NaCl. However, rheological properties and microstructures of curds induced by pepsin hydrolysis depended markedly on the addition of salts. Addition of $CaCl_2$ up to 17.5 m*M* facilitated coagulation, with decreases in coagulation time and critical hydrolysis degree, and increases in firming rate and maximum storage modulus (G'_{max}); further addition of $CaCl_2$ (22.5 m*M*) resulted in a lower G'_{max}. Increased ionic strength to 52.5 m*M* by adding NaCl retarded the coagulation and resulted in a looser curd structure. In a human gastric simulator, MC, without the addition of $CaCl_2$, did not coagulate until the pH decreased to ~ 5.0 after ~ 50 min of digestion. Addition of $CaCl_2$ facilitated coagulation of casein micelles and resulted in more cohesive curds with dense structures during digestion, which slowed the emptying rate of caseins. At the same $CaCl_2$ concentration, a sample with higher ionic strength coagulated more slowly. This study provides further understanding on the effect of divalent (Ca^{2+}) ions and ionic strength on the coagulation of casein micelles and the digestion behavior of milk.

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6.1. Introduction

The calcium in milk exists as free/ionized calcium, calcium complexed with inorganic anions (*e.g.*, phosphate and citrate), or colloidal calcium [as colloidal calcium phosphate (CCP) and bound calcium] (Bauland et al., 2020; Holt, 1997). To improve the nutritional and functional properties of milk, enrichment with calcium salts, *e.g.*, calcium chloride, calcium carbonate or calcium phosphate, has been common practice (Gastaldi et al., 1994; Ramasubramanian et al., 2014; Vyas & Tong, 2004). Previous studies have suggested that the coagulation of milk in a gastric environment at pH > 6 is due to specific hydrolysis of κ-casein by the action of pepsin, which broadly resembles the action of chymosin on κ-casein that occurs during cheese manufacture (Huppertz & Chia, 2021; Yang et al., 2022a; Ye, 2021). The addition of Ca^{2+}, which is known to improve the texture of chymosin-induced milk curd (cheese), might also affect the pepsin-induced milk coagulation and the behavior during gastric digestion.

The addition of $CaCl_2$ contributes to an increase in the calcium content in all forms accompanied by a decrease in milk pH (Bringe & Kinsella, 1986). The reported effects of $CaCl_2$ addition on the enzymatic hydrolysis of casein micelles are contradictory. According to Holt (1997) and Bauland et al. (2020) the kinetics of κ-casein hydrolysis is negatively correlated with the concentration of Ca^{2+} in the soluble phase of milk at pH 6.6; Bringe & Kinsella (1986) found that the hydrolysis rate increased with the addition of $CaCl_2$ below 8 m*M* but decreased with further addition of $CaCl_2$ at pH 6.7. Several other studies concluded that neither dialysis nor the addition of calcium salts directly affected the hydrolysis of κ-casein when kept at a constant pH (Sandra & Corredig, 2013; Sandra et al., 2012; Walstra & Jenness, 1984a); however, as hydrolysis proceeds, coagulation of casein micelles occurred because of electrostatic effects (Horne & Lucey, 2014). It was reported that increases in ionic calcium concentration ($[Ca^{2+}]$) promoted coagulation of casein micelles (Bringe & Kinsella,

1986) by decreasing the zeta-potential of para-casein micelles and formation of calcium bridges between casein micelles (Dalgleish, 1983; Sandra et al., 2012; van Hooydonk, 1987). In addition, Ono, Kaminogawa, Odagiri, & Yamauchi (1976) reported that an increase in calcium concentration changed the conformation of para-κ-casein such that hydrophobic binding sites became more exposed, thus facilitating micelle coagulation, and resulting in a progressive decrease in coagulation time (Hardy & Fanni, 1981; Kowalchyk & Olson, 1978; Marshall & Green, 1980) and an increase in firming rate (Bringe & Kinsella, 1986), final curd firmness, and yield stress (Gastaldi et al., 1994). However, beyond a certain calcium concentration (10 m*M*), some studies reported that milk coagulation was retarded (McMahon & Brown, 1983; Sanner & Kovács-Proszt, 1973; Udabage, McKinnon, & Augustin, 2001). As the addition of $CaCl_2$ increases the ionic strength of milk and in reconstituted milk powders, NaCl or KCl could be used as a positive control in an assessment of the effect of an increase in ionic strength that occurs upon $CaCl_2$ addition (Bauland et al., 2020). The addition of NaCl affected hydrolysis of κ-casein and coagulation of casein micelles, and this was dependent upon the added amount and the pH (Guinee, 2004; Payens & Both, 1980; van Hooydonk, 1987; Zoon, 1988). According to Zoon (1988), addition of 100 m*M* NaCl at constant rennet concentration increased the modulus whereas higher NaCl concentrations retarded curd formation-and-ageing at pH 6.65. However, the effect of NaCl on the modulus at pH 6.25 was different from that at pH 6.65.

Many *in vitro* dynamic gastric digestion studies have been carried out and the standout feature reported from the digestion of cow milk is the formation of a curd at a fairly high pH (~ 6.0) because of hydrolysis of κ-casein by pepsin and subsequent coagulation of the destabilized casein micelles (Huppertz & Chia, 2021; Huppertz & Lambers, 2020; Mulet-Cabero et al., 2020a; Ye, 2021; Ye et al., 2016b). To our knowledge, few studies have investigated the effect of Ca^{2+} on pepsin-induced coagulation of milk. Since casein micelles are the main component for coagulation and Ca^{2+} is removed during the production of micellar casein (MC) powder, this was dissolved in simulated milk ultrafiltrate (SMUF) prior to coagulation studies. In this current study, the effect of $CaCl_2$ and NaCl on the hydrolysis of κ-casein and the coagulation behavior of micellar MC solution was investigated at pH 6.0 without stirring. Furthermore, to better understand the effects of Ca^{2+} and ionic strength on the digestion behavior of MC solutions, the digestibility of MC solutions was investigated using a dynamic digestion model.

6.2. Materials and methods

6.2.1. Materials

MC powder [84.2% protein, 2.1% fat, 6.9% ash, 3.5% moisture (wt/wt)] was obtained from FrieslandCampina (Amersfoort, The Netherlands). Calcium chloride dihydrate ($CaCl_2·2H_2O$) was purchased from Ajax Fine Chemicals (Auckland, New Zealand). Sodium chloride (NaCl) was purchased from Sigma-Aldrich (Auckland, New Zealand). Stock solutions of $CaCl_2$ and NaCl at 1 *M* were prepared using Milli-Q water. Pepsin from porcine gastric mucosa (EC 3.4.23.1) with an enzymatic activity of 541 U mg^{-1} was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals (analytical grade) were obtained from Sigma-Aldrich unless otherwise specified. Simulated salivary fluid (SSF) and simulated gastric fluid (SGF) were prepared according to Brodkorb et al. (2019), with a slight modification. SSF contained KCl (15.1 m*M*), KH_2PO_4 (3.7 m*M*), NaCl (13.6 m*M*), $MgCl_2·6H_2O$ (0.15 m*M*), $(NH_4)_2CO_3$ (0.06 m*M*), $CaCl_2·2H_2O$ (1.5 m*M*), with pH was adjusted to 7.0 using 1 *M* HCl. SGF contained KCl (6.9 m*M*), KH_2PO_4 (0.9 m*M*), NaCl (72.2 m*M*), $MgCl_2·6H_2O$ (0.12 m*M*), $(NH_4)_2CO_3$ (0.5 m*M*), $CaCl_2·2H_2O$ (0.15 m*M*), with pH was adjusted to 1.5 using 1 *M* HCl. Note that SGF was prepared at 1.25× concentration and supplemented with pepsin solution to achieve a 1× concentration before addition into the Human gastric simulator (HGS).

6.2.2. Preparation and physicochemical analysis of MC solutions

SMUF was prepared following the method of Dumpler (2017) but without the addition of $CaCl_2$. MC solution at a concentration of 3.2% (wt/wt) protein was prepared by dispersing the MC in SMUF for ~ 10 h (Yang et al., 2023b). In this study, the six samples of Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ of 0.00, 7.50, 10.0, 12.5, 17.5, and 22.5 m*M*, respectively, resulting in ionic strengths of 0.00, 22.5, 30.0, 37.5, 52.5, and 67.5 m*M*, respectively (calculated from the added $CaCl_2$). The samples of Na-7.50, Na-15.0 and Na-30.0 represent sample Ca-7.50 after the addition of NaCl at 7.50, 15.0, or 30.0 m*M*, respectively, which increased the ionic strength to 30.0, 37.5, and 52.5 m*M*, respectively (calculated from the added $CaCl_2$ and NaCl). The samples were stirred at room temperature for ~ 5 h, after which, [Ca^{2+}] was measured using a calcium-selective electrode (Orion 9720BNWP; Thermo Fisher Scientific, Rochester, NY, USA), as described previously (Yang et al., 2023b).

6.2.3. Measurement of pepsin-induced hydrolysis and coagulation of MC solutions

To evaluate the independent effect of $CaCl_2$, the pH of all samples was adjusted to ~ 6.0, by the gradual addition of SGF (1 × concentrate, pH 1.5) under vigorous stirring conditions (Yang et al., 2023b). To obtain a pepsin activity of 1082 U/mL, 2 mg of porcine pepsin was dissolved in 5 mL of Milli-Q water (pH ~ 7.0). The pepsin solution was added to the samples at a ratio of 10 µL per 1 mL of solution and resulted in a final pepsin activity of ~ 1.10 U (mL solution)$^{-1}$.

6.2.3.1. Measurement of κ-casein hydrolysis

The release of para-κ-casein was quantified by reverse-phase high-performance liquid chromatography (RP-HPLC) (Yang et al., 2022a). After the addition of pepsin and shaking for 20 s, the mixed samples were transferred into nine different test tubes (0.2 mL per tube) and incubated in a water bath at 37 °C. HPLC buffer solution (0.8 mL, 6 *M* guanidinium hydrochloride, 0.1 *M* bis-Tris, 19.5 m*M* dl-dithiothreitol, and 5.37 m*M* sodium citrate, pH 7) was added at different time points (1, 2, 5, 10, 20, 30, 50, 70, and 120 min) to stop the hydrolysis reaction. Each sample was shaken for 10 s and then incubated for 1 h at room temperature before the HPLC injection step. The analysis was carried out using a Shimadzu Nexera-X2 ultra-HPLC instrument equipped with an SPD-M20A diode array detector (Shimadzu Corporation, Kyoto, Japan), and followed the method described by Yang et al. (2022a) The hydrolysis of κ-casein was calculated basing on the amount of para-κ-casein released, using LabSolutions Main software (Shimadzu Corporation).

6.2.3.2. Measurement of casein coagulation

The nondestructive rheological properties of the samples were determined by low-amplitude dynamic oscillation by measuring the storage modulus (*G′*) using an Anton Paar (Graz, Austria) MCR301 rheometer equipped with a Couette geometry (CC27, with a 28.93-mm cup diameter and a 26.64-mm bob diameter). A 200 µL aliquot of pepsin was added to a 20 mL sample which had been equilibrated at 37 °C for 15 min. After stirring for 30 s, the sample was loaded and oscillated at a constant frequency of 0.1 Hz with a strain of 1% at 37 °C for 120 min (Yang et al., 2022a). Subsequently, the large deformation properties of the curd were determined by applying a constant shear rate of 0.00185 s^{-1} to the curd (Lucey et al., 1997). The shear stress was recorded and the value at which it started to decrease was defined as the yield stress.

6.2.3.3. Microstructural characterization of curd

The microstructure of the pepsin-induced curd was examined using confocal laser scanning microscopy (CLSM) (Yang et al., 2022a) and scanning electron microscopy (SEM). Fast Green fluorescent dye [30 µL; 1% (wt/vol), in Milli-Q water] was added to the pre-warmed sample (37 °C, 1 mL) in an Eppendorf tube. After the addition of pepsin, part of the sample–pepsin mixture was immediately transferred to the cavity of a glass microscope slide, covered and sealed with a glass cover slip, and incubated at 37 °C in an oven for 120 min. The prepared sample was examined using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 63× magnification lens. An aliquot of the sample–pepsin mixture was also incubated at 37 °C for 120 min and prepared for SEM. Briefly, samples were freeze-dried and affixed with double-faced adhesive tape on a metal stub. The samples were sputtered (SCD 050, Balzers, Liechtenstein) with gold for 200 s. The samples were observed using an FEI Quanta 200 environmental scanning electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) with an accelerating voltage of 20 kV at Manawatū Microscopy and Imaging Center (Palmerston North, New Zealand).

6.2.4. Dynamic *in vitro* gastric digestion

Dynamic gastric digestion of samples Ca-0, Ca-7.50, Ca-17.5, Ca-22.5, and Na-30.0 (to avoid protein dilution, there was no pH adjustment in these samples) was carried out with a human gastric simulator (HGS) (Kong & Singh, 2010) using the method described by Li et al. (2022c) In brief, the oral phase of digestion was carried out by mixing 20 g of SSF into 200 g of sample. Before the start of the gastric digestion phase, 20 mL of SGF (1 × concentration, including pepsin) was added to the sample–SSF mixture, as the base level in the fasted state. The digestion was carried out for 180 min at 37 °C with a simulated peristaltic movement in the stomach chamber at a frequency of three times per minute. A 1.25 × concentrated electrolyte SGF (pH 1.5) was prepared and added at 2.0 mL min^{-1}. A solution with pepsin [2000 U (mL final SGF)$^{-1}$] and $CaCl_2$ (0.15 m*M* in the final SGF) was prepared and added at 0.5 mL min^{-1}. Thus, the addition rate of the final SGF was 2.5 mL min^{-1} and the digesta were emptied every 20 min (3 mL min^{-1}) through a 1-mm sieve to mimic gastric sieving. The pH and [Ca^{2+}] profiles during digestion were determined every 20 min by measuring the freshly emptied digesta at 25 °C using a pH meter and a calcium-selective electrode (Orion 9720BNWP; Thermo Scientific).

The solid curds were collected by passing the contents of the gastric chamber through a 1-mm sieve after 20, 60, and 180 min of digestion, in triplicate. The wet curds were weighed immediately after sampling. A sub-sample of each curd was freeze dried and ground into a powder for further compositional analysis, and another sub-sample was dried at 105 °C for 24 h. The dry weight and the moisture content were determined by gravimetric analysis, based on the wet weight ratio between the whole curd and the portion taken for oven drying (Li et al., 2022b).

6.2.4.1. Macro- and microstructures of the digesta and curds

Photographs of the fresh curds and the digesta were taken for comparison of the macrostructures. CLSM imaging was carried out to compare fresh curd microstructures (Ye et al., 2017).

6.2.4.2. Texture analysis of curds

Fresh curds were cut into 1.0 cm^3 cubes with a wire cutter immediately after sampling. The hardness of the curd was measured (Roy et al., 2021) using a TA-XT Plus texture analyzer (Stable Micro Systems, Surrey, UK), fitted with a 5.0-kg load cell and a 2.0-mm diameter lightweight stainless-steel flat probe. Exponent software (version 6.1.15.0, Stable Micro Systems) was used for the TA-XT Plus analysis. Sample were penetrated to a specified depth of 5.0 mm at a constant speed of 2.0 mm s^{-1}. The value of the maximum force (N) was obtained from the force–time curve of each measurement.

6.2.4.3. Protein profiles of curds and emptied digesta

Changes in the casein composition of the curds and the emptied digesta were determined using RP-HPLC as described in Section **6.2.3.1**. Sample buffer (0.8 mL) was added to 0.2 mL of digesta (immediately collected at 20, 40, 60, 120, and 180 min) or 8 mg of curd powder (collected at 20, 60, and 180 min and freeze dried). The casein content was determined by comparing the peak area of the caseins in the curds or digesta at each digestion time point with that of the MC solution.

6.2.5. Statistical analysis

Samples were prepared in triplicate and measurements carried out in triplicate; eight time points were examined by RP-HPLC and four of them (1, 5, 20, and 120 min) were measured in triplicate; values (or data points) are expressed as mean ± standard deviation. Experimental data were analyzed by running analysis of variance (ANOVA) tests using Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The hydrolysis kinetics and the coagulation results were analyzed using one-way ANOVA with a Tukey post-hoc test. The gastric digestion results were analyzed using two-way ANOVA, with the concentration of $CaCl_2$ or NaCl, the digestion time, and their interaction as fixed effects. If significant interaction effects were found, further analysis was carried out with Tukey post-hoc tests. Differences were considered to be significant at $P < 0.05$.

6.3. Results and discussion

6.3.1. Effect of $CaCl_2$ and NaCl addition on the physicochemical properties of MC

The addition of $CaCl_2$ to MC solution increased the $[Ca^{2+}]$ from ~ 1.4 to 12.3 m*M*, and decreased the pH from ~ 6.69 to 6.07 (**Table 6-1**), which was similar to the results reported in Ramasubramanian et al. (2014). The measured $[Ca^{2+}]$ was lower than the added $CaCl_2$ concentration, with a linear relationship (**Figure S 6-1**), suggesting that the equilibrium between Ca^{2+} and phosphate shifted towards the formation of insoluble calcium phosphate, which can potentially enter the micellar phase as CCP (Huppertz & Lambers, 2020). In addition, the release of protons from the formation of insoluble calcium phosphate resulted in a decrease in pH, which has been reported previously (Crowley, Kelly, & O'Mahony, 2014;

Lewis, 2011; Pyne & McGann, 1960). Therefore, to reach pH 6.0, lesser amounts of SGF were required for samples at higher [Ca^{2+}] (resulting in less dilution of the samples). After the pH adjustment by adding SGF, the ionic strength of all samples changed, as shown in **Table 6-1**). besides, the protein concentration slightly decreased to be 2.57 ± 0.24% (calculated according to the added amounts of SGF in **Table 6-1**). A decrease in the pH led to an increase in [Ca^{2+}] for each sample because of the dissolution of colloidal calcium phosphate. As shown in **Table 6-1**, , the addition of NaCl up to 30 m*M* decreased the sample pH (from ~ 6.51 to 6.44), probably because of an exchange of Na^+ for H^+ attached to charged groups of the caseins (Grufferty & Fox, 1985). It was reported that the addition of NaCl to milk increased the concentration of soluble calcium by ~ 0.5 m*M* for 100 m*M* of added NaCl (Zhao & Corredig, 2015); however, up to 30 m*M* NaCl did not significantly affect the [Ca^{2+}] in our study (**Table 6-1**). Therefore, the positive control for ionic strength did not significantly affect the mineral partition.

Table 6-1 Physicochemical properties of micellar casein (MC) solution samples after the addition of $CaCl_2$ or NaCl without stirring.

Sample*	Addition of $CaCl_2$ (m*M*)	Addition of NaCl (m*M*)	Increased ionic strength (m*M*) **	pH after the addition of $CaCl_2$ or NaCl	$[Ca^{2+}]$ (m*M*) after the addition of $CaCl_2$ or NaCl	Addition of SGF to reach pH 6.0 [mL (100 mL solution)$^{-1}$]	$[Ca^{2+}]$ (m*M*) after the addition of SGF	Increased ionic strength after pH adjustment by SGF (m*M*) ***	Ionic strength of prepared samples (m*M*) ****
Ca-0	0.00	–	0	6.69±0.02	1.4±0.7 ^{e}	≈ 35	2.7±0.5 ^{e}	24.4	128.6
Ca-7.50	7.50	–	22.5	6.51±0.01	3.2±0.4 ^{d}	≈ 31	4.8±0.2 ^{d}	44.8	149.0
Ca-10.0	10.0	–	30.0	6.44±0.05	4.4±0.3 ^{cd}	≈ 29	5.2±0.8 ^{cd}	51.2	155.4
Ca-12.5	12.5	–	37.5	6.26±0.01	5.6±0.5 ^{c}	≈ 17	6.7±0.5 ^{c}	51.2	155.4
Ca-17.5	17.5	–	52.5	6.17±0.02	9.3±1.1 ^{b}	≈ 14	9.0±0.1 ^{b}	64.1	168.3
Ca-22.5	22.5	–	67.5	6.07±0.03	12.3±1.2 ^{a}	≈ 5	12.8±1.0 ^{a}	72.0	176.2
Na-7.50	7.50	7.50	30.0	6.51±0.01	3.7±0.1 ^{d}	≈ 34	5.7±0.1 ^{cd}	53.9	158.1
Na-15.0	7.50	15.0	37.5	6.48±0.02	3.9±0.2 ^{cd}	≈ 31	5.8±0.4 ^{cd}	59.8	164.0
Na-30.0	7.50	30.0	52.5	6.44±0.01	4.3±0.4 ^{cd}	≈ 33	5.9±0.2 ^{cd}	75.9	180.1

* Samples Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 0.00, 7.50, 10.0, 12.5, 17.5, and 22.5 m*M*, respectively. Samples Na-7.50 to Na-30.0 represent sample Ca-7.50 after the addition of NaCl at 7.50, 15.0, and 30.0 m*M*, respectively.

** Ionic strength is due to the addition of $CaCl_2$ and NaCl and does not include the ionic strength contributed by the salts originally present in the MC solution.

*** Increased ionic strength after pH adjustment is due to the addition of $CaCl_2$, NaCl and simulated gastric fluid (SGF) and does not include the ionic strength contributed by the salts originally present in the MC solution.

**** Ionic strength of prepared samples is due to the addition of $CaCl_2$, NaCl, simulated gastric fluid (SGF) and the ionic strength contributed by the salts originally present in the MC solution (104.2 m*M*).

$^{a–d}$ Mean values between samples in the same row with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of the mean ($n = 3$).

6.3.2. Effect of $CaCl_2$ and NaCl addition on the hydrolysis kinetics of κ-casein

After the addition of pepsin, the degree of hydrolysis was determined by calculating the change in peak area of para-κ-casein (Yang et al., 2022a) (the casein profiles for sample Ca-17.5 are shown in **Figure S 6-2** as an example). The degree of hydrolysis increased with increasing reaction time, as shown in **Figure 6-1**. By fitting the experimental data into the hydrolysis model of κ-casein,

$$ln\left(1-\frac{H_t}{100}\right)=\frac{K_{enz}\cdot C}{K_{den}}\cdot[exp(-K_{den}\cdot t)-1] \qquad \textbf{(Equation 6-1)}$$

the hydrolysis rate constant K ($K_{\mathrm{enz}}\cdot C$) and the pepsin denaturation constant K_{den} were calculated (**Table 6-2**). There were no significant differences ($P > 0.05$) in the values of K or K_{den} for the samples with added $CaCl_2$ or NaCl. Although there are contradictory reports about the effects of $CaCl_2$ addition on the enzymatic hydrolysis of κ-casein using chymosin, based on the fitting shown in **Figure 6-1**, pepsin-induced hydrolysis of κ-casein was little affected by the increased concentration of $CaCl_2$ (up to 22.5 m*M*) or NaCl (up to 30 m*M*). This finding is in agreement with Mehaia & Cheryan (1983) who reported that $CaCl_2$ did not affect the rate of hydrolysis of κ-casein by pepsin.

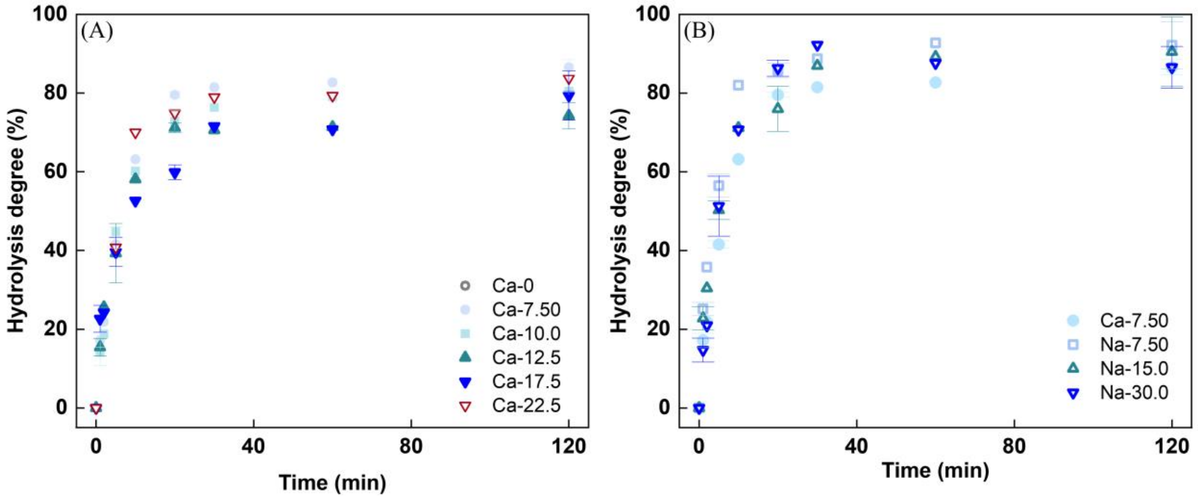

Figure 6-1 (A) Degree of hydrolysis of κ-casein in micellar casein (MC) samples with added $CaCl_2$ as a function of time after the addition of 1.10 U pepsin mL^{-1} without stirring (○ Ca-0; ● Ca-7.5; ■ Ca-10; ▲ Ca-12.5; ▼ Ca-17.5; ▽ Ca-22.5). (B) Degree of hydrolysis of κ-casein in samples with added NaCl as a function of time after the addition of 1.10 U pepsin mL^{-1} (● Ca-7.50; □ Na-7.50; △ Na-15.0; ▽ Na-30.0). Samples Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 0.00, 7.50, 10.0, 12.5, 17.5, and 22.5 m*M*, respectively. Samples Na-7.50 to Na-30.0 represent sample Ca-7.50 after the addition of NaCl at 7.50, 15.0, and 30.0 m*M*, respectively. (Note: samples with added NaCl all contain 7.50 m*M* $CaCl_2$.) Error bars represent the standard deviations from triplicate measurements.

Table 6-2 Effect of $CaCl_2$ and NaCl on the coagulation properties of micellar casein (MC) solution at pH 6.0 with the addition of 1.10 U pepsin mL^{-1} without stirring.

Sample*	K (min^{-1})	K_{den} (min^{-1})	Coagulation time (min)	dG'/dt (Pa min^{-1})	G'_{max} (Pa)	H_{ct} (%)	Yield stress (Pa)
Ca-0	0.114 ± 0.001^{a}	0.059 ± 0.003^{b}	> 120	–	–	–	–
Ca-7.50	0.138 ± 0.003^{a}	0.069 ± 0.007^{ab}	17.2 ± 0.4^{bc}	0.39 ± 0.23^{b}	10.3 ± 1.0^{d}	$75 \pm 1\%^{a}$	2.0 ± 0.3^{cd}
Ca-10.0	0.143 ± 0.003^{a}	0.089 ± 0.004^{ab}	6.9 ± 0.5^{cd}	1.18 ± 0.62^{b}	20.5 ± 1.5^{c}	$52 \pm 2\%^{d}$	3.0 ± 0.7^{c}
Ca-12.5	0.148 ± 0.036^{a}	0.110 ± 0.027^{a}	3.2 ± 0.1^{d}	1.62 ± 0.70^{b}	29.7 ± 2.6^{b}	$32 \pm 6\%^{c}$	5.5 ± 1.0^{b}
Ca-17.5	0.115 ± 0.010^{a}	0.086 ± 0.012^{ab}	2.0 ± 1.0^{d}	5.17 ± 1.22^{a}	46.0 ± 3.1^{a}	$18 \pm 7\%^{cd}$	9.2 ± 0.2^{a}
Ca-22.5	0.112 ± 0.002^{a}	0.060 ± 0.001^{b}	1.0 ± 0.1^{d}	2.42 ± 1.71^{b}	22.0 ± 7.2^{bc}	$11 \pm 1\%^{d}$	1.9 ± 0.6^{cd}
Na-7.50	0.220 ± 0.042^{a}	0.112 ± 0.012^{a}	25.7 ± 3.0^{b}	0.10 ± 0.02^{b}	5.8 ± 0.9^{de}	$85 \pm 4\%^{a}$	1.5 ± 0.2^{d}
Na-15.0	0.192 ± 0.021^{a}	0.083 ± 0.023^{ab}	52.2 ± 11.4^{a}	0.04 ± 0.01^{b}	1.2 ± 0.5^{e}	$90 \pm 5\%^{a}$	0.6 ± 0.1^{d}
Na-30.0	0.173 ± 0.044^{a}	0.082 ± 0.029^{ab}	> 120	–	–	–	–

* Samples Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 0.00, 7.50, 10.0, 12.5, 17.5, and 22.5 m*M*, respectively. Samples Na-7.50 to Na-30.0 represent sample Ca-7.50 after the addition of NaCl at 7.50, 15.0, and 30.0 m*M*, respectively.

K, hydrolysis rate constant; K_{den}, pepsin denaturation constant; dG'/dt, firming rate; G'_{max}, maximum storage modulus value during the measuring time; H_{ct}, degree of hydrolysis of κ-casein at the coagulation time.

$^{a–d}$ Mean values between samples in the same row with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of the mean ($n = 3$).

6.3.3. Effect of $CaCl_2$ and NaCl addition on the rheological properties of pepsin-induced casein curd

The evolution of the storage modulus (*G′*) after the addition of pepsin (**Figure 6-2 A, C**) showed a marked dependence on the concentrations of $CaCl_2$ and NaCl. *G′* shows a lag phase but as time progressed, *G′* increased significantly. *G′* then reached a plateau value, or slightly decreased due to syneresis (Yang et al., 2022a). The shear stress profiles as a function of strain are shown in **Figure 6-2 B, D**. The coagulation time, the curd firming rate (dG'/dt), G'_{max}, and the yield stress are calculated from a previous method (Lucey et al., 1997; Yang et al., 2022a) and summarized in **Table 6-2**.

The *G′* of the sample without the addition of $CaCl_2$ (Ca-0) was not measurable over 120 min. This indicates that there was a limiting [Ca^{2+}], below which coagulation did not occur even when the degree of hydrolysis of κ-casein was > 80%. According to Dalgleish (1983), the limiting [Ca^{2+}] is 3 m*M*. When the $CaCl_2$ concentration was increased from 7.50 to 22.5 m*M*, the coagulation time decreased from ~ 17 to 1 min. The degree of hydrolysis of κ-casein at the coagulation time (H_{ct}) was obtained by substituting the coagulation time into the hydrolysis equation (**Equation 6-1**), as reported in **Table 6-2**; H_{ct} was negatively correlated with $CaCl_2$ concentration. This indicates that coagulation of the casein micelles was facilitated by the addition of $CaCl_2$. The positive effect of $CaCl_2$ was similar to that reported for chymosin-induced milk coagulation (Sandra & Corredig, 2013; Udabage et al., 2001), which occurs because the addition of $CaCl_2$ increases [Ca^{2+}] and enhances bridging between casein micelles. In addition, ion-specific interactions between Ca^{2+} and caseins may occur; these interactions cannot occur with Na^+ due to its monovalent nature. For example, added $CaCl_2$ can crosslink casein molecules by calcium bridging or can form calcium phosphate salt at high pH values and crosslink casein molecules via CCP (Choi et al., 2007; Ramasubramanian et al., 2014). In addition, as the ionic strength increases with the addition of salts, the negative charges on the casein micelles can be screened. However, as the positive control for ionic strength had a negative effect on the coagulation behavior in our study (discussed below), the positive effects of $CaCl_2$ addition can be attributed to the strong specific effect of Ca^{2+}. The values of dG'/dt, G'_{max}, and yield stress were positively correlated with the addition of $CaCl_2$ in the range from 7.50 to 17.5 m*M*. However, the values decreased at 22.5 m*M* $CaCl_2$ addition, even though Ca-22.5 had the highest protein concentration due to less dilution of SGF during pH adjustment. As reported for chymosin-induced milk curd (van Hooydonk, 1987), the increased calcium bridging between micelles increased the rate of firming and resulted in the formation of stiffer curds (the microstructure of the curds will be discussed in **Section 6.3.4**). However, excessive calcium has been shown to have a negative effect on curd formation, due to increasing the positive charges on the surface of the micelle, which contributes to charge repulsion and a weaker curd (Choi et al., 2007; Fox et al., 2017a; Lucey & Fox, 1993; Patel & Reuter, 1986; Udabage et al., 2001).

Except for sample Na-30.0, which did not coagulate within 120 min, coagulation time was positively correlated with NaCl concentration, and the values of dG'/dt, G'_{max}, and yield stress were negatively correlated with the NaCl concentration (**Table 6-2**). In agreement with chymosin-induced coagulation (Karlsson, Ipsen, & Ardö, 2007), the degree of κ-casein hydrolysis at the coagulation time (H_{ct}) was similar for the samples at both NaCl concentrations. Although an increase in ionic strength upon addition of NaCl reduces electrostatic repulsion between casein micelles, according to Dalgleish (1983), the apparent activation energy for coagulation increases, and the coagulation rate decreases. van Hooydonk (1987) reported that the charge screening influence of electrolytes on charged groups can impair ionic bond formation and thereby decrease the number of effective collisions, despite a reduction in electrostatic repulsion. In contrast, Famelart, Gauvin, Pâquet, & Brulé (2009) found that the binding of hydrated Na^+ to negatively charged casein micelles via electrostatic interactions results in greater hydration and solubility of the casein (Bringe & Kinsella, 1991; Saluja & Kalonia, 2008), thereby reducing the tendency for coagulation (Lin, Wong, Deeth, & Oh, 2020). Therefore, the addition of NaCl presumably reduces the rate of coagulation (d*G′*/d*t*) (Karlsson et al., 2007). At the similar ionic strength, *e.g.*, Ca-12.5 compared with Na-7.50, the retarded coagulation in the samples with added NaCl indicated that the effect of ionic strength was dependent upon the type of salt added (Ca^{2+} or Na^+).

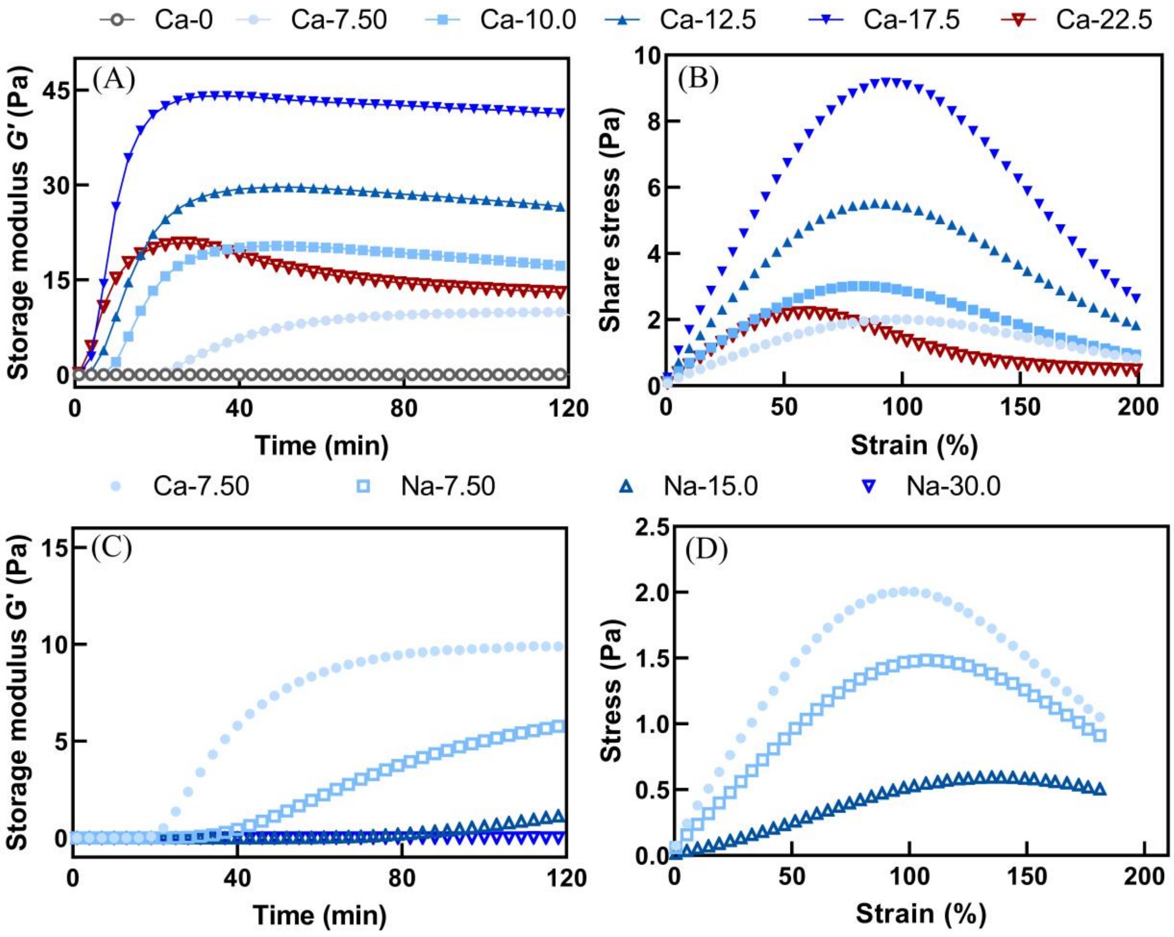

Figure 6-2 (A) Storage modulus (*G′*) of the pepsin-induced coagulum in micellar casein (MC) samples with different amounts of added of $CaCl_2$ at pH 6.0 under small amplitude oscillation. (B) Shear stress as a function of applied deformation at a constant shear rate (0.00185 s^{-1}) for the formed coagulum (○ Ca-0; ● Ca-7.50; ■ Ca-10.0; ▲ Ca-12.5; ▼ Ca-17.5; ▽ Ca-22.5). (C) *G′* of the pepsin-induced coagulum in samples with different additions of NaCl at pH 6.0. (D) Shear stress as a function of applied deformation at a constant shear rate (0.00185 s^{-1}) for the formed coagulum (● Ca-7.50; □ Na-7.50; △ Na-15.0; ▽ Na-30.0) (Note: NaCl added samples all contain 7.50 m*M* $CaCl_2$.). Samples Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 0.00, 7.50, 10.0, 12.5, 17.5, and 22.5 m*M*, respectively. Samples Na-7.50 to Na-30.0 represent sample Ca-7.50 after the addition of NaCl at 7.50, 15.0, and 30.0 m*M*, respectively.

6.3.4. Effect of $CaCl_2$ and NaCl addition on the microstructure of pepsin-induced casein curd

The microstructures of the curds formed from samples Ca-7.50, Ca-12.5, Ca-22.5, and Na-15.0 at 120 min were observed using CLSM and SEM, as shown in **Figure 6-3 A1–A4** and **Figure 6-3 B1–B4**, respectively (the microstructures of the curds from samples Ca-0 and Na-30.0 were not compared as there was no curd formation in these samples).Compared with sample Ca-7.50, Ca-12.5 sample showed denser and more homogeneous protein network structures, with smaller pores and less spacing between the aggregates, as shown in Fig. 3 (A2) (CLSM) and **Figure 6-3 B2** (SEM). This behavior is consistent with the effect of calcium on chymosin-induced curd structures, presumably due to increased calcium bridging between casein proteins (Ong et al., 2013a; Priyashantha et al., 2019). The curd structure formed from sample Ca-22.5 was different from those of the curds formed from the other samples, in that distinct protein aggregates and extremely large pores could be seen in the CLSM image (**Figure 6-3 A3**). Although no large pores were observed in the SEM image (**Figure 6-3 B3**), the protein strands were irregular and uneven. After excessive addition of $CaCl_2$ (22.5 m*M*), as mentioned above, it is probable that the calcium and phosphate from the serum phase transferred into the casein micelles as CCP (Walstra, 1999b). An increase in CCP at this level would increase crosslinking of the casein molecules and contribute to formation of denser protein aggregates and a larger pore size, which is consistent with the rheological results in **Figure 6-2 A**. When sample Ca-7.50 (**Figure 6-3 A1** and **Figure 6-3 B1**) was compared with sample Na-15.0 [the same addition of $CaCl_2$ at 7.50 m*M* (**Figure 6-3 A4** and **Figure 6-3 B4**), the latter has a more homogeneous but looser microstructure in the CLSM image and a finer grained appearance in the SEM image. According to Paulson, Mcmahon, & Oberg (1998), NaCl increases the water-holding capacity of the cheese matrix by increasing hydration of the casein molecules, thereby reducing hydrophobic interactions between casein molecules. Therefore, the Na-15.0 sample has more pores but a more evenly distributed protein arrangement.

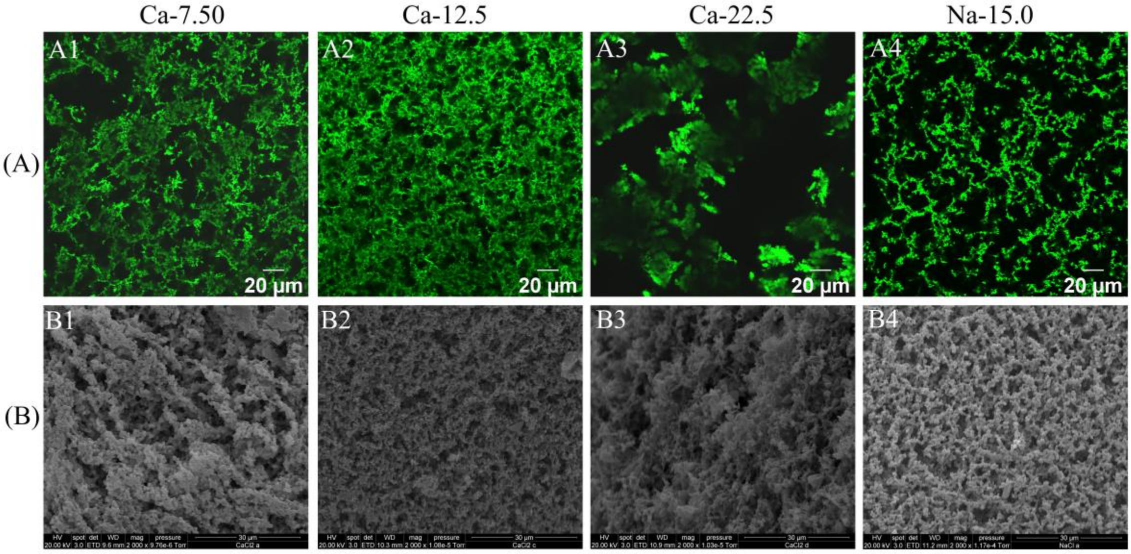

Figure 6-3 Microstructures of pepsin-induced coagula formed from micellar casein (MC) solutions at 120 min and 37 °C without stirring as analyzed by (A) confocal laser scanning microscopy (CLSM) (Protein network appearing as green and the serum pores appearing as black) and (B) scanning electron microscopy (SEM) (Protein network appearing as grey and the serum pores appearing as black). Samples Ca-7.50 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 7.50, 12.5, and 22.5 m*M*, respectively. Sample Na-15.0 represents sample Ca-7.50 after the addition of NaCl at 15.0 m*M*. The scale bars are 20 µm in length for the CLSM micrographs and 30 µm in length for the SEM micrographs.

6.3.5. Effect of $CaCl_2$ and NaCl addition on digestion

6.3.5.1. Gastric pH profile and [Ca^{2+}]

As there was no pH adjustment of the MC solutions before digestion, the initial pH of each sample was slightly different (a lower initial pH in the samples with higher $CaCl_2$ concentration, as mentioned in **Section 6.3.1)**. The changes in pH were significantly affected by the addition of $CaCl_2$ or NaCl, the digestion time, and their interactions ($P < 0.05$, **Figure 6-4 A**). During the first 100 min of digestion, the pH of sample Ca-0 was significantly higher ($P < 0.05$) than those of the other samples, followed by samples Ca-7.50, Na-30.0, and Ca-17.5 and Ca-22.5. The differences in pH may have also been related to the formation of curds with different structures. Because of the rapid formation of solid curds in samples Ca-17.5

and Ca-22.5 (as discussed later), the diffusion of molecules and ions from the SGF into and out of the curds would be slower. In the later stages of digestion (> 100 min), the pH changes amongst all samples were similar ($P > 0.05$) and the pH decreased to ~ 2.0 after 180 min of digestion.

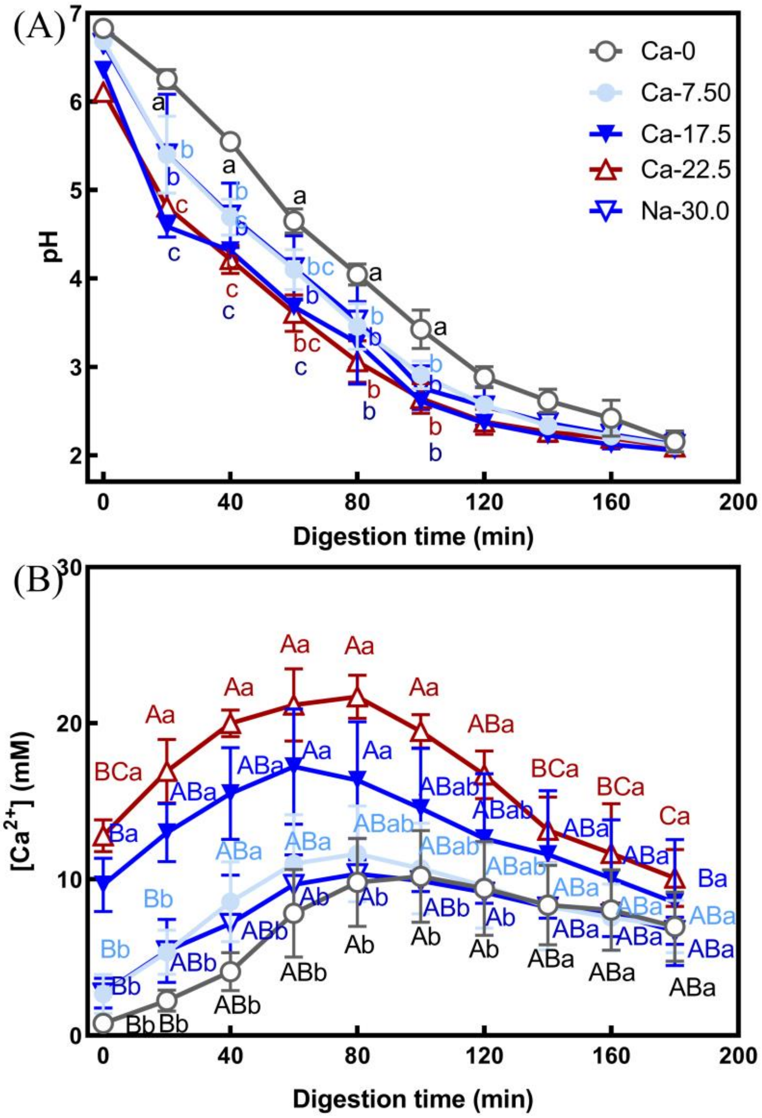

Figure 6-4 Measured (A) pH profiles, and (B) calcium ion concentrations ($[Ca^{2+}]$) of the gastric digesta during 180 min digestion of micellar casein (MC) solutions (○ Ca-0; ● Ca-7.50; ▼ Ca-17.5; △ Ca-22.5; ▽ Na-30.0). (Note: Na-30.0 contains 7.50 m*M* $CaCl_2$.). Samples Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 0.0, 7.50, 17.5, and 22.5 m*M*, respectively. Sample Na-30.0 represents sample Ca-7.50 after the addition of NaCl at 30.0 m*M*. $^{A–C}$ Mean values for the same sample at different digestion times with different superscripts are significantly different ($P < 0.05$). $^{a–c}$ Mean values between samples with different superscripts are significantly different ($P < 0.05$). Error bars represent the standard deviations from triplicate measurements.

The changes in [Ca^{2+}] during digestion, shown in **Figure 6-4 B**, were significantly affected by the addition of $CaCl_2$ or NaCl, the digestion time, and their interactions ($P < 0.05$). In general, comparatively higher [Ca^{2+}] concentrations were found in samples Ca-22.5 and Ca-17.5 during the first 40 min. The [Ca^{2+}] in all samples increased during the first ~ 80 min and then decreased thereafter. As digestion progressed, the increase in [Ca^{2+}] can be attributed to the addition of SGF, which contains 0.15 m*M* $CaCl_2$, and the gradual solubilization of the colloidal calcium because of the decrease in pH (Gaucheron, 2005). In contrast, because of the dilution effect during the digestion process (the addition of SGF and the emptying of the digesta), there was an expected decreasing trend in [Ca^{2+}] in the later stages of digestion.

6.3.5.2. Formation of gastric curds

The samples Ca-17.5 and Ca-22.5 coagulated immediately when ingested in the HGS. Samples Ca-7.50 and Na-30.0 coagulated more slowly but still within the first 10 min. No significant structured curd was observed for sample Ca-0 until 50 min of digestion, when the pH was ~ 5, close to the pI of the casein proteins. At the beginning of *in vitro* gastric digestion, the pepsin concentration in the HGS was ~ 33 U (mL solution)$^{-1}$ and increased with the digestion time (from the protocol described in **Section 6.2.4**). Most of the κ-casein was hydrolyzed to para-κ-casein and (glyco)macropeptide in the first few minutes (**Figure S 6-1**); however, even when the degree of hydrolysis of κ-casein was sufficiently high, a minimum calcium concentration was required for pepsin-induced casein coagulation. The slow coagulation of sample Ca-0 was similar to the digestion behavior of calcium-depleted milk protein concentrate, as described by Wang et al. (2018). These authors concluded that this slow coagulation of calcium-depleted milk protein concentrate could be due to a low proportion of intact casein micelles. However, in our study, as coagulation occurs with the addition of $CaCl_2$, the Ca-0 MC solution probably contained intact micelles. Therefore, below a minimum calcium concentration, coagulation occurred not as a result of the hydrolysis κ-casein, but primarily due to the acidic pH. At lower pH (< 5), the CCP started to solubilize, and the native casein micelles are dissociated, which allows increased hydrophobic interactions between casein proteins. Accordingly, the pH values at coagulation for samples Ca-7.50, Ca-17.5, Ca-22.5, and Na-30.0 were all above 6. Therefore, coagulation was primarily due to the action of pepsin on the κ-casein at the surface of the casein micelles, resulting in their destabilization (Mulet-Cabero et al., 2019; Ye et al., 2016a, 2016b; Ye et al., 2019b). Although the *G′* of sample Na-30.0 did not measurably increase during the 120 min of digestion at pH 6.0 (**Figure 6-2 A**), a curd was observed in the gastric environment (**Figure 6-5 Am**).

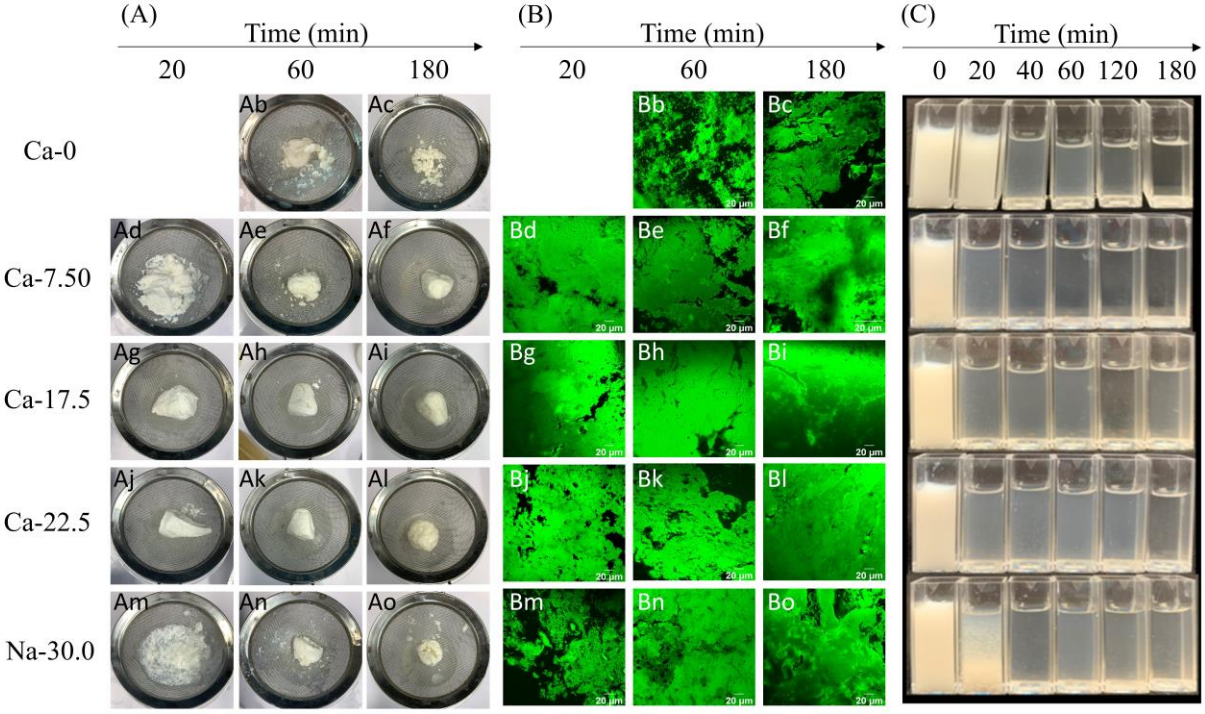

Figure 6-5 (A) Photographs (macrostructure) and (B) confocal micrographs (microstructure) of curds formed from micellar casein (MC) solution samples at 20, 60, and 180 min of gastric digestion in a human gastric simulator. (C) Photographs of the emptied digesta from 20 to 180 min of digestion. Samples Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 0.00, 7.50, 17.5, and 22.5 m*M*, respectively. Sample Na-30.0 represents sample Ca-7.50 after the addition of NaCl at 30.0 m*M*.

The effects of $CaCl_2$ and NaCl addition on the macro- and microstructures of the curds formed during the gastric digestion of the MC solution are presented in **Figure 6-5 A** and **B**, respectively. At 20 min, sample Ca-7.50 formed a crumbled curd (**Figure 6-5 Ad**), with a wet and soft appearance similar to that of cottage cheese. With the same addition of $CaCl_2$ (7.50 m*M*), the curd of sample Na-30.0 (**Figure 6-5 Am**) appeared to be more fragmented with smaller granules than the curd formed from sample Ca-7.50 (**Figure 6-5 Ad)**. The microstructure of the sample Na-30.0 (**Figure 6-5 Bm**) curd was more open and porous, with smaller continuous solid structures surrounded by more and larger gaps. In contrast, the Ca-17.5 curd (**Figure 6-5 Ag**) and the Ca-22.5 curd (**Figure 6-5 Aj**) were intact with relatively smooth surfaces. The microstructure of the sample Ca-17.5 curd (**Figure 6-5 Bg**), showed small pores within the curd matrix. More gaps were found in the matrix of the sample Ca-22.5 curd (**Figure 6-5 Bj**). This could be attributed to easier rearrangement and subsequent syneresis of the protein network in sample Ca-22.5, as mentioned in **Section 6.3.3**. At 60 min, there was obvious curd formation for sample Ca-0 (**Figure 6-5 Ab**). The curds formed from samples Ca-7.50 (**Figure 6-5 Ae**) and Na-30.0 (**Figure 6-5 An**) were denser, with relatively smooth surfaces. After 180 min of digestion, the curds of all samples became smaller in weight and the curd of sample Ca-0 (**Figure 6-5 Ac**) consisted of small granules. The protein matrix of the sample Ca-0 curd was the most fragmented with the most pores amongst all samples, which was similar to the microstructure of calcium-depleted milk protein concentrate after 220 min of digestion in a HGS (Wang et al., 2018).

The liquid digesta were collected every 20 min, as reported in **Figure 6-5 C** (the digesta at 0 min is the MC solution). At 20 min, the digesta for samples Ca-0 and Na-30.0 were turbid, as most of the casein proteins were still in the liquid phase. The digesta for samples Ca-7.50, Ca-17.5, and Ca-22.5 were less turbid with few sedimented particles because most of the casein proteins were coagulated in the early stage of digestion, as shown in **Figure 6-5 A**. All the digesta appeared to be less turbid after 180 min of digestion.

6.3.5.3. Weight and texture analysis of the curds

The dry matter content, water content, and total weight (the wet weight of the curd weighed immediately after sampling) of the curds at each time point are presented in **Figure 6-6 A** (except for sample Ca-0 at 20 min). All values were significantly affected by the addition of $CaCl_2$ or NaCl, the digestion time, and their interactions ($P < 0.05$). At the beginning of the digestion (20 min), the total weight of the curds followed the sample order Ca-7.50 > Ca-17.5 ≈ Ca-22.5 > Na-30.0. The total weights of the sample Ca-7.50, Ca-17.5, and Ca-22.5 curds decreased significantly after 180 min of digestion due to hydrolysis of proteins by pepsin (decreased dry matter content) and the drained water content (decreased water content). For the curd samples Ca-0 and Na-30.0, the total weights were low (or not available) at 20 min, increased at 60 min, and then decreased at 180 min. At 20 min, some of the curd particles were still less than 1 mm in size and were present in the digesta and the chyme (**Figure 6-5 C**). The increased weight of the curd (both in dry matter and water content) at 60 min was due to coagulation of the caseins between 20 to 60 min. As discussed in **Section 6.3.5.2**, the delayed coagulation of sample Ca-0 was due to the limited [Ca^{2+}]. There was curd formation at 20 min for sample Na-30.0, as shown in **Figure 6-5 Am**; however, because of inhibited coagulation at higher ionic strengths, some of the casein proteins was not incorporated into the curd at 20 min. From 20 to 60 min, when the pH was closer to the pI (4.6) of the casein proteins, CCP would be expected to completely dissolve, which may allow more water to be incorporated into the sample Ca-0 and Na-30.0 curds at 60 min compared to 20 and 180 min.

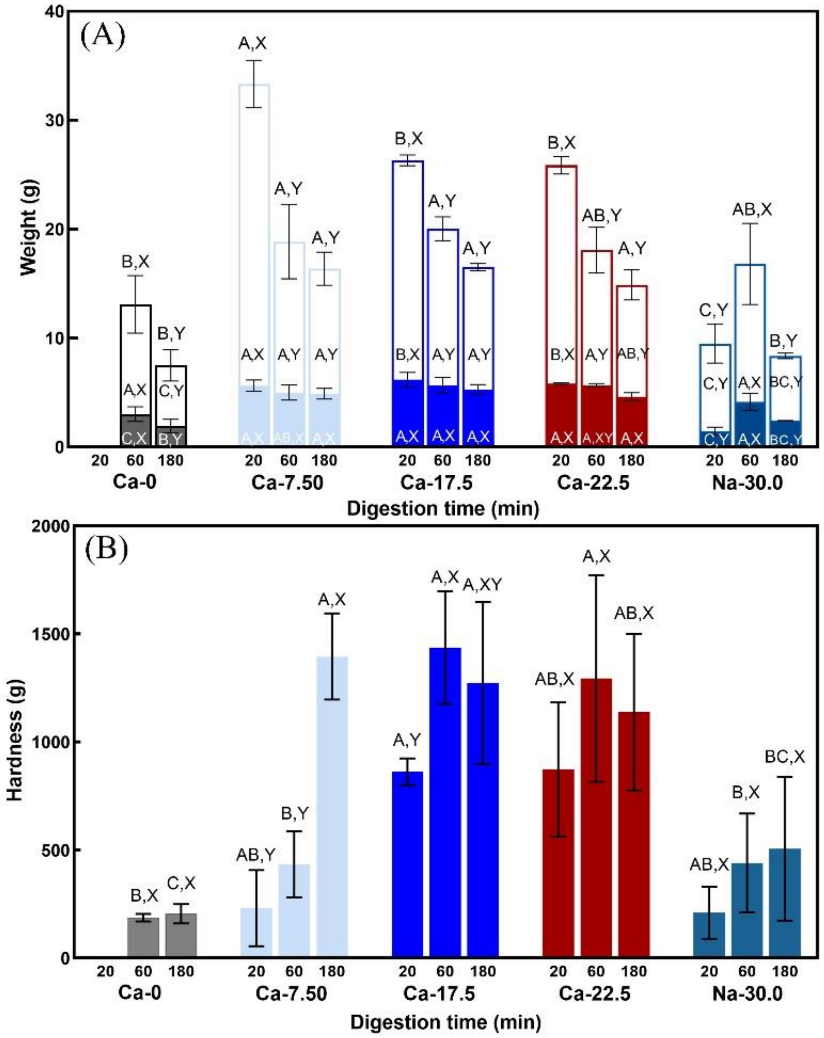

Figure 6-6 (A) Dry matter weight (bottom colored bar) and water weight (top white bar) of fresh curds formed during gastric digestion of micellar casein (MC) solution samples at 20, 60, and 180 min. (B) Hardness of the curd formed during the gastric digestion at 20, 60, and 180 min. Samples Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 0.00, 7.50, 17.5, and 22.5 m*M*, respectively. Sample Na-30.0 represents sample Ca-7.50 after the addition of NaCl at 30.0 m*M*. $^{X–Z}$ Mean values for the same sample at different digestion times with different superscripts are significantly different ($P < 0.05$). $^{A–C}$ Mean values between samples at the same digestion time with different superscripts are significantly different ($P < 0.05$). Error bars represent standard deviations from triplicate measurements.

The hardness values of the curds at 20, 60, and 180 min are shown in **Figure 6-6** (**B**). The changes in the hardness of each sample with digestion time could be related to the structural rearrangement of the protein network (due to the dissolution of CCP at low pH) and the mechanical peristaltic movement of the stomach chamber during the dynamic digestion process. The hardness of the sample Ca-7.50 curd increased markedly at 180 min and became similar to those of the sample Ca-17.5 and Ca-22.5 curds. All the different hardness values of the curds could be related to the effect of $CaCl_2$ and NaCl on the coagulation behavior of the casein micelles and the structure of the curds, as discussed above. The coagulation of sample Ca-0 was believed to be caused by the acidic environment, with weak internal bonds in the casein micelles (because of the dissolution of CCP) during coagulation and a looser structure. Thus, the hardness of the sample Ca-0 curd was lower than those of the other curds, especially the sample Ca-7.50, Ca-17.5, and Ca-22.5 curds at 60 and 120 min. Greater addition of $CaCl_2$ resulted in faster coagulation with relatively denser structures and higher hardness values. Because of the different coagulation behavior of sample Na-30.0, its curd hardness at 180 min was lower than that of sample Ca-7.50 [both samples had the same concentration of $CaCl_2$ (7.50 m*M*)] and was lower than that of sample Ca-17.5 [both samples were at the same ionic strength (52.5 m*M*)].

6.3.5.5. Protein profiles of curds and emptied digesta

The protein patterns during digestion were determined using RP-HPLC (the protein pattern for sample Ca-17.5 during digestion is shown in **Figure S 6-3**, as an example). The amount of each major casein (αs_1-casein, αs_2-casein, β-casein, and κ-casein) was calculated according to the peak area compared to that in the MC solution. The total casein content in the curds and the digesta are compared in **Figure 6-7 A** and **B**, respectively. Both were significantly affected by the addition of $CaCl_2$ or NaCl, the digestion time, and their interactions ($P < 0.05$). A considerable amount of casein was found in the digesta of sample Ca-0 at 20 min; this decreased significantly with an increase in digestion time. As the pH was still above 4 (in the first 80 min), two reasons for this decrease in casein content of the digesta are: (1) protein coagulation involving more protein in the curd, and (2) the dilution effect during the digestion process (the addition of SGF and the emptying of the digesta). For samples Ca-7.5, Ca-17.5, and Ca-22.5, most of the protein was incorporated into the curds (**Figure 6-7 B**) because of faster coagulation of the casein micelles.

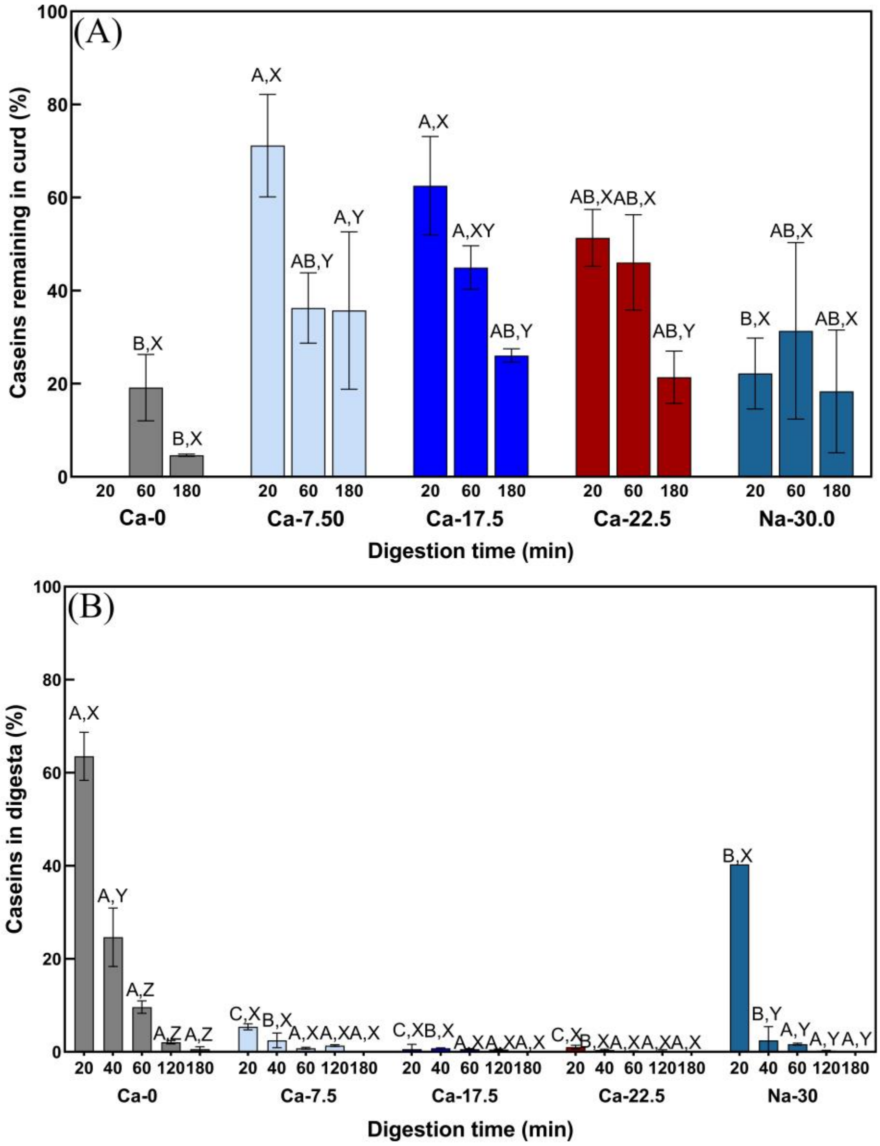

Figure 6-7 (A) Percentage of caseins remaining in the curd formed from micellar casein (MC) solutions at 20, 60, and 180 min of digestion. (B) Percentage of caseins detected in the 60 g of emptied digesta at 20, 40, 60, 120, and 180 min of digestion. Samples Ca-0 to Ca-22.5 represent MC solutions with the addition of $CaCl_2$ at 0.00, 7.50, 17.5, and 22.5 m*M*, respectively. Sample Na-30.0 represents sample Ca-7.50 after the addition of NaCl at 30.0 m*M*. $^{X–Z}$ Mean values for the same sample at different digestion times with different superscripts are significantly different ($P < 0.05$). $^{A–C}$ Mean values between samples at the same digestion time with different superscripts are significantly different ($P < 0.05$). Error bars represent standard deviations from triplicate measurements.

As digestion progressed, the decreased intensity of all proteins in both the curds and the digesta, especially after 120 min, was due to hydrolysis by pepsin when the pH was < 4 (Wang et al., 2018). After 180 min of digestion, the lowest casein content was found in the Ca-0 curd, whereas differences amongst the other samples were not significant ($P > 0.05$). This indicates that the greatest release rate of the proteins occurred in sample Ca-0 during gastric digestion. According to Ye et al. (2017), faster degradation of proteins is caused by a looser and more crumbly structure of the curds in the early stages of digestion, which allows pepsin to rapidly diffuse into the curd and hydrolyze the proteins. However, more proteins were emptied into the digesta at each time point for samples with a looser curd structure in the early stages of digestion, resulting in a greater release rate of the proteins. Therefore, both Ca^{2+} and ionic strength are important to the coagulation and digestion behavior of the casein micelles; however, for the samples with added $CaCl_2$, Ca^{2+} plays a more important role in digestion than ionic strength.

6.4. Conclusions

The present study has demonstrated the important role of Ca^{2+} in pepsin-induced coagulation of casein during gastric digestion, despite the pepsin-induced hydrolysis of κ-casein being independent of both calcium concentration and ionic strength. Even though the hydrolysis of κ-casein was extensive, there was a critical calcium concentration (~ 4.8 m*M* [Ca^{2+}] at pH 6.0 in this study) required for coagulation of casein micelles. The addition of Ca^{2+} promoted the coagulation of the casein micelles and enhanced the strength and structure of the curd through inter-micellar Ca bridging. The degree of κ-casein hydrolysis required for coagulation decreased markedly from 75% to 11% when the [Ca^{2+}] was increased from 7.50 to 22.5 m*M*. The highest maximum storage modulus of the curd occurred at 17.5 m*M* $CaCl_2$ and decreased at 22.5 m*M* $CaCl_2$. However, increasing the ionic strength by adding NaCl impaired the coagulation of the casein proteins, resulting in a lower storage modulus at 120 min.

In the dynamic *in vitro* gastric digestion, the coagulation behavior of MC was dependent on the level of $CaCl_2$ or NaCl, which in turn impacted the release rate of proteins during digestion. Except for the MC solution without $CaCl_2$ addition, which coagulated at pH ~ 5 (> 50 min), the other samples formed structured curds at pH > 6.0 (< 10 min). The curds formed from samples with higher $[Ca^{2+}]$ (either higher or equal ionic strength) had a much denser and firmer structure, with a slower release of proteins. At the same $[Ca^{2+}]$, the sample with the higher ionic strength (by the addition of NaCl) formed a looser and fractured structure, leading to a quicker release of proteins during the initial stage. However, there was no significant difference in protein content within the curd after 180 min of digestion. This study has highlighted the role of Ca^{2+} in the structural and rheological properties of pepsin-induced casein protein curds and the importance of Ca^{2+} in affecting the breakdown and gastric emptying of proteins under gastric conditions.

6.5. Acknowledgments

This study was funded by the New Zealand Milks Mean More (NZ3M) program and the Riddet Institute Centre of Research Excellence, Tertiary Education Commission, New Zealand. The author Mengxiao Yang thanks the Australian Institute of Nuclear Science and Engineering (AINSE) for a Post Graduate Research Award. We would like to acknowledge the support of Michael Agnew (AgResearch Limited, New Zealand) for helping with the RP-HPLC and Claire Woodhall (Havelock North, New Zealand) for proofreading the manuscript.

6.6. Supplementary materials

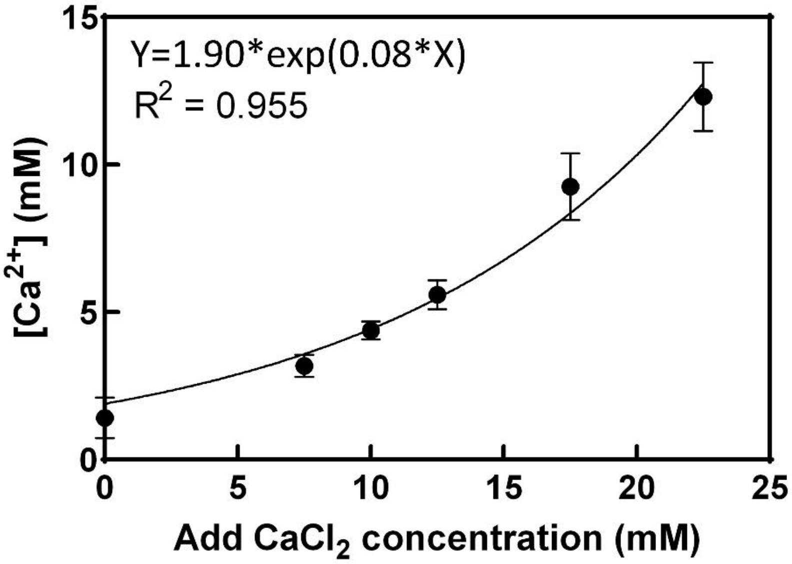

Figure S 6-1 The relationship between increased [Ca^{2+}] after the addition of $CaCl_2$.

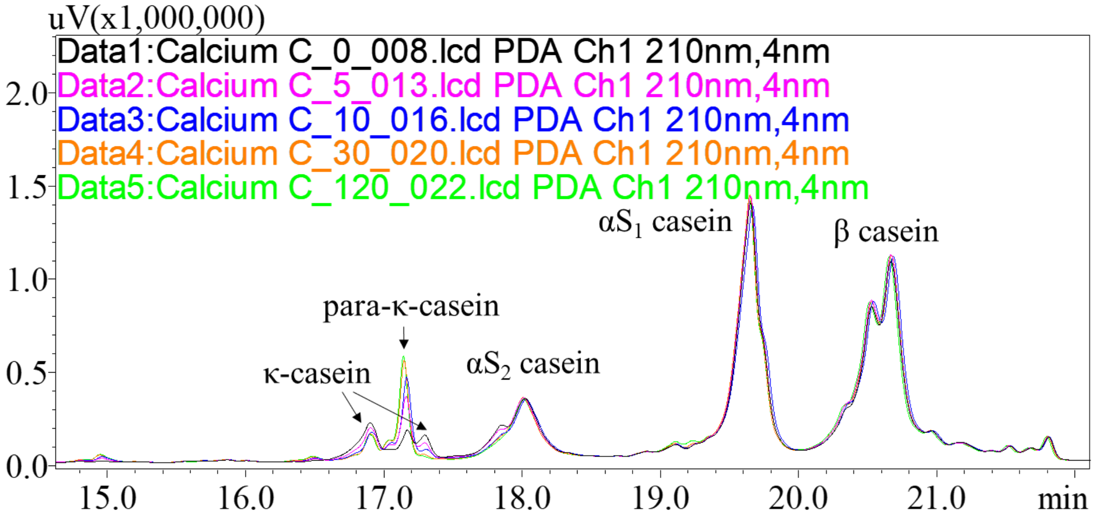

Figure S 6-2 Example of an overlay of UV chromatograms analyzing protein profiles before (−) and after the addition of pepsin at 5 min (−), 10 min (−), 30 min (−), and 120 min (−) for a micellar casein (MC) sample with the addition of $CaCl_2$ at 17.5 m*M* at pH 6.0. All major milk proteins were identified.

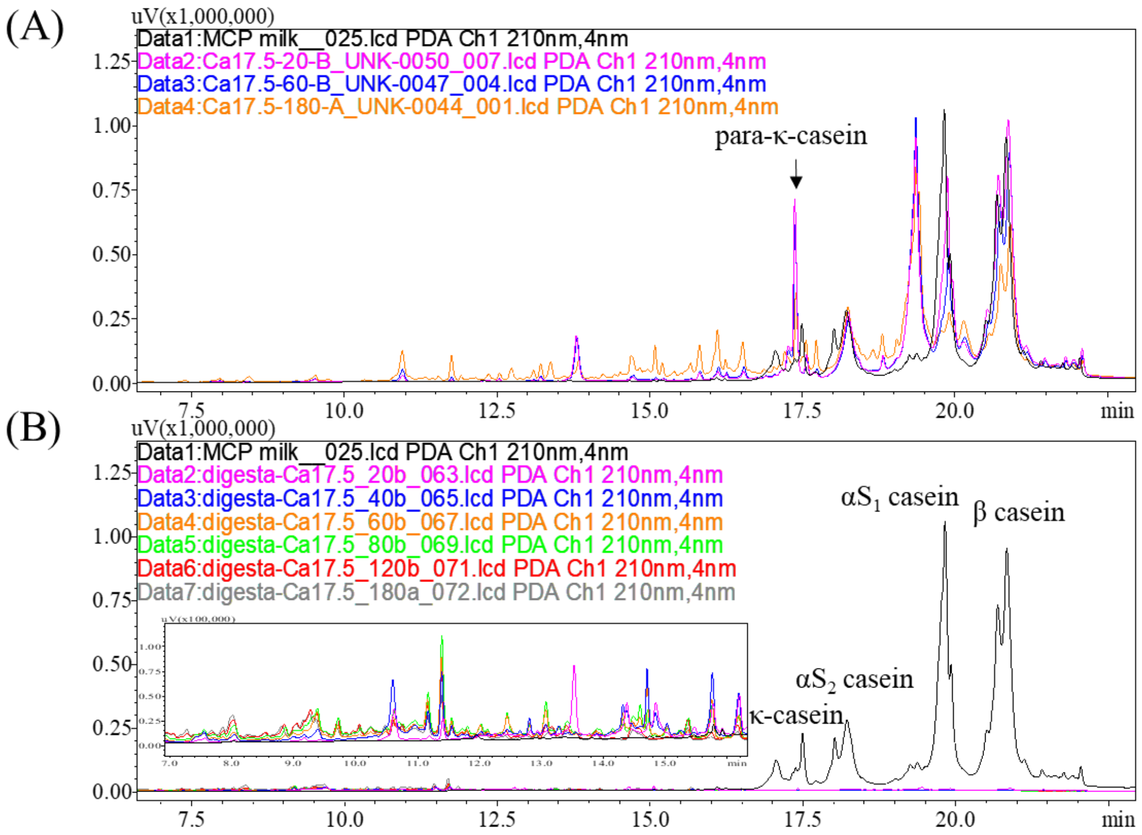

Figure S 6-3 (A) Overlay of UV chromatograms analyzing the protein profiles of a micellar casein (MC) solution (−) and the curd formed from an MC sample with the addition of $CaCl_2$ at 17.5 m*M* (Ca-17.5): 20 min (−), 60 min (−), and 180 min (−). (B) Overlay of UV chromatograms analyzing the protein profiles of the MC solution (−) and the Ca-17.5 emptied digesta: 20 min (−), 40 min (−), 60 min (−), 120 min (−), 180 min (−).

STATEMENT OF CONTRIBUTION
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Student name:	Mengxiao Yang
Name and title of main supervisor:	Professor Aiqian Ye
In which chapter is the manuscript/published work?	Chapter 7

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

Mengxiao Yang: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft.
Aiqian Ye: Conceptualization, Funding acquisition, Supervision, Resources, Writing – review & editing. Zhi Yang: Supervision, Writing – review & editing. David W. Everett: Supervision, Writing – review & editing. Elliot Paul Gilbert: Supervision, Writing – review & editing. Harjinder Singh: Supervision, Resources, Writing – review & editing.

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Chapter 7 Pepsin-induced hydrolysis and coagulation of proteins in goat, sheep and cow milk[6]

Abstract

The kinetics of pepsin-induced hydrolysis of κ-casein and the consequent coagulation of casein proteins in cow, goat, and sheep milk were investigated at 37 °C and pH 6.3 and 6.0. At the same pepsin-to-κ-casein ratio (0.1 U/mg), the hydrolysis rate of κ-casein was found to be the highest in sheep milk, followed by cow milk and goat milk as assessed by quantifying the release of para-κ-casein using reverse phase high performance liquid chromatography (RP-HPLC). At both pH 6.3 and 6.0, sheep milk coagulated most rapidly at the lowest extent of κ-casein hydrolysis of 64% and 59%, respectively; goat milk coagulated most slowly and required the highest extent of κ-casein hydrolysis at 90% and 86%, respectively. The rheological properties and the microstructure of the formed coagulum were significantly different for milk of different species. Sheep milk formed a curd with denser microstructure whereas the curd formed from goat milk exhibited a more porous microstructure. Small-angle neutron scattering (SANS) on the freeze-dried milk powders reconstituted to a same casein concentration, indicated differences in rates of increase in the size of aggregates within different species. These results suggest that the differences in the rate of hydrolysis of κ-casein and coagulation of casein micelles from various milk types can not only be attributed to differences in their casein content, but other physicochemical characteristics, such as casein micelle size may play a role. The knowledge obtained from this study provides further understanding on the mechanism of sheep and goat milk coagulation compared to that of cow milk, during the initial stages of gastric digestion.

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[6] This chapter will be submitted for publication: Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Pepsin-induced hydrolysis and coagulation of proteins in goat, sheep and cow milk (In preparation).

7.1. Introduction

For nutritional value and health benefits, milk from non-cow species such as buffalo, goat and sheep has been widely accepted by consumers (Claeys et al., 2014; Roy et al., 2020a). There are distinct differences in physicochemical characteristics between cow, goat and sheep milk (Park et al., 2007). It is known that the fat, protein, and total solids content of sheep milk is higher than that in cow and goat milk. Milk from different species all contain α_{s1}-, α_{s2}-, β-, κ-caseins albeit with slight variations in their relative proportion and amino acid sequence (Selvaggi & Tufarelli, 2012). For instance, compared with cow and sheep caseins, goat caseins contain less α_s-casein (particularly α_{s1}) (Li, Delger, Dave, Singh, & Ye, 2022a; Park, 2007). The casein micelle size of cow, sheep and goat milk also differs. Casein micelle diameters of 142–229 nm, 173–207, and 220–270 nm have been reported for cow, sheep and goat milk, respectively (Pellegrini, Remeuf, & Rivemale, 1994; Pierre, Michel, Le Graët, & Zahoute, 1998; Remeuf, 1993; Remeuf, Cossin, Dervin, Lenoir, & Tomassone, 1991). In addition, the size also depend upon the lactation cycle. However, the internal structure of casein micelles among different species of milk shows strong similarities (Ingham, Smialowska, Kirby, Wang, & Carr, 2018; Yang et al., 2023d).

Coagulation of milk is a fundamental process that occurs in both the manufacture of dairy products (e.g., yoghurt, cheese) (Lucey, 2002) and in the digestion of milk products (Huppertz & Chia, 2021; Ye, 2021). In the presence of digestive enzymes (e.g., chymosin and pepsin), the κ-casein in the boundary layer of casein micelles is hydrolyzed, resulting in coagulation of casein micelles. Compared to cow milk, a shorter coagulation time and weaker curd consistency of goat milk during chymosin-induced coagulation has been observed, which was explained by a lower casein content, lower α_{s1}-casein content and larger casein micelle size (Park et al., 2007; Remeuf & Lenoir, 1986). With its higher β/α_s-casein ratio, sheep milk is very sensitive to chymosin and tends to coagulate faster than cow or goat milk

(Muir et al., 1993; Park, 2007). In addition, sheep milk generally produces firmer curds and has a slower rate of syneresis due to a higher casein and colloidal calcium content (Muir et al., 1993; Storry & Ford, 1982).

During *in vivo* and *in vitro* gastric digestion, regardless of dairy species (i.e., cow, goat and sheep), milk coagulates and separates into curd and liquid phases (Li et al., 2022c; Pan et al., 2021; Roy et al., 2022; Roy et al., 2021). However, the coagulation and digestion behavior are different among these species, e.g., the firmness of the sheep skim milk curd is higher than that of cow and goat milk curds at the end of gastric digestion (Li et al., 2022b). As a result, the stomach emptying time, nutrient bioavailability and the functional properties of the milk greatly depends on the coagulation behavior of the milk during digestion (Huppertz & Chia, 2021; Ye, 2021). For example, the proteins in goat milk are hydrolyzed faster and more extensively than cow milk proteins (Rutella, Solieri, Martini, & Tagliazucchi, 2016; Tagliazucchi, Shamsia, Helal, & Conte, 2017).

It is known that the coagulation of milk at pH ~ 6.1 to 6.3 in the stomach is due to the action of pepsin (Huppertz & Chia, 2021; Tam & Whitaker, 1972; Ye, 2021). The kinetics of pepsin-induced hydrolysis of κ-casein and the consequent coagulation of cow milk have been extensively investigated (Yang et al., 2022a). To measure the microstructure of the milk curds, several techniques have been applied, including microscopy, diffusing wave spectroscopy, and (ultra) small-angle neutron scattering ((U)SANS). Neutron scattering methods are valuable tools for the study of food colloids (Gilbert, 2019). Indeed, according to previous research, (U)SANS has been shown as a powerful technique to probe the microstructure of milk curd induced either by acid, tamarillo protease, or rennet (Callaghan-Patrachar et al., 2021; Li et al., 2018; Yang et al., 2023d). However, information about the kinetics of pepsin-induced hydrolysis of κ-casein and the consequent coagulation of sheep and goat milk is limited.

Since casein is the main component in milk that forms the primary structure of the curd, the coagulation process of milk will greatly depend upon the kinetics of hydrolysis of κ-casein. To extend knowledge on pepsin-induced hydrolysis of κ-casein and the consequent coagulation of proteins in different milk (cow, sheep, and goat), raw fresh milk samples were treated with pepsin at pH 6.3 and 6.0. These two pH values were specifically selected because acid-induced coagulation occurs at pH < 5, and also hydrolysis of κ-casein and coagulation are affected by varying pH levels (Yang et al., 2022a). As the κ-casein content differs in milk from different species, the kinetics of the pepsin-induced hydrolysis of κ-casein was investigated at a same pepsin-to-κ-casein ratio (0.1 U/mg) by measuring the release of para-κ-casein using RP-HPLC. The consequent coagulation behavior and curd microstructures were examined by rheology and confocal laser scanning microscopy (CLSM), respectively. To exclude the effect of different coagulation behavior being related to the casein content in the different types of milk, cow, sheep and goat milk samples were reconstituted to the same casein concentration (~ 2.3 %). Subsequently, at the same pepsin activity, the structural changes of curds from different milk species were compared using SANS. The knowledge obtained provides a deeper understanding of pepsin-induced coagulation of raw cow, sheep and goat milk.

7.2. Materials and methods

7.2.1. Materials

Three different batches of raw whole milk were collected from milk suppliers between January and May 2022. The cow milk was collected from Massey University No. 4 dairy farm (Palmerston North, New Zealand), sheep milk was obtained from Fernglen Ltd. (Masterton, New Zealand), and goat milk was collected from Central Dairy Goats Ltd. (Levin, New Zealand). Upon receipt of the milk samples, 0.02% (wt/wt) sodium azide (Merck KGaA, Darmstadt, Germany) was added to prevent microbial growth. By centrifugation of raw whole milk at 3,000×*g*, 15 min, 4°C with a Thermo Fisher Scientific Multifuge Heraeus 3SR+ centrifuge (Thermo Electron LED GmbH, Osterode, Germany), the fat layer was removed. The initial pH of the skim milk samples was measured and then adjusted to 6.3 or 6.0 at 25 °C by the gradual addition of 1 *M* HCl under vigorous stirring conditions. The raw skim milk samples were stored at 4 °C for up to 5 days until used. Small pH adjustments were made to achieve the desired pH value, as required, before further experiments.

Porcine pepsin (EC 3.4.23.1; Sigma-Aldrich, St. Louis, MO, USA) with stated activity of 541 U/mg protein was dissolved in Milli-Q water (5 mg per 5 mL; activity 541 U/mL). All other chemicals (analytical grade) were obtained from Sigma-Aldrich unless otherwise specified.

7.2.2. Determination of milk composition

The composition of the cow, goat and sheep milk was analyzed using a Milkoscan (Foss, Hilleroed, Denmark). The ionic calcium concentration [Ca^{2+}] in the milk samples was determined by a calcium-selective electrode (Orion 9720BNWP; Thermo Scientific), as described by Yang et al. (2023b). The [Ca^{2+}] of milk samples was measured at room temperature (~25 °C) before and after pH adjustment.

7.2.3. Determination of casein micelle diameter by dynamic light scattering

The diameters of casein micelles in raw milks were measured by a Malvern Zetasizer (Nano ZS; Worcestershire, UK) using disposable cells (DTS0012) at 25°C. The milk samples were diluted 50× with calcium imidazole buffer solution (20 m*M* imidazole, 5 m*M* $CaCl_2$, 30 m*M* NaCl, pH 7.0) and analyzed under the ‘milk protein size analysis’ option (Anema & Li, 2003). The diameters of casein micelles were measured before and after pH adjustment. In addition, a Mastersizer Hydro 2000 (Malvern Instruments Ltd, Worcestershire, U.K.) was used to determine the specific surface area (m^2/g) of the casein micelles.

7.2.4. Pepsin-induced hydrolysis and coagulation at the same pepsin-to-κ-casein ratio

To treat the three milk samples at the same pepsin-to-κ-casein ratio (0.1 U/mg), the pepsin solution (541 U/mL) was diluted 7.7×, 6.3×, and 11.7× with water. The diluted pepsin solutions were added to milk at a ratio of 10 µL per 1 mL milk. As a result, the final pepsin concentrations in cow, sheep and goat milk were ~ 0.7, 0.9 and 0.5 U/mL, respectively.

7.2.4.1. Measurement of hydrolysis of κ-casein

The action of pepsin on κ-casein was tracked by measuring changes in the amount of para-κ-casein using RP-HPLC (Yang et al., 2022a). An aliquot of diluted pepsin solution (20 µL) was added to 2 mL samples, which was then immediately transferred into eight different test tubes (0.2 mL per tube) and incubated in a water bath at 37 °C. The reaction was stopped at different times (0, 2, 5, 10, 20, 30, 60, and 90 min) with HPLC buffer solution (0.8 mL, 6 *M* guanidinium-HCl, 0.1 *M* Bis-Tris, 19.5 m*M* DL-dithiothreitol and 5.37 m*M* sodium citrate, pH 7). Note, the sample at 0 min corresponds to the milk samples without pepsin addition. Each sample was shaken for 10 s and left at room temperature for 1 h before HPLC injection. Four time points (5, 10, 30, and 90 min) were examined by HPLC in triplicate.

The analysis was carried out using a Nexera-X2 ultra-HPLC instrument equipped with an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). The protein composition of milk samples (0 min, without pepsin addition) was indicated by percentages based on the peak areas from the individual proteins to the total peak area of all proteins using LabSolutions Main (Shimadzu) software. The amount of para-κ-casein was quantified based on the peak areas with an established method described previously (Yang et al., 2022a). The degree of hydrolysis of κ-casein at each time point was thus determined, calculated as the ratio between the obtained chromatographic peak area of para-κ-casein at a given time and the chromatographic peak area of para-κ-casein at complete hydrolysis (Yang et al., 2022a).

7.2.4.2. Rheological measurements

The coagulation process was determined by measuring the storage modulus (*G′*) as a function of time using a stress-controlled rheometer (MCR301 Anton Paar, Graz, Austria) equipped with a Couette geometry (Anton Paar; CC27, with 28.93 mm cup diameter and 26.64 mm bob diameter) (Yang et al., 2022a). An aliquot of diluted pepsin solution (200 μL) was added into 20 mL samples – i.e., the same ratio as used above - and stirred for 30 s before loading. By carrying out a time sweep measurement with a strain of 1% and a constant frequency 0.1 Hz at 37 °C for 90 min, *G′* was recorded every three minutes. The onset of coagulation (coagulation time) was defined as the intercept of a linear regression of the first four *G′* points that consistently increase above the time axis. The firming rate was defined as the maximum slope of *G′* as a function of time (d*G′*/dt) (Yang et al., 2022a).

After the time sweep measurement over 90 min, the large deformation properties of coagulum were determined by applying a constant shear rate of 0.00185 s^{-1}. The yield stress was defined as the point when the shear stress started to decrease (Lucey et al., 1997; Choi et al., 2007). All measurements were carried out in triplicate.

7.2.4.3. Confocal laser scanning microscopy (CLSM)

The microstructure of the pepsin-induced coagulum was examined by CLSM (Yang et al., 2022a). Fast Green fluorescent dye (1% wt/vol in water) was used to stain proteins (3 μL/100 μL). An aliquot of diluted pepsin solution was added into the pre-stained samples with the pepsin concentration as stated above. The sample–pepsin mixture was transferred to the cavity of a microscope glass slide, covered with a glass cover slip, and incubated at 37 °C for 90 min. The fluorescence images were observed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 63× magnification lens at room temperature.

7.2.5. Small-angle neutron scattering (SANS)

Aliquots of raw skim milk from different species (mentioned in **7.2.1**, before pH adjustment) were freeze-dried. All freeze-dried milk powders were reconstituted into Milli-Q H_2O at ~ 2.3 wt% casein content under gentle magnetic stirring for ~ 10 h to allow protein hydration at room temperature (~ 25 °C). The pH of the skim milk samples were then adjusted to 6.3 or 6.0 at 25 °C by the gradual addition of 1 *M* HCl under vigorous stirring conditions. The pepsin solution (541 U/mL) was diluted with water and added to milk at a ratio of 10 μL per 1 mL milk, resulting in a similar pepsin-to-κ-casein ratio at 0.1 U/mg (0.4 U/mL milk) for milk samples from all species. After shaking for 30 s, the samples at pH 6.0 were transferred into the SANS sample cells and incubated in an oven at 37 °C, 2 hours, to reach equilibration. The samples at pH 6.3 were transferred into the SANS sample cells to start the neutron scattering experiments on the QUOKKA beamline at the OPAL reactor (Lucas Heights, NSW, Australia) (Wood et al., 2018) for real-time measurements (averaged over 2 min intervals for 120 min). A thermally controlled sample changer was used to maintain the sample temperature at 37 °C. Measurements on all samples (milk sample without pepsin addition, equilibrated samples at pH 6.0, and samples at pH 6.3) covered a q-range from 0.0007 to 0.03 $Å^{-1}$ where q is the magnitude of the scattering vector and is equal to $(4\pi/\lambda)\sin(\theta)$, and where λ is the wavelength and 2θ is the scattering angle. All data were reduced using IGOR Pro 8.02 software modified for the QUOKKA instrument with the scattering from H_2O subtracted as background (Kline, 2006). Data fitting was carried out using SasView software (version 5.0.5, **https://www.sasview.org/**).

A Guinier–Porod model describing the radius of gyration, and the power-law behavior of a scattering system was used to fit the SANS data. The scattered intensity is given by:

$$\mathrm{I}(q) = \frac{\mathrm{G}}{q^{s}} \exp\left[\frac{-\mathrm{q}^2\mathrm{Rg}^2}{3-\mathrm{S}}\right], \quad q \leq q_1$$

$$I(q) = \frac{D}{q^m}, \quad q \geq q_1 \qquad \textbf{(Equation 7-1)}$$

where q is the magnitude of the scattering vector, $I(q)$ is the scattered intensity, R_g is the radius of gyration, m is the Porod exponent, G and D are the Guinier and Porod scale factors, respectively. S is equal to zero for spheres in this study. The Guinier form is used for $q \leq q_1$ and the Porod form is used for $q \geq q_1$. Enforcing the continuity of the Guinier and Porod functions and their derivatives yields:

$$q1 = \frac{1}{Rg}\sqrt{\frac{3m}{2}}$$

$$D = G \exp\left(\frac{-q1^2 R_g^2}{3}\right) q1^m = G \exp\left(-\frac{m}{2}\right)\left(-\frac{3m}{2}\right)^{\frac{m}{2}} \frac{1}{R_g{}^m} \qquad \textbf{(Equation 7-2)}$$

7.3. Results and discussion

7.3.1. Physicochemical properties of cow, sheep and goat milk

Differences in the physicochemical properties of cow, goat and sheep milk are listed in **Table 7-1**. The highest protein content, casein content, and total solids content were found in sheep milk, consistent with previous reports (Li et al., 2022a; Nguyen, Afsar, & Day, 2018; Roy et al., 2020b). The initial pH was similar for the three milk samples, at ~ 6.7. At the initial pH, the average casein micelle sizes were ~ 161, ~ 186, and ~ 210 nm for cow, sheep, and goat milk, respectively, in accordance with previous studies (Pellegrini et al., 1994; Pierre et al., 1998; Remeuf, 1993; Remeuf et al., 1991). The micelle size distributions can vary widely between different breeds and even between individual animals (de Kruif & Huppertz, 2012); in this study, the largest casein micelle diameter was found in goat milk, followed by sheep milk and cow milk, in agreement with Park et al. (2007). After pH adjustment, to pH 6.3 or 6.0, there was little change in casein micelle size. According to **Table 7-1**, there is no significant difference found in [Ca^{2+}] between cow, sheep and goat milk at pH ~ 6.7. Due to the dissolution of colloidal calcium phosphate (CCP), higher [Ca^{2+}] was observed at lower pH values for each species. The observed [Ca^{2+}] in sheep milk was significantly greater than goat and cow milk at pH 6.0. Although the total calcium concentration was not measured in the current study, according to Li et al. (2022a) and Park et al. (2007), the total calcium concentration in sheep milk is greater than that in goat and cow milk; therefore, it can be concluded that more calcium transformed from the colloidal state to ionic state in sheep milk at pH 6.0.

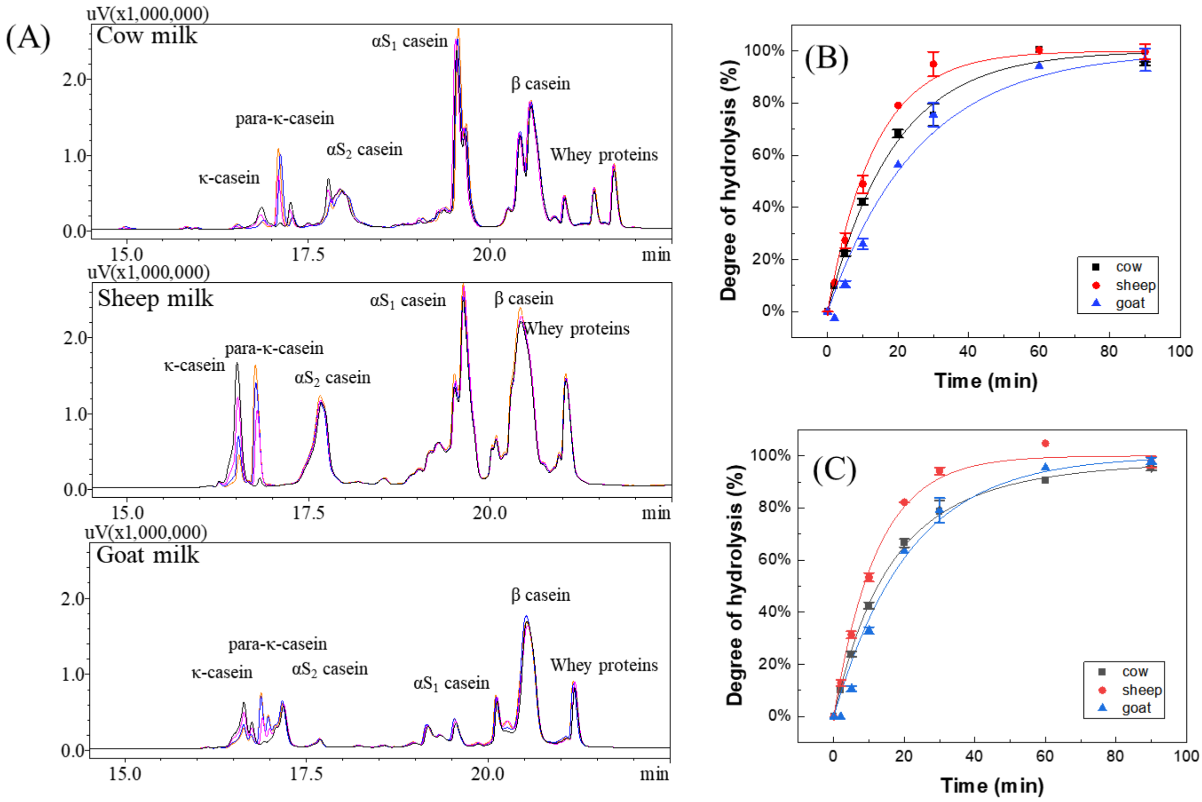

Figure 7-1 (A) Representative example of overlay of UV chromatograms analyzing protein profiles before (–) and after addition of pepsin into cow, sheep and goat milk at pH 6.3, (–) 30 min, (–) 60 min, (–) 90 min. (B) Degree of hydrolysis of κ-casein in each milk as a function of time after the addition of pepsin at pH 6.3. (C) Degree of hydrolysis of κ-casein in each milk as a function of time after the addition of pepsin at pH 6.0. The curves are fits to **Equation 7-3**. Error bars represent SD from triplicate.

Table 7-1 Physicochemical properties of raw cow, goat and sheep skim milk.

	Cow milk	**Sheep milk**	**Goat milk**
Total solid, *wt%*	9.81 ± 0.27	13.28 ± 0.62	8.08 ± 0.21
Lactose, *wt%*	5.07 ± 0.26	5.09 ± 0.19	4.53 ± 0.14
Fat, *wt%*	0.10 ± 0.10	0.43 ± 0.19	0.12 ± 0.14
Protein, *wt%*	3.90 ± 0.18	6.31 ± 0.52	2.91 ± 0.12
Casein, *wt%*	3.16 ± 0.04	5.35 ± 0.06	2.29 ± 0.05
κ-casein, *wt%*	0.56 ± 0.11 (15%) *	0.69 ± 0.08 (11%) *	0.37 ± 0.02 (13%) *
α_{s1}-casein, *wt%*	1.15 ± 0.05 (33%) *	2.06 ± 0.17 (36%) *	0.33 ± 0.01 (13%) *
α_{s2}-casein, *wt%*	0.34 ± 0.02 (10%) *	0.77 ± 0.06 (13%) *	0.33 ± 0.01 (13%) *
β-casein, *wt%*	1.39 ± 0.06 (40%) *	2.25 ± 0.19 (39%) *	1.57 ± 0.07 (60%) *
Initial pH	6.70 ± 0.01	6.74 ± 0.06	6.70 ± 0.04
At initial pH			
Diameter, *nm*	161.0 ± 1.0 ^{AZ}	185.6 ± 4.0 ^{AY}	209.8 ± 10.0 ^{AX}
Specific surface area, *m^2/g*	~ 48.8	~ 53.1	~ 35.0
[Ca^{2+}], *mM*	1.7 ± 0.3 ^{CX}	2.3 ± 0.6 ^{CX}	2.7 ± 0.1 ^{CX}
At pH 6.3			
Diameter, *nm*	160.8 ± 5.3 ^{AZ}	185.6 ± 2.5 ^{AY}	210.4 ± 13.3 ^{AX}
[Ca^{2+}], *mM*	5.5 ± 1.2 ^{BX}	6.2 ± 0.7 ^{BX}	5.3 ± 0.6 ^{BX}
At pH6.0			
Size, *nm*	159.2 ± 0.7 ^{AY}	190.8 ± 2.6 ^{AX}	199.5 ± 2.2 ^{AX}
[Ca^{2+}], *mM*	7.3 ± 1.0 ^{AY}	9.8 ± 0.9 ^{AX}	7.1 ± 0.1 ^{AY}
Pepsin concentration, *U/mL milk*	0.7	0.9	0.5
Pepsin-to-κ-casein ratio, *U/mg*	0.1	0.1	0.1
Pepsin-to-casein surface area ratio, *U/m^2***	0.014	0.016	0.013

*, the weight percent of each casein in total casein.

******, calculated as pepsin concentration divided by specific surface area.

$^{A-B}$ Mean values for the same sample at different pH with different superscripts are significantly different ($P < 0.05$). $^{X-Z}$ Mean values between samples at the same pH with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of the mean ($n = 3$).

The protein composition of milk samples (before the addition of pepsin) is shown in the HPLC pattern in **Figure 7-1 A** (black curves). A slight shift in retention times of the major casein peaks (α_{s1}, α_{s2}, β and κ) occurred in the cow, sheep and goat milk samples, which could be due to the protein genotypes (Li et al., 2022a). Based on the HPLC results, the casein compositions were calculated and presented in Table 1. Consistent with previous reports (Li et al., 2022a; Park et al., 2007), the most abundant protein in goat milk was β-casein and, compared to cow and sheep milk, α_{s1}-casein was at the lowest level in goat milk. The calculated κ-casein in each milk was similar at around 10–15% of casein proteins.

7.3.2. Pepsin-induced hydrolysis and coagulation

7.3.2.1. The hydrolysis of κ-casein in cow, sheep and goat milk

After the addition of pepsin, the peak area of κ-casein decreased and the para-κ-casein peak area increased, as shown in **Figure 7-1 A**. The hydrolysis degree of κ-casein increased with reaction time in the three milk samples at pH 6.3 and 6.0 as shown in **Figure 7-1 B** and **C**, respectively. The data were fitted to **Equation (7-3)** that was previously used for the pepsin-induced hydrolysis of κ-casein in cow milk (Yang et al., 2022a):

$$ln\left(1-\frac{H_t}{100}\right)=\frac{K_{enz}\cdot C}{K_{den}}\cdot\left[exp(-K_{den}\cdot t)-1\right]$$ (**Equation 7-3**)

The resulting reaction constants, K ($K_{\mathrm{enz}}\cdot$C), and denaturation constants, K_{den}, are shown in **Table 7-2**.

Table 7-2 The hydrolysis kinetics of κ-casein in different systems after the addition of pepsin. Results are expressed as the mean ± SD of the mean (n = 3).

	Hydrolysis kinetics of samples at pH 6.3 according to Eq. (7-3)		**Hydrolysis kinetics of samples at pH 6.0 according to Eq. (7-3)**	
Sample	**(K_{enz}·C) (min^{-1})**	**$\mathrm{K_{den}}$(min^{-1})**	**(K_{enz}·C) (min^{-1})**	**$\mathrm{K_{den}}$(min^{-1})**
Cow milk	0.059±0.003 $^{\mathrm{Ba}}$	0.01 ± 0.01 $^{\mathrm{Aa}}$	0.056 ± 0.001 $^{\mathrm{Ba}}$	1.19E-2 ± 2.36E-3 $^{\mathrm{Aa}}$
Sheep milk	0.074±0.005 $^{\mathrm{Aa}}$	4.97E-9 ± 5.59E-9 $^{\mathrm{Ba}}$	0.080 ± 0.002 $^{\mathrm{Aa}}$	1.84E-9 ± 1.31E-9 $^{\mathrm{Ba}}$
Goat milk	0.039±0.002 $^{\mathrm{Ca}}$	2.85E-9 ± 3.97E-10 $^{\mathrm{Ba}}$	0.045 ± 0.004 $^{\mathrm{Ca}}$	4.45E-9 ± 5.55E-9 $^{\mathrm{Ba}}$

$^{\mathrm{a–b}}$ Mean values for the same sample at different pH with different superscripts are significantly different (P < 0.05). $^{\mathrm{A–C}}$ Mean values between samples at the same pH with different superscripts are significantly different (P < 0.05). The results are expressed as the mean ± the standard deviation of the mean (n = 3).

The differences in K and K_{den} between pH 6.3 and 6.0 were not significant ($P > 0.05$), regardless of the milk species. The K for sheep milk was significantly higher than cow and goat milk at either pH 6.3 or 6.0; this indicates that, at the same pepsin-to-κ-casein ratio, the hydrolysis reaction was fastest in sheep milk, followed by cow milk and goat milk. The reason for this difference is unclear, but it could be related to the genetic variations of κ-casein in different milks. It has been reported that the Phe^{105}-Met^{106} bond, which is the specific bond hydrolyzed by pepsin, is present in all three milk types; however, according to Fox & Mulvihill (1990), it is the sequence around the Phe-Met bond, rather than the bond itself, that is the important determinant for hydrolysis. The alignment of the sequences of goat, sheep and cow κ-casein has been compared by Brignon, Chtourou, & Ribadeau-Dumas (1985) and differs in some of the amino residues, e.g., the assignment of residues 102, 103. Although the total surface area to volume ratio is inversely related to the casein micelle size, the casein micelle content varies between samples. Assuming the casein micelles are spherical particles, the total surface areas (specific surface areas) of casein micelles were determined to be ~ 48, 53 and 35 m^2/g for cow, sheep and goat milk, respectively (**Table 7-1**). Assuming that the κ-casein is evenly located at the surface, for the same pepsin-to-κ-casein ratio, the highest pepsin-to-casein micelle surface area ratio would be for goat milk, followed with cow and sheep milk (i.e., ~ 0.014, 0.016 and 0.013 U/m^2 for cow, sheep and goat milk, respectively). Therefore, compared to cow milk, the higher pepsin to casein micelle surface area ratio in sheep milk should result in faster hydrolysis of κ-casein by pepsin; the lower pepsin to casein micelle surface area ratio in goat milk should result in a slower hydrolysis of κ-casein by pepsin. In addition, glycosylated κ-casein carries hydrophilic sugar moieties that both increase the negative charge and reduce the hydrophobicity of κ-casein (Holland & Boland, 2014; Vreeman et al., 1977). According to Park et al. (2007), compared to 60% of κ-casein in cow milk, about 30% of κ-casein in sheep and goat are glycosylated. As pepsin (pI 2.6) is also negatively charged at either pH 6.3 or 6.0, the hydrolysis of κ-casein by pepsin could be more extensive in milk with higher glycosylated κ-casein due to reduced electrostatic repulsion between pepsin and glycosylated κ-casein. With these different properties of casein micelles in different milk species in mind, and based on the results in this study, the hydrolysis rate of κ-casein by pepsin followed the order: sheep milk > cow milk > goat milk.

7.3.2.2. The coagulation behavior of pepsin-induced cow, sheep and goat milk

After the addition of pepsin, the storage modulus (G') and loss modulus (G'') as a function of time under small oscillation rheology at pH 6.3 and 6.0 are shown in **Figure 7-2** (**A**) and (**B**), respectively. Consistent with Yang et al. (2022a), for all milk species, G' showed a lag phase in the early stages; and after the onset of coagulation, G' increased significantly and reached a plateau, or dropped slightly for some of the samples. This could be attributed to the syneresis of milk curd at a lower pH or a reduction in adhesion of the curd to the stainless steel in the rheometer. Values for the coagulation time, the firming rate (dG'/dt), and G'_{max} are summarized in **Table 7-3**; all values were significantly affected by milk species ($P < 0.05$).

At both pH 6.3 and 6.0, shorter coagulation time was found in sheep milk, followed by cow milk and goat milk. By substituting the coagulation time into the hydrolysis equation, the hydrolysis degree at coagulation time was calculated in **Table 7-3**. The cow milk coagulated when 81% of κ-casein on the casein micelle surface was hydrolyzed at pH 6.3, and 67% at pH 6.0. The sheep milk coagulated when 64% of κ-casein on the casein micelle surface was hydrolyzed at pH 6.3, and 59% at pH 6.0. For goat milk, the highest hydrolysis degree was required before coagulation could occur, corresponding to 90% and 86% hydrolysis at pH 6.3 and pH 6.0, respectively. Therefore, the shortest coagulation time for sheep milk is not only due to the fast hydrolysis of κ-casein, but also the extent to which the sheep casein micelles are prone to aggregation [lower requirement in the amount of κ-casein being hydrolyzed for coagulation (H_{ct})]. The evolution of G' was also significantly different for cow, goat and sheep milks. The values of dG'/dt and G'_{max} of the sheep milk was higher than cow milk and goat milk at both pH 6.3 and 6.0.

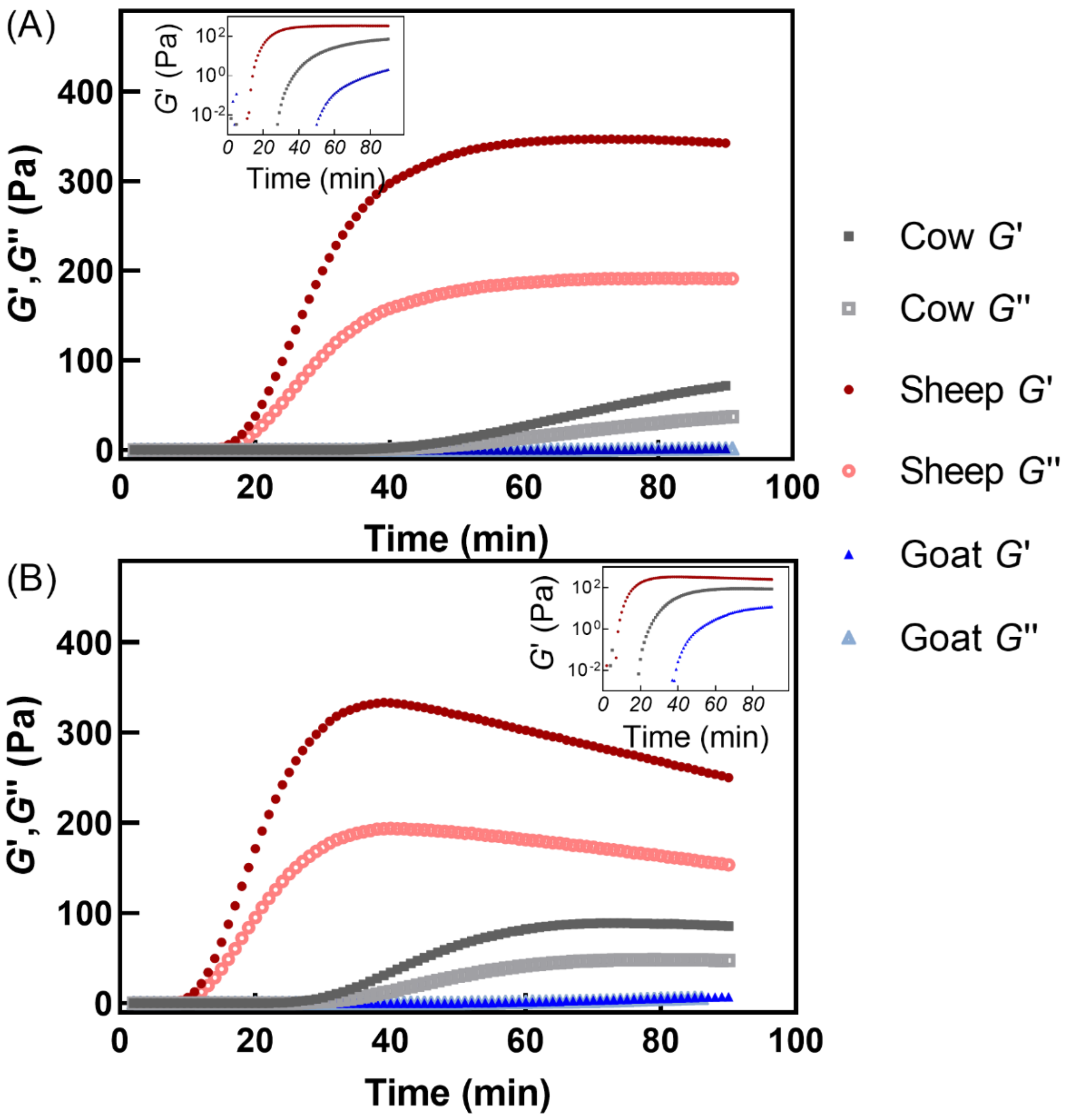

Figure 7-2 Evolution of G' (solid symbols) and G'' (open symbols) for different milk types over time after the addition of pepsin at 37 °C: (A) pH 6.3 (B) pH 6.0, with an inset to show the G' plotted on a logarithmic scale.

The different coagulation behavior in different milk types could be related to the total solid content and casein content, which is associated with the number of crosslinks between casein micelles in the network. However, it was reported that even when casein concentrations or protein concentrations are equal, chymosin-induced goat curd is softer than its bovine counterparts (Miocinovic et al., 2016; Remeuf & Lenoir, 1986; Storry, Grandison, Millard, Owen, & Ford, 1983). This suggests that the specific properties of casein micelles from different species, such as the proportion of caseins fractions (α_{s1}-, α_{s2}-, β-, κ-) (**Figure 7-1** A), size of casein micelles (**Table 7-1**), and hydration could have a more significant effect on the coagulation of casein micelles than the quantity/content of the proteins (Park, 2007). According to Dalgleish, Brinkhuis, & Payens (1981), small micelles start to aggregate at a slightly lower degree of proteolysis than large micelles. Therefore, one of the reasons for the highest requirement of H_{ct} for goat milk could be related to its largest casein micellar size. The lower proportion of α_{s1}-casein in goat milk (13%) compared to that in cow (33%) and sheep (36%) milk has been reported to contribute to the weak gel properties in goat yoghurt (Miocinovic et al., 2016; Morgan et al., 2003; Nguyen et al., 2018), which could also be the case for pepsin-induced milk curd. As for sheep milk, even though the casein micelle size is larger than that of cow milk, the lowest requirement in H_{ct} could be related to the relatively higher total calcium concentration among the three milk types (Li, Delger, Dave, Singh, & Ye, 2023). It was reported that calcium promotes coagulation, inducing cross-linking and aggregation of para-casein (Guinee, O'Brien, Law, & Tamime, 2010; Park, 2007). Besides, the average mineral content (e.g., Ca, Mg, etc.) in sheep milk is higher than in cow and goat milk (Park, 2007). There is an inverse relationship between the mineralization of the micelle and its hydration, which also means that sheep milk is less hydrated than cow and goat milk (Remeuf & Lenoir, 1986; Sood, Gaind, & Dewan, 1979). The rate of aggregation increased significantly under conditions where limited hydration of the casein micelles is expected to occur.

Table 7-3 Coagulation properties of skim milk at pH 6.3 and pH 6.0 with the addition of pepsin.

		Cow milk	**Sheep milk**	**Goat milk**
pH 6.3	**Coagulation time (min)**	34.9±7.2 ^{Ba}	13.6±0.2 ^{Ba}	61.6±13.1 ^{Aa}
	***H*ct (%)**	81 ± 4 ^{Ba}	64 ± 3 ^{Ca}	90 ± 3 ^{Aa}
	Firming rate (d*G′*/dt, Pa/min)	2.3±0.6 ^{Ba}	18.0±1.0 ^{Aa}	0.2±0.2 ^{Ca}
	Maximum storage modulus (*G*', Pa)	71.73±32.6 ^{Aa}	350.00±50.6 ^{Aa}	2.1±3.1 ^{Aa}
	Shear stress (Pa)	16.5±3.0 ^{Ba}	117.3±129.7 ^{Aa}	1.0±0.9 ^{Ca}
pH 6.0	**Coagulation time (min)**	22.2±3.0 ^{Ba}	11.2±1.2 ^{Ca}	44.2±6.8 ^{Ab}
	***H*ct (%)**	67 ± 4 ^{Bb}	59 ± 3 ^{Ba}	86 ± 3 ^{Aa}
	Firming rate (d*G′*/dt, Pa/min)	3.7±0.6 ^{Ba}	22.3±1.2 ^{Aa}	0.3±0.2 ^{Ca}
	Maximum storage modulus (*G*', Pa)	89.8±9.1 ^{Aa}	334.7±14.5 ^{Aa}	7.3±4.4 ^{Aa}
	Shear stress (Pa)	9.9±2.0 ^{Ba}	47.4±37.3 ^{Ba}	1.1±0.3 ^{Ba}

$^{a–b}$ Mean values for the same sample at different pH with different superscripts are significantly different ($P < 0.05$). A–C Mean values between samples at the same pH with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of the mean ($n = 3$).

Comparing samples at pH 6.3 and 6.0, the coagulation time and required hydrolysis degree for coagulation is lower at a lower pH, which is in agreement with Yang et al. (2022a). This is due to partial charge neutralization of the negatively charged para-casein micelles and the increased ionic calcium concentration at lower pH (van Hooydonk, 1987; Yang et al., 2022a). After 90 min, large deformation test was carried out on the milk curd; the associated yield stress data is summarized in **Table 7-3**. The highest yield stress was found for sheep milk, followed by cow milk and goat milk. The lowest yield stress for goat milk may be attributed to the occurrence of large pores and weaker interactions between casein particles (Lucey et al., 1997). The differences in firmness and the yield stress of the samples could be related to the different microstructures (Tamime, Kalab, Davies, & Mahdi, 1991), which will be discussed below.

7.3.2.3. The microstructure of cow, sheep, goat milk curd

The microstructures of cow, goat and sheep milk curd were examined 90 min after the addition of pepsin (**Figure 7-3**). The CLSM micrographs of all three curds consists of a protein network (appearing as green) and the pores (appearing as black). The protein network was different among cow, goat, and sheep casein curds. At both pH 6.3 and 6.0, the goat milk curd (**Figure 7-3 C, F**) exhibited the most porous microstructure and least dense protein network. Casein micelles in sheep and cow milk curd assembled into chains with obvious pores (**Figure 7-3 A, B, D, E**), whereas sheep milk curd contained the most homogenous and compact structure with small pores (**Figure 7-3 B, E**). Compared to sheep milk curd thicker protein strands were observed in cow milk curd (**Figure 7-3 A, D**). Limited work has been done comparing the enzyme-induced microstructure of coagula in different skim milk types. According to Hamad, Ismail, & El-Menawy (2016) and Ong, Dagastine, Kentish, & Gras (2013b), the dense protein structure of sheep cheese curd (chymosin-induced curd) could be due to the higher protein content. The high concentration of protein in the sheep milk samples decreases the mean free distance between casein micelles, resulting in a faster rate of coagulation and densely aggregated casein micellar network. The porous microstructure in goat curd illustrates the weak network which results in a lower G' value (**Figure 7-2**).

Comparing the curd micrographs at pH 6.3 and 6.0, the average pore size observed within the cow and sheep milk at pH 6.0 was larger than that in the goat milk at pH 6.3. It has been reported that the rearrangement of the casein curd network occurs more extensively with decreasing pH (Mellema et al., 2002) and facilitates syneresis. At later stages, a pronounced decrease in G' was consequently observed for sheep milk at pH 6.0 (**Figure 7-2 B**). For the goat milk, the curd formed a denser and more compact microstructure at pH 6.0 than at pH 6.3. This observation is consistent with rennet curd at different pH values by Ong et al., 2013.

As shown in **Figure 7-2 C**, *G*' of the goat milk at pH 6.0 is higher than at pH 6.3, and kept increasing during 90 min of reaction (there was no drop in *G*' caused by syneresis).

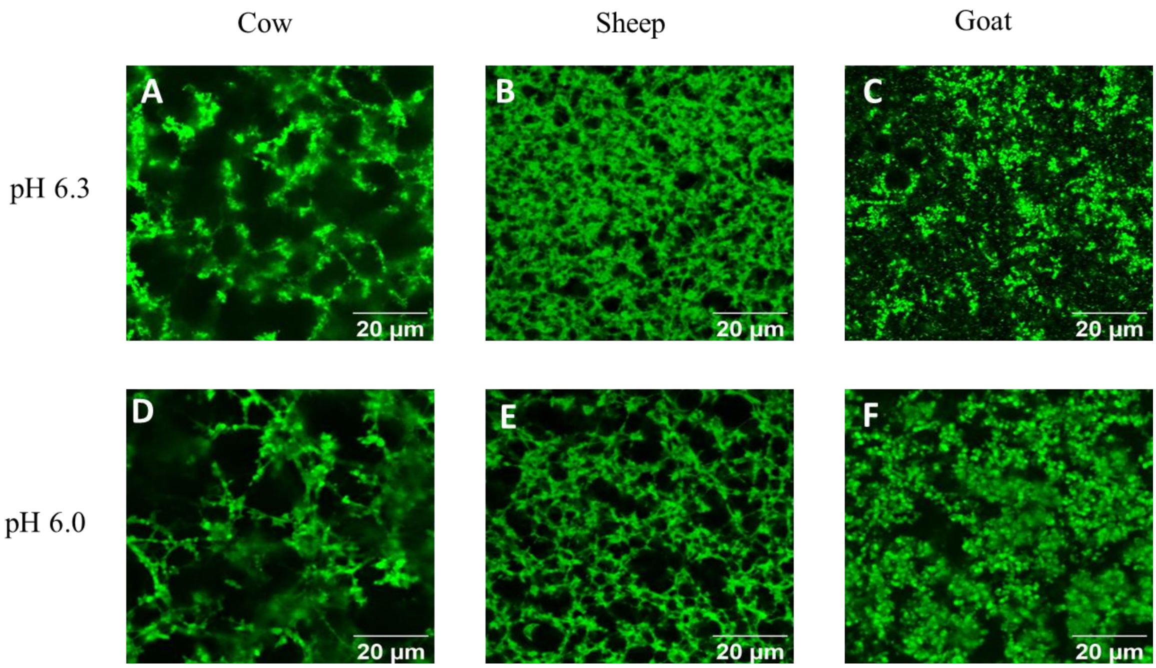

Figure 7-3 Microstructures of the pepsin-induced cow, sheep and goat coagulum at 90 min at same pepsin-to-κ-casein ratio as analysed by confocal laser scanning microscopy. The scale bars are 20 μm in length for all micrographs.

7.3.3. SANS study of microstructures of different milk species with and without pepsin addition

To better compare and understand the changes in the internal structure of the casein micelles after the addition of pepsin, SANS was employed to probe the structure of samples reconstituted at same casein content (**Table 7-4**). The dimensions of casein micelles in these samples were slightly different from those in native milk, but still follows the order: cow milk < sheep milk < goat milk. Although deuterated solvents are typically employed in neutron scattering measurements to facilitate contrast variation (Lopez-Rubio & Gilbert, 2009), according to Bayrak et al. (2021), the microstructure of casein curd in D_2O is markedly different than in H_2O dissolved samples. As a result, H_2O was used as a solvent in this study to avoid potential artefacts in the microstructure of milk proteins in these coagulated systems.

The measurement covered a *q* range from ~ 0.0007 to 0.02 Å^{-1}, corresponding to a length scale of approximately 40 to 1000 nm.

Table 7-4 physicochemical characteristics of different reconstituted milk milks used for small angle neutron scattering (SANS) studies. TS = total solids.

	Casein micelle size (nm)	**TS (%)**	**Fat (%)**	**Lactose (%)**	**Protein (%)**	**Casein (%)**	**κ-casein (%)**	**Pepsin concentration (U/g milk)**	**Pepsin-to-κ-casein ratio (U/mg)**
Cow milk	138	7.54	0.15	3.78	2.97	2.37	0.33	~ 0.4	~ 0.1
Sheep milk	158	6.24	0.32	2.46	3.06	2.39	0.27	~ 0.4	~ 0.1
Goat milk	209	8.35	0.37	4.51	3.00	2.30	0.30	~ 0.4	~ 0.1

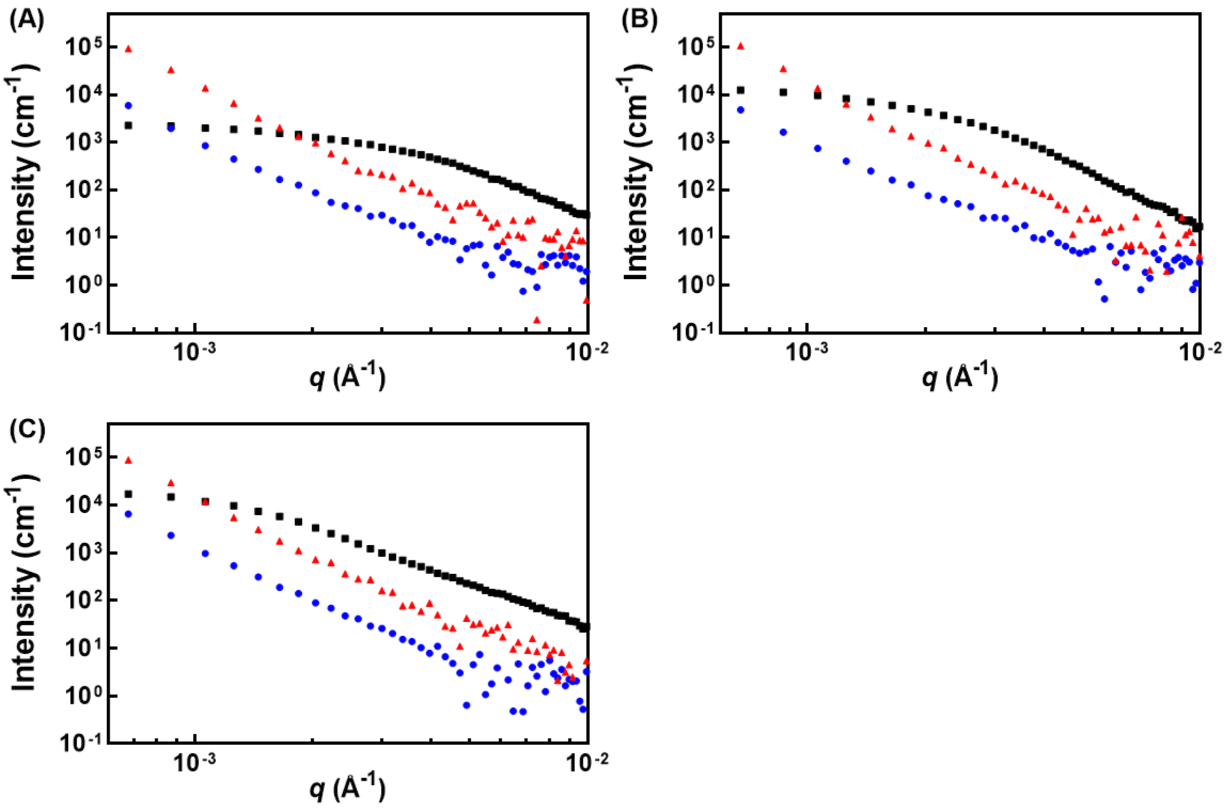

Figure 7-4 SANS data from initial milk samples (■) and equilibrated pepsin-induced coagulated samples (▲, pH 6.3; ●, pH 6.0), (A) cow; (B) sheep; (C) goat.

Before the addition of pepsin, the SANS profiles of milk samples were measured, as shown in **Figure 7-4** (blue curves). The curvature in the scattering curve as one approaches the lowest q is consistent with the presence of a Guinier region; this can be observed at q ~ 0.004 Å^{-1}, 0.003 Å^{-1}, and 0.0015 Å^{-1} for cow, sheep, and goat milk, respectively, and enables an assessment of the size of casein micelles (Gilbert, 2019; Holt, de Kruif, Tuinier, & Timmins, 2003; Singh, Hemar, Gilbert, Wu, & Yang, 2020). The Guinier–Porod model was used to fit the curves (Fig. S1) and the fitting parameters are summarized in **Table 7-5** (milk control). In this model, the casein micelles are regarded as spheres, and the hydrodynamic diameter D can be calculated using $(D/2)^2 = 5/3 \times R_g^2$ (Witten et al., 2004). Fits to each set of milk data gave a micelle diameter of ~ 129, ~ 201 and ~ 307 nm for cow, sheep and goat milk, respectively; these values are within the expected range from the literature (Day, Raynes, Leis, Liu, & Williams, 2017; de Kruif, Huppertz, Urban, & Petukhov, 2012; Ingham et al., 2018; Yang et al., 2023d).

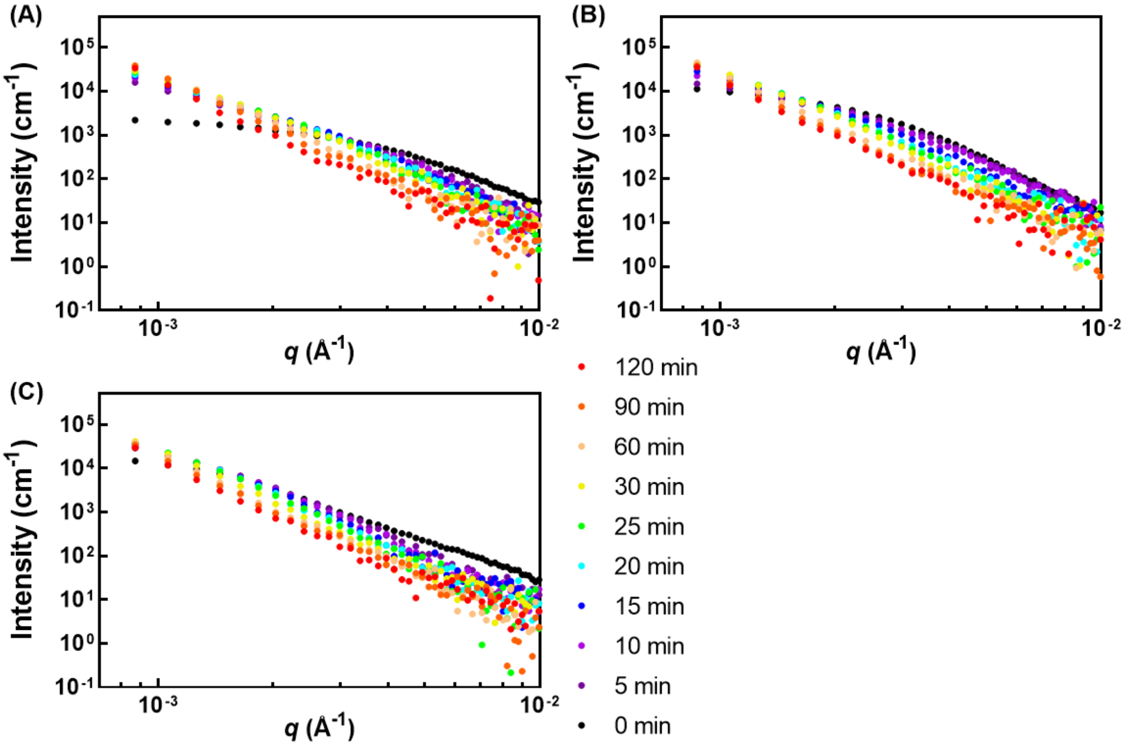

Figure 7-5 Evolution of time-resolved SANS intensity data during coagulation of various milk samples (pH 6.3) up to 120 min (A: cow milk, B: sheep milk, C: goat milk).

Table 7-5 Parameters obtained from the fit of the SANS experimental data (associated with **Figure 7-5**) to Guinier–Porod equation and power law equation.

			Cow			**Sheep**			**Goat**		
	Time (min)	**Model**	**Scale**	**Rg (Å)**	**Porod/Poewer exponent**	**Scale**	**Rg (Å)**	**Porod/Poewer exponent**	**Scale**	**Rg (Å)**	**Porod/Poewer exponent**
Milk control	**0**	Guineer Porod	1826(17)	501(3)	2.99(0.02)	10848(85)	779(3)	3.75(0.01)	19644(282)	1208(8)	2.93(0.01)
Milk at pH 6.3	**5**	Guineer Porod	28544(2121)	1580(50)	2.83(0.03)	14298(390)	954(12)	3.38(0.04)	27207(1096)	1416(26)	3.09(0.03)
	10	Guineer Porod	37887(2567)	1647(44)	3.00(0.03)	29818(1178)	1298(22)	3.16(0.03)	25526(946)	1332(22)	3.08(0.03)
	15	Guineer Porod	46407(3287)	1714(46)	3.15(0.03)	42311(2215)	1460(29)	3.46(0.03)	27738(986)	1317(21)	3.13(0.03)
	20	Guineer Porod	57874(4576)	1840(53)	3.21(0.03)	73395(4961)	1749(39)	3.69(0.04)	33474(1034)	1341(18)	3.32(0.03)
	30	Guineer Porod	71788(5961)	1895(54)	3.48(0.03)	142330(13308)	2163(62)	3.81(0.04)	51558(2269)	1505(25)	3.65(0.04)
	60	Guineer Porod	150750(15481)	2344(73)	3.87(0.04)	439930(98804)	3191(204)	3.93(0.04)	370910(75301)	3182(182)	3.96(0.05)
	90	Guineer Porod	259090(42527)	2805(130)	3.91(0.04)	741760(287830)	3916(410)	3.91(0.05)	449340(127160)	3456(264)	3.97(0.05)
	120	Power law	5.2E-8(1.4E-8)	-	3.80(0.04)	3.2E-8(8.9E-9)	-	3.88(0.04)	2.8E-8(8.0E-9)	-	3.87(0.04)
Milk at pH 6.0	**120**	Power law	1.9E-7(5.6E-8)	-	3.21(0.04)	3.9E-7(1.1E-7)	-	3.10(0.04)	5.6E-8(1.7E-8)	-	3.42(0.05)

Fitted *q* range: 0.0006-0.01 Å^{-1}. Fixed parameters: background: 0.1 cm^{-1}, s: 0 (for spherical particles). The results are expressed as the mean (standard deviation).

To investigate the changes in the structure of casein micelles as a function of reaction time at pH 6.3, SANS patterns obtained at 5, 10, 15, 20, 30, 60, 90 and 120 min after the addition of pepsin are provided in **Figure 7-5**. As the reaction progressed, a significant increase in the scattering intensity was observed at $q < 0.001$ Å^{-1}, indicating the formation and growth of large-scale aggregates. Guinier shoulder features can still be observed but are shifted to smaller q values, representing the formation of protein aggregates with dimensions of the order of q^{-1} (Li et al., 2018; Wang et al., 2019). Similar observations were found in previous studies of rennet- (Callaghan-Patrachar et al., 2021) and plant protease- (tamarillin from Tamarillo fruits) (Li et al., 2018) induced coagulation of cow milk. The curves obtained from 5 to 90 min were fitted with a Guinier–Porod model, and the refined parameters are recorded in **Table 7-5**. The change of R_g was plotted as a function of reaction time in **Figure 7-6**. Overall, the size of aggregates (Rg) is positively related to the reaction time; however, the kinetics differs within the species. Extensive increase in R_g was found in cow milk in the first 5 min, while a gradual increase was found from 5 min to 90 min, with a smallest aggregate size at 90 min (the diameter of aggregates in cow, sheep and goat milk curd were calculated at 691, 1011 and 724 nm, respectively). The aggregate size of goat milk showed little changes in the first 20 min, then increased markedly from 30 min. The sheep milk kept increasing from 5 min to 90 min and grew to the largest size at 90 min. At 120 min, the Guinier feature, i.e., plateau region, was not observed, and the scattering intensity continued the increase in the low q region. A power law model was used to fit the curve at 120 min: the power law exponent at $0.0006 < q < 0.015$ Å^{-1} was ~ 3.80, 3.88 and 3.87 in cow, sheep, and goat milk curd, respectively (**Table 7-5**). This is consistent with the casein micelles arranging as fractal-like structures to form large aggregates in the milk curds (Chodankar et al., 2009; Vogtt, Javid, Alvarez, Sefcik, & Bellissent-Funel, 2011). The protein aggregate size was too large to be determined in the limited *q* range although ultra-SANS measurements would be valuable to extend the range to lower *q*.

A real-time SANS measurement was not conducted for samples at pH 6.0. However, the equilibrated milk curd, incubated for 120 min at 37 °C in an oven, was measured and is presented in **Figure 7-5**. There are no Guinier shoulder features observed for all samples in the studied *q* range as well, because of the large size of aggregates in the samples. The power law exponent at $0.0006 < q < 0.015$ Å^{-1} of these samples were ~3.21, ~3.10 and ~3.42 for the cow milk curd, sheep milk curd, and goat milk curd, respectively. Although Porod-type exponents can be interpreted in terms of the degree of surface roughness, in the current study, such an approach has not been pursued as it is more likely that the scattering is a manifestation of a broad size distribution of aggregates (Schmidt, 1982). Based on the fitting parameters (**Table 7-5**), there was a significant decrease in the power law exponent when comparing the pepsin-induced curd at pH 6.3 and 6.0. The difference in power law exponent may be related to either surface roughness or associated with changes in size distribution of the aggregates that may be facilitated to a greater extent at pH 6.0 compared to pH 6.3 (although the aggregate size at 120 min could not be determined in the studied *q* range).

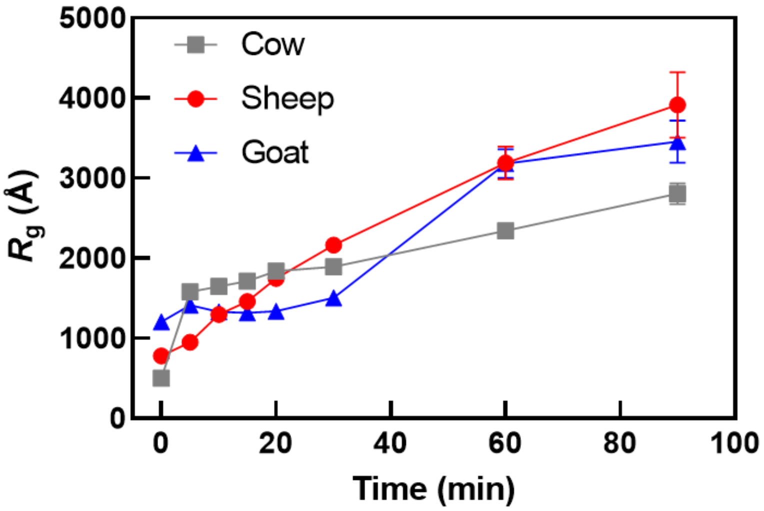

Figure 7-6 Evolution of R_g of various milk samples (pH 6.3) after the addition of pepsin (associated with **Figure 7-5**, fitted with Guinier–Porod model).

7.4. Conclusions

At the same pepsin-to-κ-casein ratio, the hydrolysis of κ-casein in sheep milk was most rapid, followed by cow κ-casein and goat κ-casein at both pH 6.3 and pH 6.0. These observations may be related to the sequence around the Phe-Met bond, the glycosylation degree and conformation of κ-casein, and the differences in casein micelle dimensions among different species of milk. The consequent coagulation processes of cow, goat and sheep milk were also different. Sheep milk coagulated when ~ 64% of the κ-casein was hydrolyzed at pH 6.3 and when ~ 59% of the κ-casein was hydrolyzed at pH 6.0; sheep milk coagula possessed the highest firmness and densest structure. The coagulation of goat milk was slow and required the highest extent of hydrolysis of κ-casein, namely ~ 90% at pH 6.3 and ~ 86% at pH 6.0. The curd formed from goat milk was softer (lower storage modulus), and the microstructure contained more large pores with fewer crosslinks between the protein aggregates compared to cow and sheep curds.

Even at a same casein concentration, the curd microstructure varied across different milk species. At pH 6.3, the casein aggregate size in cow, sheep and goat milk curd all increased with reaction time, whilst differing in the rate of increase and possibly surface roughness. However, to compare the aggregate size of equilibrated curds in different milk species, access to lower q is required. In general, the aggregates in all milk species at pH 6.0 are larger and possibly exhibit greater surface roughness than that at pH 6.3. Such differences in the coagulation processes in curds formation are not only due to the casein content, but also related to other physicochemical properties of different types of milk. Such factors could include differences in κ-casein chemistry (e.g., degree of glycosylation, amino acid sequences) casein micelle size, casein micelle density, and mineralization. The fundamental insights generated from this study provide an improved understanding of pepsin-induced coagulation behavior of milk from different species.

7.5. Acknowledgments

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7.6. Supplementary material

Guinier–Porod model: https://www.sasview.org/docs/user/models/guinier_porod.html

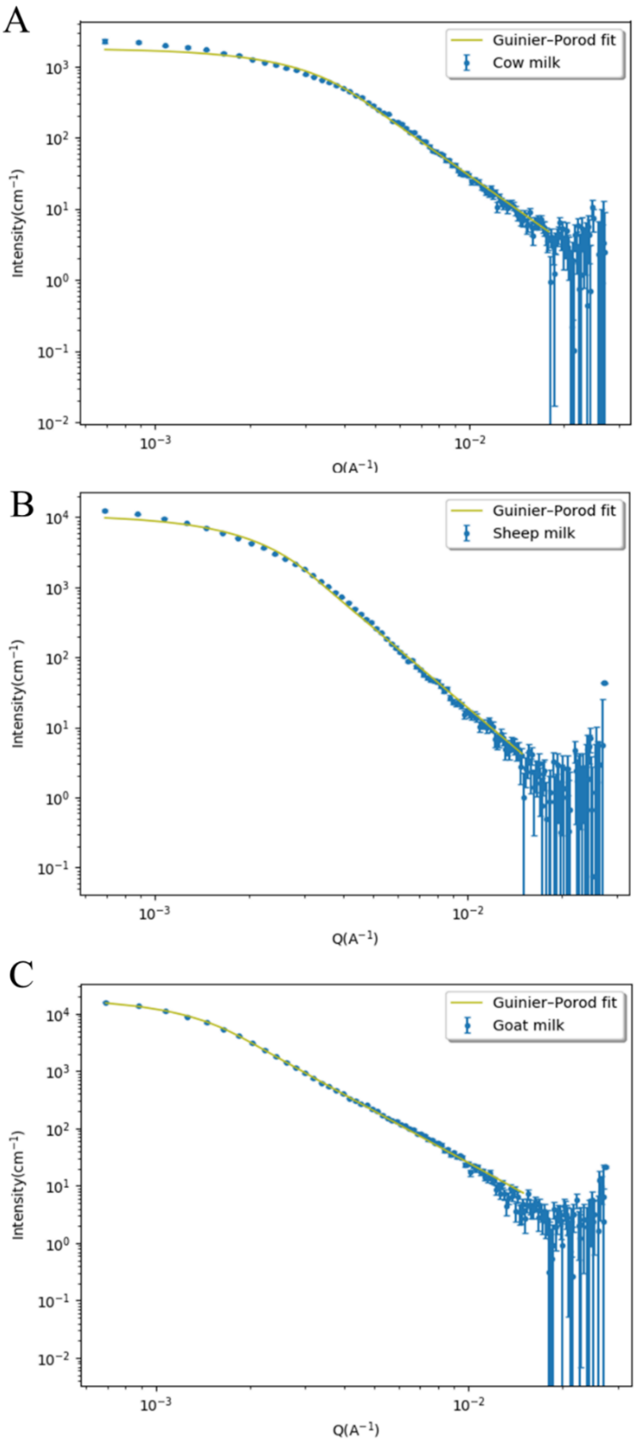

Figure S 7-1 SANS profiles from cow (A), goat (B), and sheep (C) milk. The solid curves represent the fits of Guinier–Porod equation in the q range of $0.0006 < q$ (Å^{-1}) < 0.01.

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Chapter 8 Small and ultra-small angle neutron scattering investigation of acid- and/or pepsin-induced milk coagulation and degradation of milk curd by pepsin[7]

Abstract

This study aimed to understand the real time structural evolution of unheated and heated cow milk (80 °C for 30 min) under acidic conditions or/and in the presence of low and high concentration of pepsin using ultra-small (USANS) and small-angle neutron scattering (SANS). Combining with small oscillation rheometric measurements and confocal scanning laser microscopy observations, the heat treatment of milk had different effects on acid (glucono-δ-lactone, GDL)-, pepsin- and combination (both GDL and pepsin)-induced coagulation. The heated milk demonstrated a faster increase in *G*' and scattering intensity rate than the unheated milk in GDL-induced coagulation and combination coagulation. At pH 6.3, heat treatment retarded the pepsin (1.10 U/mL)-induced coagulation of milk, with slower increases rate in *G*' and scattering intensity. At a high concentration of pepsin (2000 U/mL), protein coagulation and proteolysis occurred. Heat treatment retarded coagulation but accelerated the proteolysis of the curd. This study demonstrates the potential of using time-resolved USANS and SANS to investigate the structural evolution of protein coagulation and degradation under gastric environment conditions at the nano- and micron length scales.

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[7] This Chapter will be published as: Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). (Ultra) Small-angle neutron scattering investigation of acid- and/or pepsin-induced milk coagulation and degradation of milk curd (In preparation).

8.1. Introduction

In the gastric environment, the secretion of hydrochloric acid, the enzyme pepsin, and their combined action results in the coagulation of milk proteins (Ye, 2021). To better understand the coagulation behavior of milk proteins *in vitro*, considerable research has taken place, including on pepsin-induced coagulation (Yang et al., 2022a, 2023b), acid-induced coagulation (Lucey, 2017), and ‘combination coagulation’ (Roy et al., 2020b) of casein micelles. At pH > 5, the specific hydrolysis of the Phe^{105}–Met^{106} bond of κ-casein by pepsin yields the C-terminal glycosylated caseinomacropeptide and para-κ-casein (Plowman & Creamer, 1995; Yang et al., 2022a). Casein micelles subsequently aggregate by hydrophobic association together with ionic electrostatic effects (Horne & Lucey, 2014), which occur when a minimum amount of the caseinomacropeptide has been removed (i.e. at a critical degree of hydrolysis). Acid-induced coagulation occurs when the isoelectric point (pH ~ 4.6) is approached with glucono-δ-lactone (GDL) normally being used as an acidulant (Lucey et al., 1997; Wang et al., 2019; Yang et al., 2023d). In the presence of both GDL and pepsin, acidification and pepsin action occur simultaneously, which results in the rheological and structural properties differing with respect to the other two coagulation mechanisms (Roy et al., 2020b).

The high penetrating power and non-destructive nature of neutron radiation makes small-angle neutron scattering (SANS) and ultra small-angle neutron scattering (USANS) attractive techniques to conduct *in situ* structural analysis of protein gelation (Chodankar et al., 2009; Gilbert, 2019). To eliminate potential structural artefacts during extensive sample preparation in electron microscopy, USANS has been employed to study the rennet- (Callaghan-Patrachar et al., 2021) and plant protease- (tamarillin from Tamarillo fruit) (Li et al., 2018) induced coagulation of cow milk, as well as the GDL-induced coagulation of skim cow, sheep and goat milks (Wang et al., 2019; Yang et al., 2023d). According to Yang et al. (2023d), the microstructural (fractal dimension) evolution kinetics probed by USANS correlates well with the development of curd strength (*G′*) determined by rheological measurements. In addition, small-angle neutron scattering (SANS) has been shown as a powerful technique to probe the internal structures of casein micelles and colloidal calcium phosphate (CCP) in casein micelles. Pasquier et al. (2019) firstly employed small-angle neutron scattering (SANS) to probe structural changes of various vegetable (cruciferin and napin) protein gels protein gels during *in vitro* gastric and intestinal digestion. Recently, Bayrak et al. (2023) investigated the structural evolution of casein gels throughout simulated gastric digestion with (2000 U/ml, 8000 U/ml) and without pepsin by SANS and USANS.

Thermal processing is extensively employed in the dairy industry to extend product shelf life, and to modify the textural properties of milk products (Lewis, 1994). According to Ye et al. (2017) and Ye et al. (2019b), in a gastric environment (with the associated decreases in pH and increases in pepsin concentration), heated (pasteurized and UHT) cow milk forms curds with fragmented and crumbly structures; whereas unheated milk forms more cohesive curds. As a result, a faster rate of protein hydrolysis and fat globule release rate has been observed during gastric digestion of heated cow whole milk. The effects of heat treatment on rheological properties and microstructural characteristics are significantly different between rennet-induced, acid-induced and combination coagulation gels (Anema et al., 2007; Anema, Lee, & Klostermeyer, 2011; Lucey, Tamehana, Singh, & Munro, 2001; Lucey, Wilbanks, & Horne, 2022; Yang et al., 2023b). However, the effect of heat treatment on pepsin-induced milk coagulation has not been compared with acid-induced and combination coagulation with respect to rheological properties and microstructural characteristics.

In this study, the acid (GDL)-induced coagulation (AC), pepsin-induced coagulation (PC) and combination [both acid (GDL) and pepsin] coagulation (CC) of unheated and heated milk were compared. Moreover, to investigate the structural changes occurring during casein curd degradation by pepsin, GDL and a high pepsin concentration [2000 U/ml, mimicking gastric conditions (Brodkorb et al., 2019)] were added to unheated and heated milk. Time-resolved SANS and USANS were employed to probe milk protein structural evolution at length scales from a few nanometers to several micrometers. The structural parameters obtained from these scattering studies (e.g., fractal dimensions and aggregate size), combined with the results of rheological characterization and confocal laser scanning microscopy observations. shed light on protein structural evolution of unheated and heated cow milk during coagulation and proteolysis.

8.2. Materials and methods

8.2.1. Materials

Bulk raw whole cow milk was collected from Massey University No. 4 dairy farm (Palmerston North, New Zealand) in March 2022. Upon receipt of the milk samples, 0.02% (wt/wt) sodium azide (Merck KGaA, Darmstadt, Germany) was added to prevent microbial growth. By centrifugation of raw whole milk at 3,000×*g*, 15 min, 4 °C in a bench centrifuge (Heraeus X3R; Thermo Fisher Scientific, Inc; USA), the fat layers were carefully removed. The skimmed cow milk was then freeze dried. Porcine pepsin (EC 3.4.23.1; Sigma-Aldrich, St. Louis, MO, USA) with stated activity of 541 U/mg protein was dissolved in Milli-Q water (5 mg per 5 mL; activity 541 U/mL). All other chemicals (analytical grade) were obtained from Sigma-Aldrich unless otherwise specified.

8.2.2. Milk preparation characterization

The skim milk powders were reconstituted into 100 % H_2O or 100 % D_2O at 3 wt% protein content under gentle magnetic stirring overnight to allow protein hydration. The samples were stored at 4 °C for up to 5 days and equilibrated to 37 °C before further use. The composition of the reconstituted cow milk was analyzed using a Milkoscan (Foss, Hilleroed, Denmark), as containing 2.97% proteins (2.37% caseins), 0.15% fat, and 3.78% lactose. Part of the reconstituted milk was heated at 85 °C for 30 min in a water bath.

Particle sizes of casein micelles were determined according to the method of Anema (2018) with a Malvern Zetasizer (Nano ZS; Worcestershire, UK) using disposable cells (DTS0012) at 25 °C. The unheated and heated milks were diluted 50× with calcium-imidazole buffer solution (20 m*M*-imidazole, 5 m*M*-$CaCl_2$, 30 m*M*-NaCl, pH 7.0) and analyzed under the 'milk protein size analysis' option.

8.2.3. Sample preparation

The preparation of each sample is shown in **Table 8-1**. Samples arising from acid-induced coagulation (AC), pepsin-induced coagulation (PC), combination coagulation (CC) and high pepsin concentration (HP) of unheated milk are denoted as ACU, PCU, CCU, HPU; and for heated milk as ACH, PCH, CCH, HPH. Associated D_2O-dispersed samples, ACU-D_2O, PCU-D_2O, CCU-D_2O, were prepared for exploring the specific CCP features for SANS measurements.

Table 8-1 Preparation of acid-induced coagulation (AC), pepsin-induced coagulation (PC), combination coagulation (CC) and high pepsin concentration (HP) samples.

Sample	Initial pH	GDL (wt/wt)	Pepsin (U/mL sample)
AC	6.7	2%	-
PC	6.3*	-	1.10
CC	6.7	2%	0.24
HP	6.7	2%	2000

*Note: As pepsin-induced hydrolysis of κ-casein occurs effectively at pH 6.3, the pH of PC sample was preadjusted to 6.3 by slow addition of 1*M* HCl. The same treatment was carried out on unheated milk and heated milk.

For sample preparations, GDL powder (2 wt%) was added to milk under magnetic stirring at 25 °C for 1 min before transferring into the rheometer geometry and the USANS/SANS sample cells. Pepsin solutions were added to the samples at a ratio of 10 µL per 1 mL of milk and resulted in final pepsin concentrations of 1.10 U/mL and 0.24 U/mL for PC and CC, respectively. The pepsin concentration was selected on the basis of preliminary rheological experiments. For each pepsin concentration, CC occured at pH above 4.6; PC occured within 60 min and reached equilibrium after around 2 h. In terms of the HP samples, the milk was mixed with GDL and pre-loaded into the rheometer geometry or into sample cells for SANS/USANS, followed by the addition of pepsin solution (2000 U/mL) using either tips or a syringe, and well mixed.

8.2.4. pH measurement

During the reaction, the pH-value of each sample was determined *in situ* by dipping a HALO Bluetooth wireless pH electrode (HI11102, Hanna instruments, Smithfield, RI, USA) into the milk samples incubated in a water bath set at 37 °C at the desired time (2 h for AC, PC, CC; 8 h for HP).

8.2.5. Rheological measurements

Rheological measurements were conducted in an AR-G2 magnetic-bearing rheometer (TA Instruments, Crawley, West Sussex, UK) with standard Peltier concentric cylinder geometries (including a cup and a rotor with radii of 15 and 14 mm, respectively). Low viscosity silicone oil was added at the top of the geometry to avoid evaporative loss during measurements. A time sweep measurement was carried out at a constant strain of 1% and a constant frequency of 1 Hz to monitor the rheological properties for desired time (2 h for AC, PC, CC; 8 h for HP). The storage modulus G' and the viscous modulus G'' were recorded every minute at 37 °C.

8.2.6. Ultra small angle neutron scattering (USANS)

Sample preparation for USANS measurements was described in Section **8.2.3**. The prepared samples ~ 1.5 mL were carefully transferred into demountable USANS sample cells (thickness 1 mm) using a disposable syringe and placed on the KOOKABURRA instrument at the OPAL facility at the Australian Nuclear Science & Technology Organisation (ANSTO), Australia (Rehm, Brûlé, Freund, & Kennedy, 2013; Rehm et al., 2018). All measurements were carried out at 37 °C, using a neutron wavelength of 4.74 Å with a 29 mm Cd aperture. It should be noted that the KOOKABURRA instrument uses an analyzer crystal that must change position to collect data for each q value representing a point-by-point measurement, therefore the acquisition of data over a wider q-range at short intervals of time is not possible. To reduce the measuring time during the reaction (one full scan with acceptable statistics takes more than 30 min), the analyzer crystal angle was fixed, and a single q value (1×10^{-4} Å^{-1}) was applied to monitor absolute intensity change (corresponding to a length scale about 6 µm). In addition, the milk before coagulation was measured by USANS over an extended q range of ~ 4×10^{-5} to 5×10^{-3} Å^{-1}, corresponding to a length scale from about 130 nm to 16 µm (Yang et al., 2022c). A Guinier–Porod model describes the radius of gyration and the power law behavior of a scattering system. The untreated samples (unheated milk and heated milk) were fitted into Guinier–Porod model in SasView software (version 5.0.5, https://www.sasview.org/), given by

$$\mathrm{I}(q) = \frac{\mathrm{G}}{q^{s}} \exp\left[\frac{-\mathrm{q}^{2}\mathrm{Rg}^{2}}{3-\mathrm{S}}\right], \quad q \le q_1$$

$$I(q) = \frac{D}{q^{m}}, \quad q \ge q_1 \qquad \textbf{(Equation 8-1)}$$

where q is the magnitude of the scattering vector, $I(q)$ is the scattered intensity, R_g is the radius of gyration, m is the Porod exponent, G and D are the Guinier and Porod scale factors, respectively. S is equal to zero for spheres in this study. The Guinier form is used for $q \le q_1$ and the Porod form is used for $q \ge q_1$. Enforcing the continuity of the Guinier and Porod functions and their derivatives yields

$$q1 = \frac{1}{Rg}\sqrt{\frac{3m}{2}}$$

$$D = G \exp\left(\frac{-q1^{2}R_g^{2}}{3}\right) q1^{m} = G \exp\left(-\frac{m}{2}\right)\left(-\frac{3m}{2}\right)^{\frac{m}{2}} \frac{1}{R_g{}^{m}} \qquad \textbf{(Equation 8-2)}$$

8.2.7. Small angle neutron scattering (SANS)

Time-resolved SANS measurements were carried out on the QUOKKA SANS instrument at ANSTO, Sydney, Australia (Wood et al., 2018). The samples were prepared under the same conditions as for USANS, and ~ 350 µL of the mixed samples were loaded into the banjo cell. The measurement was carried out at L1=L2=20m, Lens 12.5, covering a q range from ~ 0.001 to 0.25 $Å^{-1}$. SANS data were reduced using the Mantid suite and the scattering from H_2O or D_2O was subtracted as background. The intensity of untreated samples (unheated milk and heated milk) in SANS were fitted into the Guinier–Porod model as well. The data at 0.007 $Å^{-1} < q <$ 0.0022 Å⁻1 during reaction at 0, 5, 10, 15, 20, 30, 60, 90, 120 (and 240, 480) min for each sample were fitted into power law model, given as:

$$I(q) = scale \times q^m \qquad \textbf{(Equation 8-3)}$$

where m is the power law exponent, from which the nature and geometry of the scattering object can be determined.

To explore the differences in CCP between AC, PC and CC samples, GDL or/and pepsin were added to the milk reconstituted in D_2O (same conditions as for the H_2O dissolved samples) to enable enhanced scattering contrast. The resulting mixture was then injected into a SANS banjo cell and incubated in an oven at 37 °C over 2 hours for equilibration. The equilibrated samples were probed by QUOKKA under the same conditions as for the H_2O dissolved samples.

8.2.8. Confocal laser scanning microscopy

The different coagulum microstructures were observed using confocal laser scanning microscopy. Fast Green fluorescent dye (3 µL; 1% wt/vol) was added to skim milk (100 µL) and mixed for 30 s. After prewarming to 37 °C, the mixture was then transferred to the cavity of a glass microscope slide and covered with a glass coverslip and incubated for 2 hours at

37 °C for each sample. The sample was examined using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems) with a 63× magnification lens.

8.3. Results and discussions

8.3.1. SANS and USANS probing of unheated and heated milk

The USANS profiles over extended range (4×10^{-5} Å^{-1} $< q < 10^{-2}$ Å^{-1}) of unheated and heated milk samples are shown in **Figure 8-1**. In the high-q region, a Guinier shoulder feature (building block size, R) can be observed at $q \sim 0.001$ to 0.002 Å^{-1}, indicating the scattering from individual casein micelles (unheated milk) or casein micelle-whey protein complexes (heated milk) (Li et al., 2018; Wang et al., 2019). The curves at 5×10^{-4} Å^{-1} $< q <$ 10^{-2} Å^{-1} were fitted with a Guinier–Porod model. A relatively larger casein micelle size was found in the heated samples: the calculated size of casein micelles (diameter) determined for unheated and heated milk was 120 ± 3 nm and 144 ± 2 nm, respectively. The Guinier-type feature of untreated milk can also be observed in the SANS results (blue curves in **Figure 8-4 A** and **B** are unheated and heated milk, respectively), representing the size of casein micelles: the diameter was calculated at 129 ± 1 nm and 143 ± 1 nm in unheated milk and heated milk, respectively, in accordance with the USANS results. The diameters of casein micelles in unheated and heated milk agree well with previous SANS/USANS studies (Day et al., 2017; de Kruif, 2014; Liu et al., 2017).

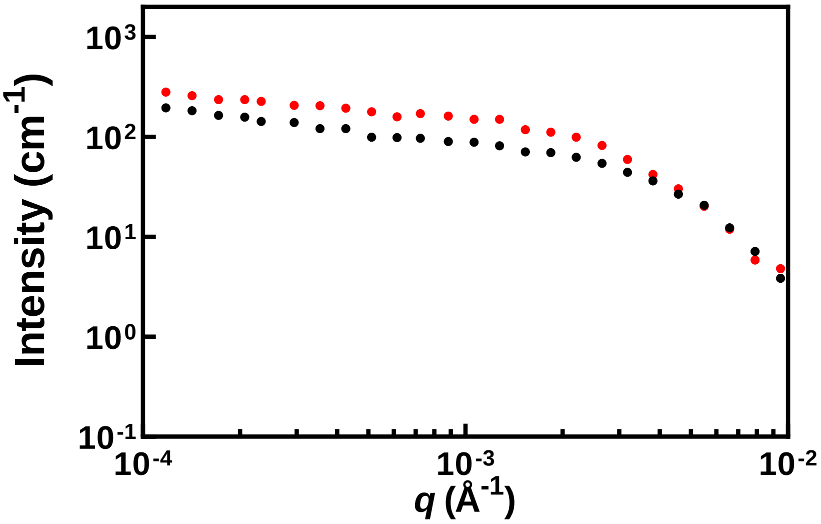

Figure 8-1 Reduced and smeared USANS I(*q*) of unheated cow skim milk (black color) and heated cow skim milk (red color).

8.3.2. SANS probing of calcium phosphate nanocluster feature in equilibrated acid-, pepsin-induced and combination coagulation of milk samples in D_2O

Variations in curd firmness or brittleness were noticeable between D_2O milk samples and H_2O samples, whereas the CCP feature was only observable in samples reconstituted in D_2O under SANS (Bayrak et al., 2021). Therefore, the SANS patterns of equilibrated ACU-D_2O, PCU-D_2O and CCU-D_2O were probed and shown in **Figure 8-2**. The D_2O dispersed milk sample was not probed in this study but compared with previous results from Yang et al. (2023d). The peak at around 0.035 Å^{-1} is most prominent when the solvent is contrast matched to protein. According to previous research, the appearance of a shoulder at around 0.035 Å^{-1} is attributed to the interference between calcium phosphate nanocluster (CCP) within the casein micelle (de Kruif, 2014; Yang et al., 2023d). The peak position is related to the average correlation distance between two neighboring CCP particles. After coagulation, the peak position in PCU-D_2O was still at *q* 0.035 Å^{-1}, whereas the peak position in ACU-D_2O, CCU-D_2O shifted to a higher *q* at 0.070 Å^{-1}. This indicates that the CCP region in PCU-D_2O did not change after the 2-hour reaction, whereas a progressive fading of the CCP feature was found in ACU-D_2O and CCU-D_2O. Native casein micelles (at normal milk pH ~ 6.7) are stabilized by negative charge and steric repulsion. When the pH is close to ~ 5.0, the CCP within casein micelles are completely dissolved (Lucey, 2017). According to Yang et al. (2023d), the acidity (pD-value) of milk was close to 4.0 after a 2-hour reaction time with the addition of GDL powder (2.5 wt%). Under these conditions (pD < 4), the CCP within casein micelles are dissolved completely in ACU-D_2O and CCU-D_2O samples. Acidification of milk could induce progressive fading of the CCP feature due to acid-induced dissociation of CCP (Moitzi, Menzel, Schurtenberger, & Stradner, 2011) in ACU-D_2O and CCU-D_2O samples; whereas at constant pD, the CCP region in PCU-D_2O did not change after a 2-hour reaction time and the casein micelles remained intact in the milk curd (Li et al., 2018; Schaefer, Martin, Wiltzius, & Cannell, 1984). The CCP in heated milk was not determined in this study, as previous studies suggest that the heat treatment does not alter the dissolution kinetics of CCP in cow milk (Singh, 1995; Yang et al., 2023d).

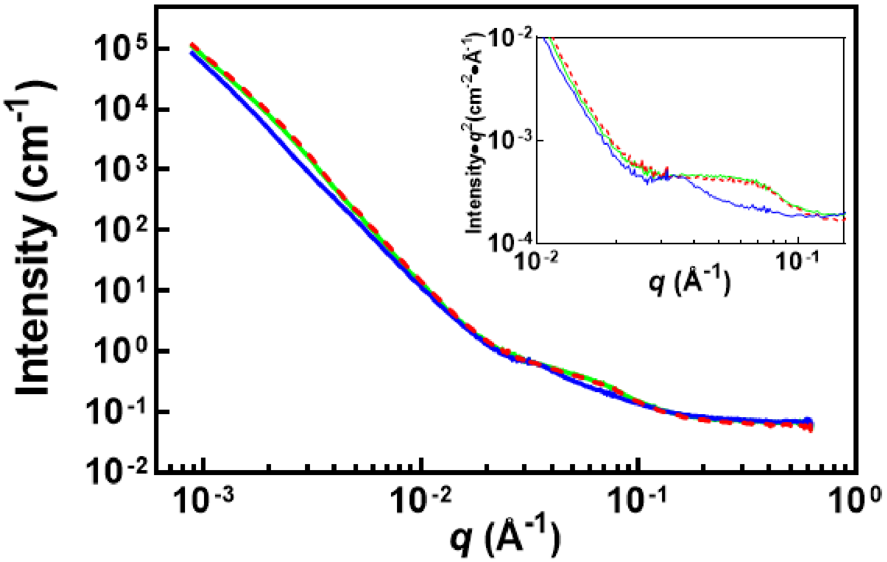

Figure 8-2 SANS patterns of D_2O dissolved milk samples coagulated by GDL (green color), pepsin (blue color) and combination (red color). Inset shows the corresponding Kratky plots highlighting the structure of CCP nanoclusters.

8.3.3. Acid-induced milk coagulation and the effect of heat treatment

With an addition of 2% GDL, the pH of ACU and ACH decreased from 6.7 to ~ 4.3 after the 2-hour reaction time (**Figure 8-3 A**). The evolution of storage modulus (*G'*) in ACU and ACH is shown in **Figure 8-3 B**: the *G'* of both samples showed a lag phase in the early stages and, after the onset of coagulation, *G'* increased significantly, indicating an enhancement in curd firmness. The coagulation properties, including the coagulation time, firming rate (d*G'* /dt) and maximum *G'* (G'_{max}) are summarized in **Table 8-2**. ACU coagulated at ~ 40 min, when the pH was ~ 4.90, near the pI of the casein proteins. ACH had shorter coagulation times (~ 34 min) and higher pH values (~ 5.07) at the coagulation point. Within 2 h of reaction, the firming rate and G'_{max} of ACH was higher than ACU. In agreement with Lucey, Tamehana, Singh, & Munro (1998), the shorter coagulation time and higher firming rate, G'_{max}, pH at coagulation point was observed in the heated sample.

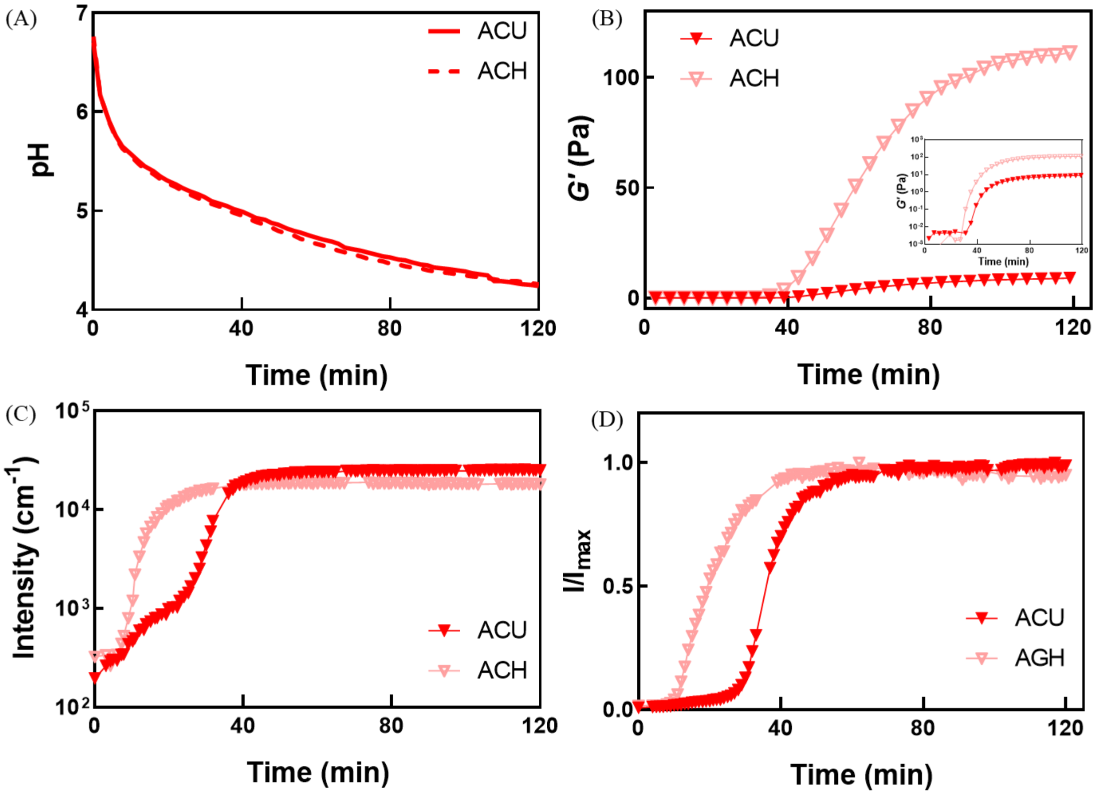

Figure 8-3 Evolution of pH (A), *G'* (B) (inset shows the *G'* on a logarithmic scale), absolute USANS intensity at $q = 1 \times 10^{-4}$ Å^{-1} (C), normalized USANS intensities (I_t/I_{max}) (D), for unheated milk (ACU) and heated milk (ACH) after the addition of 2% GDL.

Table 8-2 Coagulation properties of milk in different samples.

	ACU	ACH	PCU	PCH	CCU	CCH	HPU	HPH
t_1 (min)	40	34	20	22	27	13	2	2
t_2 (min)	122 [a]	122 [a]	122 [a]	122 [a]	38	26	3	11
t_3 (min)	–	–	–	–	48	41	80 [c]	38 [c]
pH_1	4.90	5.07	6.3	6.3	5.26	5.62	–	–
pH_2	4.24 [b]	4.26 [b]	6.3 [b]	6.3 [b]	5.11	5.12	5.59	6.16
pH_3	–	–	–	–	4.97	4.80	–	–
FR_1 (Pa/min)	0.22	2.90	1.48	0.38	0.32	2.50	–	–
FR_2 (Pa/min)	–	–	–	–	-0.06 [d]	-0.46 [d]	-1.1 [d]	-3.14 [d]
FR_3 (Pa/min)	–	–	–	–	0.37	0.83	–	–
G'_{max1} (Pa)	8.32	106.60	37.47	22.24	2.55	18.05	38.28	17.50
G'_{max2} (Pa)	–	–	–	–	18.04	42.23	–	–

ACU, PCU, CCU, HPU, samples arising from the acid-induced coagulation of unheated milk, pepsin-induced coagulation of unheated milk, combination coagulation of unheated milk, unheated milk treated with acid and high concentration of pepsin. ACH, PCH, CCH, HPH, samples arising from the acid-induced coagulation of heated milk, pepsin-induced coagulation of heated milk, combination coagulation of heated milk, heated milk treated with acid and high concentration of pepsin.

The time point in this table takes into account the time required for sample preparation, i.e., 2 minutes from the moment the sample was mixed until the first time point recorded by the rheometer. t_1 refers to the first time point when G' significantly increased, regarded as coagulation time. t_2 refers to the first time point when G' significantly decreased. t_3 refers to the second time point when G' significantly increased.

pH_1, pH_2, and pH_3 refers to the pH at times t_1, t_2, and t_3, respectively.

The maximum absolute slope of the G' curve (dG'/dt) is defined as the firming rate. FR_1 refers to the firming rate from t_1 to t_2. FR_2 refers to the firming rate from t_2 to t_3. FR_3 refers to the firming rate from t_3 to end time point.

G'_{max1} refers to the maximum storage modulus value during t_1 to t_2. G'_{max2} refers to the maximum storage modulus value during t_3 to end time point.

[a] the end time point during measurement. [b] the pH at end time points during measurement. [c] the time point when G' stops decreasing. [d] negative values correspond to decreasing rate of curd firmness.

At $q = 1 \times 10^{-4}$ Å^{-1} (corresponding to a length scale of about 6 μm), the changes in USANS absolute scattering intensity as a function of time for ACU and ACH are shown in **Figure 8-3 C**. The changes in the absolute intensity in the USANS were observed from the very beginning, which could be related to the increases in particle size and volume fraction of aggregates. At the coagulation time (t_1), the absolute intensities were ~18000 and ~17000 cm^{-1} for ACU and ACH, respectively. To better examine the intensity changes, evolution of normalized intensities (I_t/I_{max}) as a function of reaction time is shown in **Figure 8-3 D.** The I_t/I_{max} of AC samples showed a lag phase, log phase, and stationary phase. The significant increase (log phase) in ACH (~ 10 min, pH 5.55) occurred more rapidly than that in ACU (~ 30 min, pH 5.06), with a similar increase in rate (slope) during the log phase. Besides, the I_t/I_{max} of ACH reached a plateau (stationary phase) at ~ 35 min, faster than ACU at ~ 50 min. This suggests that pepsin-induced hydrolysis of κ-casein decreases the distance between para-casein micelles. Although coagulation did not occur right at the beginning according to the rheological results, the size of aggregates increased from the very beginning. However, the rate is very slow, thus a lag phase was evident in the normalized intensity (**Figure 8-3 D**).

To extend the structural characterization down to smaller scales, SANS was employed to further characterize the real-time nanostructural evolutions of milk during coagulation. Time-resolved SANS profiles of ACU and ACH are shown in **Figure 8-4 A** and **B**, respectively. In general, the SANS scattering patterns of the ACU and ACH during coagulation were similar. As time progressed, the intensity increased in the lower q range ($q < 0.003$) and decreased in the higher q range ($q > 0.003$). A Guinier feature could be observed until around 20 min (describing the size of aggregates). However, due to the increase in aggregate size, the Guinier feature disappeared (may have shifted to lower q) after about 20 min (too large to be determined). Therefore, the Guinier model fitting was not used in this study; instead, a power law model was employed to fit the curves obtained at different time points (5, 10, 15, 20, 30, 60, 90, 120 min) within the low q range ($0.007\ \text{Å}^{-1} < q < 0.003\ \text{Å}^{-1}$). The parameters obtained from the fits are listed in **Table S 8-1**. The changes in the power law exponent (m) are shown in **Figure 8-4 C**. A higher power law exponent (m) was obtained from the ACH in the initial 20-minute period, indicating a denser structure of the aggregates in ACH than ACU at each time point. A power law between 3 and 4 is usually interpreted as arising from surface geometry, with a smooth surface giving a value of 4 and values approaching 3 indicating increasing roughness (Mata, Udabage, & Gilbert, 2011). At 30 min, the lower m ~ 3 of ACH indicated a relatively rough surface compared to ACU (which has m ~ 4). Although the aggregate size was too large to be determined in the later stages from SANS results, larger aggregates were observed in ACH than ACU (**Figure 8-4 D**) at 2 h in the CLSM image. The protein stained by fast green appears as green and the serum voids are shown as black. The structure of ACU exhibited a looser protein network structure with greater porosity than for ACH. Similar observations were found in previous microscopic studies of acid gels induced by GDL (Wang et al., 2019; Yang et al., 2023d).

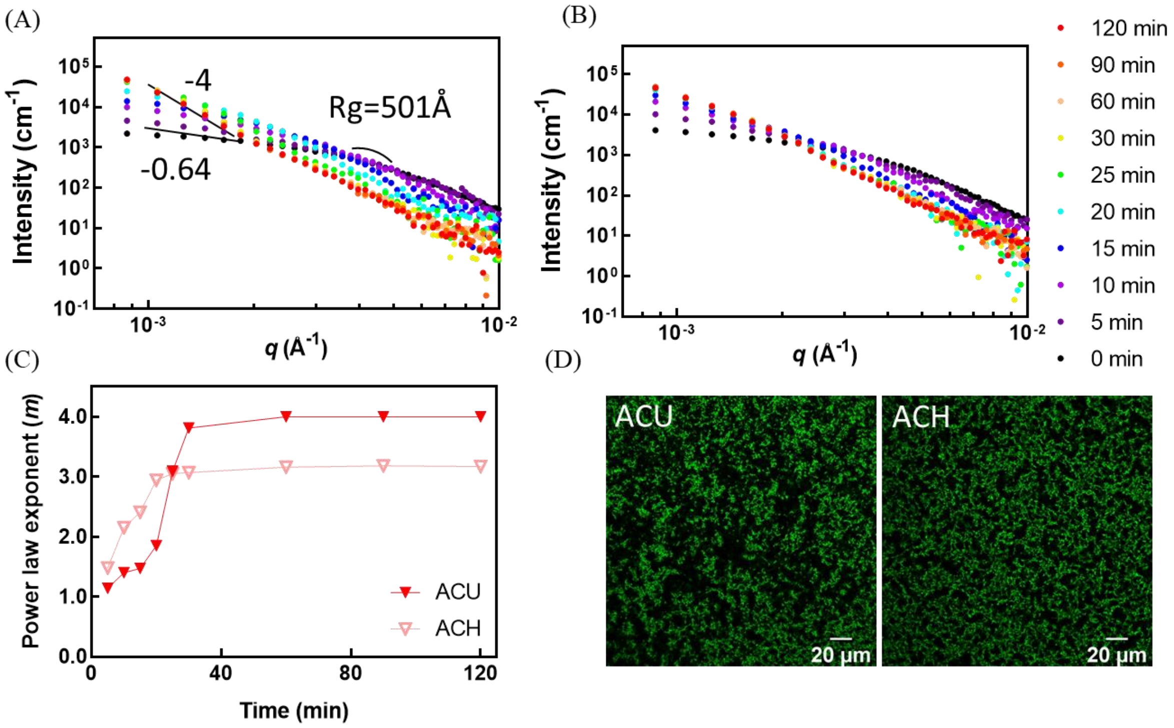

Figure 8-4 (A) Evolution of time-resolved SANS intensity data at 0.007 Å^{-1} < q < 0.0022 Å^{-1}of unheated milk (ACU). (B) Evolution of time-resolved SANS intensity data at 0.007 Å^{-1} < q < 0.0022 Å^{-1}of heated milk (ACH). (C) Power law exponent (m) obtained from the power law fitting of SANS results (0.007 Å^{-1} < q < 0.003 Å^{-1}) [associated with (A) and (B)]. (D) Confocal scanning laser micrographs of unheated (ACU) and heated milk (ACH) after 2 h. The stained protein networks are in green. The scale bars are 20 μm for all micrographs.

The heat treatment on milk at pH 6.7 at 85 °C for 30 min results in more than 80% whey proteins being denatured, and around 30% associated with casein micelles involving κ-casein (Anema et al., 2007). The denatured whey proteins associated with casein micelles become susceptible to aggregation during acidification and facilitate crosslinking of casein particles in the curd network, leading to an augmentation in the number and strength of bonds among protein particles. Besides, the isoelectric pH values of the whey proteins is ~ 5.2 to 4.8, higher than the casein proteins (De Wit, 1981) and it is likely that there are enhanced protein–protein interactions between denatured whey proteins near their isoelectric pH values owing to the heat-induced exposure of previously buried hydrophobic groups (Zhu & Damodaran, 1994).

8.3.4. Pepsin-induced coagulation of milk and the effect of heat treatment

With the addition of pepsin only, no pH changes were observed during coagulation, and the pH of both unheated and heated samples remained constant at ~ 6.3 (**Figure 8-5 A**). As shown in **Figure 8-5 B**, the *G′* showed a lag phase, and significant increases in *G′* of PCU and PCH were observed at ~ 20 min and 22 min, respectively. The *G′* of PCU reached a plateau value (~ 40 Pa) at ~ 70 min; the *G′* of PCH continually increased with reaction time over 2 h, but lower than that of PCU at 120 min (**Figure 8-5 B**). The increases in *G′* were due to the hydrolysis of κ-casein and the aggregation of the para-casein micelles (Yang et al., 2022a). The evolution of *G′* is in accordance with a previous study on the pepsin-induced milk coagulation (Yang et al., 2022b) that reported a slower firming rate for a heated milk sample.

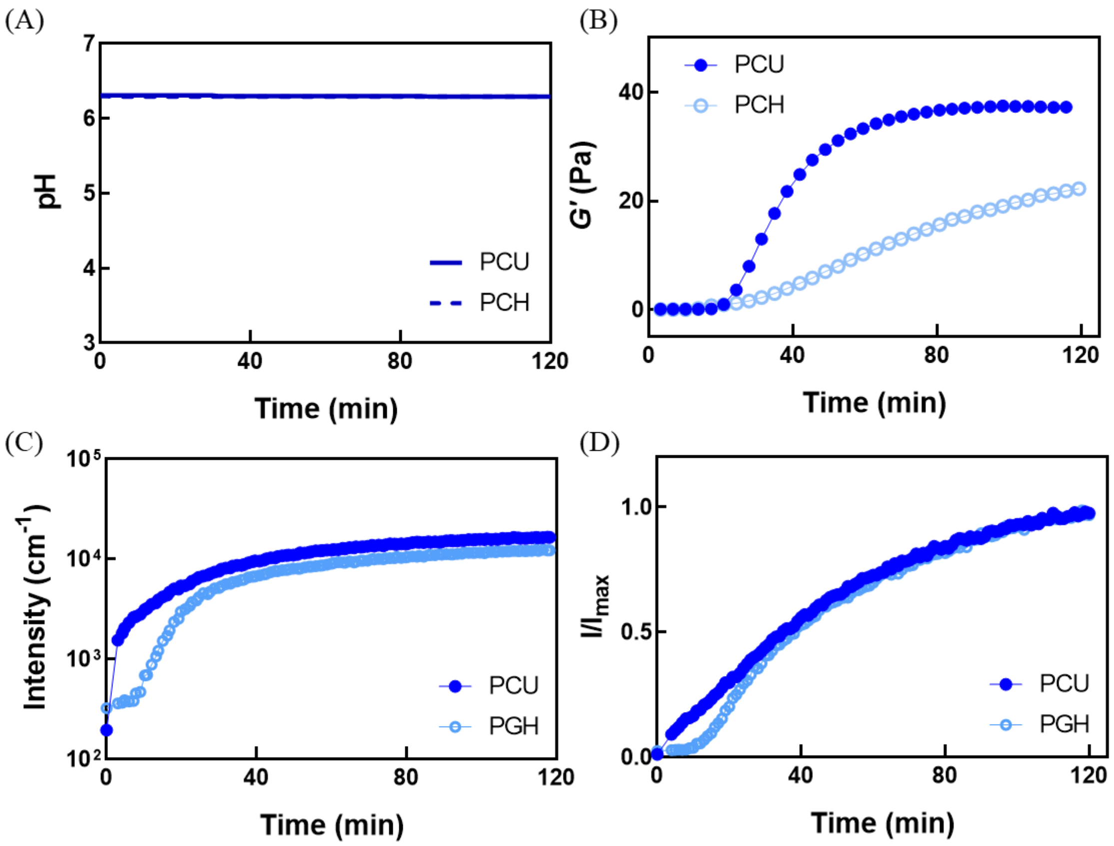

Figure 8-5 Evolution of pH (A), G' (B), absolute USANS intensity at $q = 1 \times 10^{-4}$ Å^{-1} (C), normalized USANS intensities (I_t/I_{max}) (D) for unheated milk (PCU) and heated milk (PCH) after the addition of 1.10 U/mL pepsin.

An immediate increase of absolute USANS intensity for PCU could be observed once the pepsin was added, whereas a lag phase of intensity was observed for PCH (**Figure 8-5 C**). At the coagulation time (t_1), the absolute intensities were ~ 5000 and ~ 3000 cm^{-1} for PCU and PCH, respectively. According to **Figure 8-5 D**, the I_t/I_{max} of PCU increased faster than for PCH, especially during the first 10 min. Significant increases in I_t/I_{max} of PCH was observed at 10–30 min, and from 30 min, a similar increase rate of I_t/I_{max} was observed for both samples. Time-resolved SANS profiles of PCU and PCH were shown in **Figure 8-6 A** and **B**, respectively. Due to the increases in aggregate size, the Guinier feature disappeared from ~ 20 min as well. The power law exponent (m) obtained from the power law model fits at low q range (0.007 Å^{-1} < q < 0.003 Å^{-1}) are listed in **Table S 8-1** and shown in **Figure 8-6 C**. Higher power law exponents (m) were observed in PCU during the entire reaction, and it reached ~ 4 at 120 min. The lower power law exponents (m < 3) of the PCH indicated a looser structure of the aggregates (Yu et al., 2019). According to the CLSM images at 2 h (**Figure 8-6 D**), the observed particles of protein for PCH were smaller than for PCU. As the firming rate for PCH was slower than for PCU, the presence of small particles may have arisen from the slower rate of aggregation of the proteins. Besides, the faster coagulation rate in PCU may also have resulted in the extensive rearrangement (Mellema et al., 2002), which resulted in the relatively stronger crosslink but larger voids, with a rough aggregate surface (the power law exponent (m) was ~ 4 at 2 h). Although PCU exhibited large pores, the structure of the proteins shows more interconnectivity than PCH, which could be due to more extensive protein rearrangements in PCU (Yang et al., 2023b).

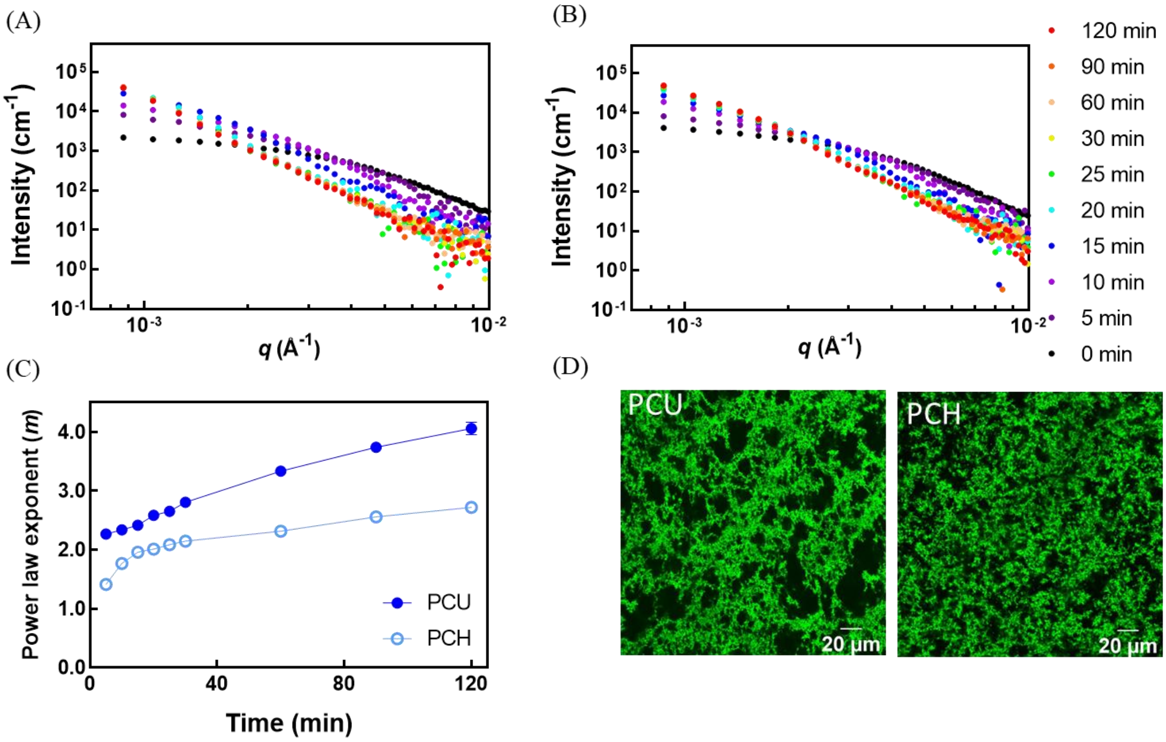

Figure 8-6 (A) Evolution of time-resolved SANS intensity data at 0.007 Å^{-1} < q < 0.0022 Å^{-1}of unheated milk (PCU). (B) Evolution of time-resolved SANS intensity data at 0.007 Å^{-1} < q < 0.0022 Å^{-1}of heated milk (PCH). (C) Power law exponent (m) obtained from the power law fitting of SANS results (0.007 Å^{-1} < q < 0.003 Å^{-1}) [associated with (A) and (B)]. (D) Confocal scanning laser micrographs of unheated (PCU) (C) and heated milk (PCH) after the addition of 1.10 U/mL pepsin for 2 h at pH 6.3. The stained protein networks are in green. The scale bars are 20 μm for all micrographs.

According to Yang et al. (2022b), the denatured whey proteins that associate with κ-casein on the micellar surface could impair the hydrolysis of κ-casein. With a slower hydrolysis rate of κ-casein, the heated milk showed a longer coagulation time (**Figure 8-5 B**). Besides, the para-casein micelles can be partially stabilized by the denatured whey proteins on the micelle surface, leading to a much slower aggregation process, i.e., longer coagulation time, lower G'_{120}, slower increase in I_t/I_{max} and slower increase in the power law exponents. This is also in agreement with reports on chymosin-induced milk coagulation at pH ~ 6.5 where heat treatment delayed the coagulation process (Anema et al., 2007; Dalgleish, 1990; Raynal & Remeuf, 1998; Singh & Waungana, 2001; Vasbinder et al., 2003).

8.4.5. Combination coagulation and the effect of heat treatment

With the addition of GDL (2%) and a low concentration of pepsin (0.24 U/mL), the pH of unheated and heated samples both decreased from 6.7 to ~ 4.3 after a 2-hour reaction time (**Figure 8-7 A**). G' exhibited a sequential pattern of a lag phase, followed by an increase phase, subsequent decrease (or flatten), and again an increase at a later time (**Figure 8-7 B**): the first significant increase in G' was at ~ 27 min, pH 5.26 for the unheated milk, and ~ 13 min, pH 5.62 for the heated milk. A decrease in G' at ~ 38 min (pH 5.11), and a shoulder at ~ 26 min (pH 5.12) were observed for unheated and heated milks, respectively (**Figure 8-7 B**). The increases in G' again occurs at ~ 48 min (pH 4.97), 41 min (pH 4.80) for unheated and heated milk, respectively (**Table 8-2**). The trends in rheological properties in the CC samples are consistent with the results for the combination coagulation induced by GDL and rennet (Herbert, Riaublanc, Bouchet, Gallant, & Dufour, 1999; Lucey, Tamehana, Singh, & Munro, 2000). Compared to the AC samples, the addition of pepsin in CC samples led to a shorter coagulation time and higher coagulation pH (**Table 8-2**), which was probably due to an accelerated pepsin-induced coagulation at pH > 6 (Lucey et al., 2000). Decreasing the pH of milk has been shown to increase the rate of the enzymatic hydrolysis stage (hydrolysis of κ-casein), with a maximum at pH 6.0 (Yang et al., 2022a); aggregation occurs at a lower extent of hydrolysis of κ-casein (van Hooydonk, 1987; Yang et al., 2022a). Thus, compared to the PC sample, the decreasing pH in CC samples could lead to the aggregation of micelles at a lower extent of hydrolysis of κ-casein and at a shorter coagulation time (van Hooydonk, 1987). With decreases in pH (< 6) and closer to the pI of whey proteins, the associated denatured whey proteins may facilitate the aggregation. Therefore, shorter coagulation time and higher *G′* was found in CCH. The first decrease/flattening in the *G′* of CC could be due to excessive large-scale rearrangements of bonds and strands in the protein network (Lucey et al., 2000). At pH < 6, there was a partial loosening of bonds within the casein particles in CC due to the solubilization of CCP (**Figure 8-2**). This solubilization may alter the balance between viscous and elastic components in the network and modify protein–protein interactions, and hence may be responsible for the decreases in *G′* (Lucey et al., 2000). With further decreases in pH, the *G′* increased again as a result of the transition of a curd exhibiting a pepsin-induced curd character, along with a curd exhibiting an acid-induced curd character (van Vliet, Lakemond, & Visschers, 2004). Reduced electrostatic repulsion at lower pH leads to an increase in hydrophobic interactions (Horne, 1999). Over time, the decreases in *G′* of CCU at ~ 110 min could be due to syneresis of the curd brought about by protein rearrangements (Mellema et al., 2002). In addition, when pH decreases to ~ 4, the general hydrolysis activity of pepsin increases (Tam & Whitaker, 1972; Ye, 2021). Most of the proteins in the curd began to be hydrolyzed by pepsin, which could also result in the decrease in *G′*.

The evolution of absolute intensity shows a ‘first order’ characteristic (**Figure 8-7 C**). Higher absolute intensity value was observed in the CCH than CCU from ~ 30 min. The I_t/I_{max} of CCU and CCH both reached a plateau at ~ 30 min, whereas relatively higher rate increases were observed for CCU in the first 30 min (**Figure 8-7 D)**. In agreement with the rheological data, higher rate increases of I_t/I_{max} for CCH during the first 30 min could be related to the denatured whey proteins, which accelerate the coagulation rate when the pH decreases. Excessively large-scale rearrangements of bonds and strands in the gel network and the curd character transition was not reflected in the USANS data.

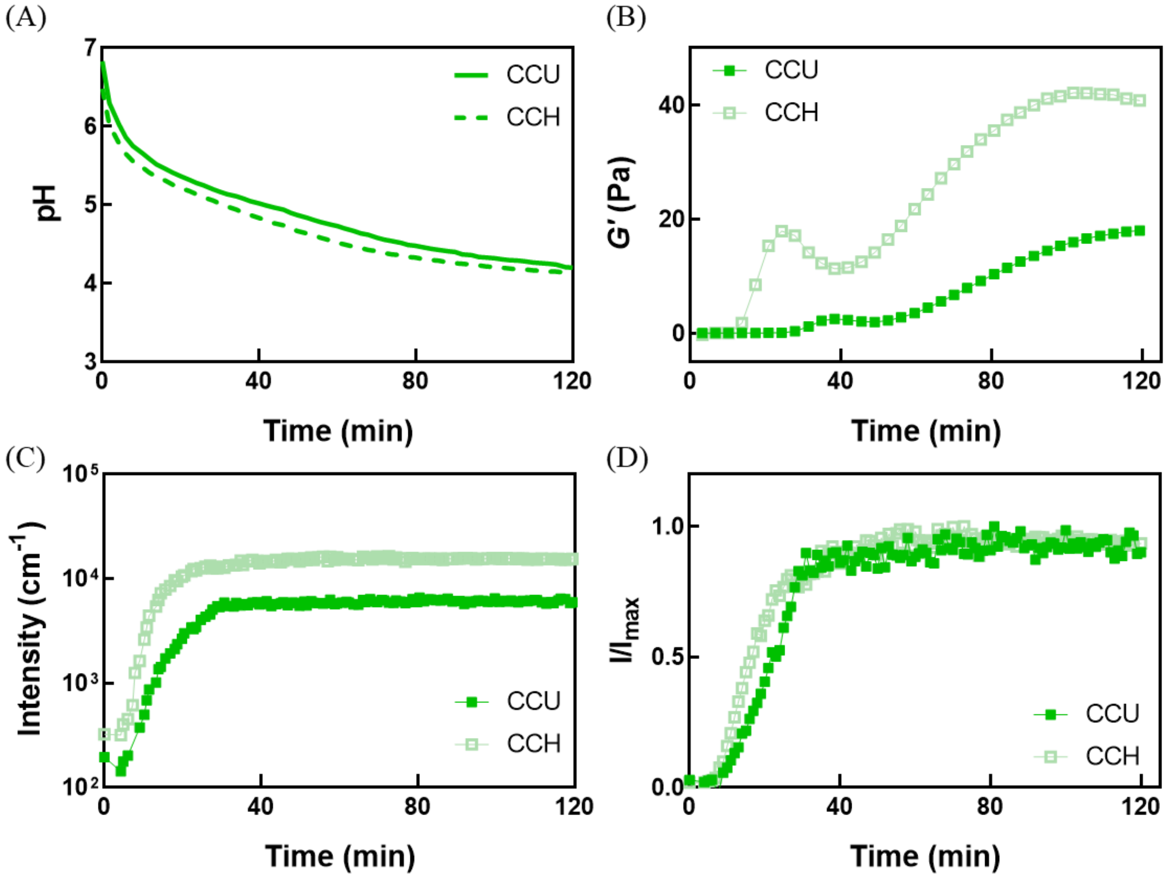

Figure 8-7 Evolution of pH (A), *G′* (B), absolute USANS intensity at $q = 1 \times 10^{-4}$ $Å^{-1}$ (C), normalized USANS intensities (I_t/I_{max}) (D) for unheated milk (PCU) and heated milk (PCH) after the addition of 2% GDL and 0.24 U/mL pepsin.

Power law model fits of the time-resolved SANS results for CCU and CCH at low q range (0.007 $Å^{-1} < q <$ 0.003 $Å^{-1}$) are listed in **Table S 8-1** and shown in **Figure 8-8 C**. Slightly lower increase rate of m was observed in CCU at the first 10 min; whereas a higher m was found for CCU from 20 min; m reached ~ 3 for CCH and ~ 4 for CCU at 30 min. This indicates that the CCH had a rougher aggregate surface than CCU from 30 min. At 2 h, the microstructure of CCU was coarse with pores up to 30 μm (**Figure 8-8 D**); whereas the structure of CCH appeared fairly homogeneous, with small pores, mostly ~ 5 μm (**Figure 8-8 D**). These differences could be related to the association of denatured whey protein incorporated into the curd network.

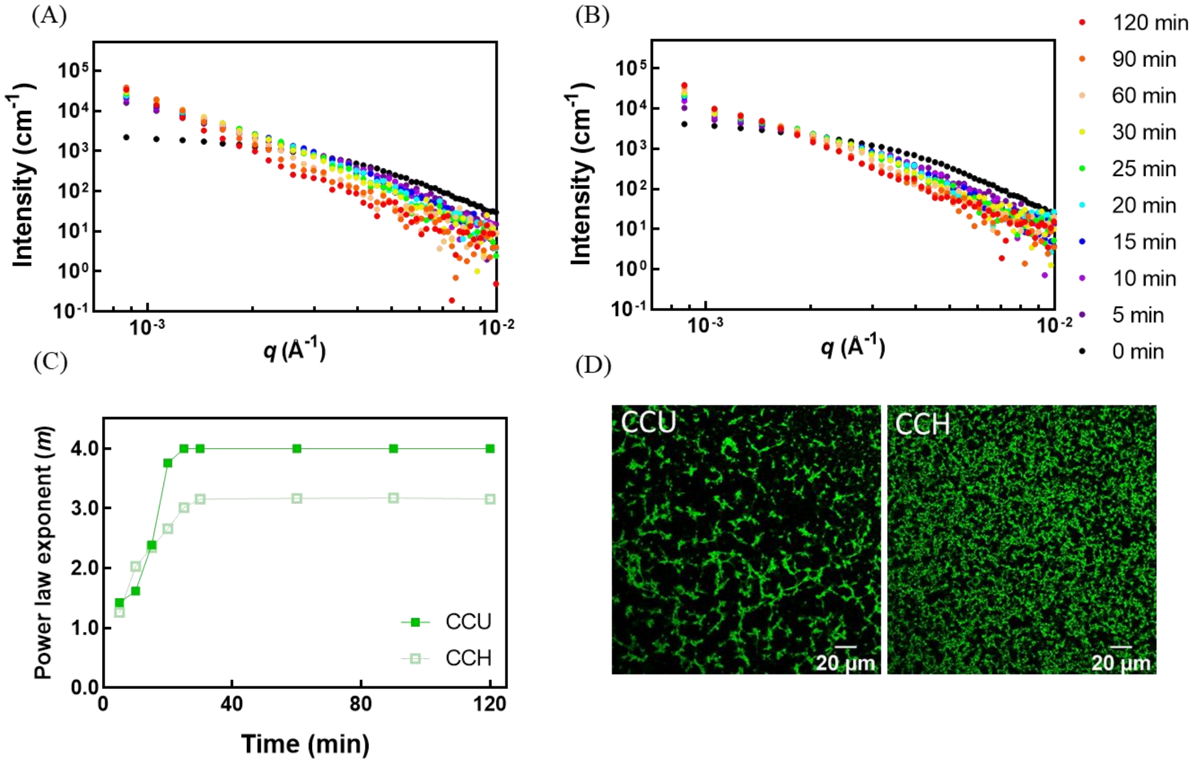

Figure 8-8 (A) Evolution of time-resolved SANS intensity data at 0.007 Å^{-1} < q < 0.0022 Å^{-1}of unheated milk (CCU). (B) Evolution of time-resolved SANS intensity data at 0.007 Å^{-1} < q < 0.0022 Å^{-1}of heated milk (CCH). (C) Power law exponent (m) obtained from the power law fitting of SANS (0.007 Å^{-1} < q < 0.003 Å^{-1}) [associated with (A) and (B)]. (D) Confocal scanning laser micrographs of unheated (CCU) and heated milk (CCH) after the addition of 2% GDL and 0.24 U/mL pepsin for 2 h. The stained protein networks are in green. The scale bars are 20 μm for all micrographs.

8.3.6. Effect of adding GDL and 2000 U/mL pepsin on the unheated and heated milk

With the addition of 2% GDL and 2000 U/mL pepsin, the pH changes aligned with the AC and CC samples in the first 2 hours and decreased to ~ 3.8 after the 8-hour reaction (**Figure 8-9 A**). During the experiment, coagulation occurred immediately after the addition of GDL and a high concentration of pepsin (2000 U/mL) (visual observation). *G'* increased immediately once the rheological measurements commenced (it took 2 minutes from the moment the sample was mixed until the first time point recorded by the rheometer), and decreased after reaching ~ 40 Pa for unheated milk and ~ 20 Pa for heated milk (**Figure 8-9 B**). With a decreasing rate of G' ~ 1.1 Pa/min for HPU, G' reached to a low value after ~ 80 min reaction. With a decreasing rate of G' ~ 3.14 Pa/min for HPH, G' reached a low value after ~ 38 min reaction (**Table 8-2**). The increases in the absolute intensity occurred at the very beginning and reached a maximum value within 20 min (**Figure 8-9 C)**. A decrease in absolute intensity was observed at ~18 min and ~ 41 min for HPH and HPU, respectively. The absolute intensity of the heated sample reached a plateau value (~ 3000 cm^{-1}) after ~ 1 h, whereas the unheated sample reached plateau value after ~ 4 h (**Figure 8-9 C**). The decreases in the intensity agreed with a previous study (Bayrak et al., 2023), which could be due to the fact that the milk curd was digested by pepsin. The rate of curd network disruption could be inferred from the decrease rate of I_t/I_{max} (**Figure 8-9 D**).

As the reaction commenced, a progressive increase in the scattering intensity can be observed at $q < 0.003$ $\mathrm{Å}^{-1}$ in HPU (**Figure 8-10 A**), which indicates the formation and growth of large-scale aggregates in the first 10 min. From 15 min (red curve), a decrease in the scattering intensity was found for HPU. A slight increase could also be observed at $q < 0.008$ $\mathrm{Å}^{-1}$ in the first 10 min for HPH (**Figure 8-10 D**), whereas a significant decrease was found as the reaction progressed (the absolute intensity of HPH was lower than the HPU at each time point). The significant decreases in intensity could be more related to the general hydrolysis of proteins by pepsin (Bayrak et al., 2023), although the possibility of (micro)phase separation cannot be excluded. As shown in **Figure 8-10 C**, an increase in the power law exponent (m) was found in the first 10 minutes for both samples. m reached a maximum value at 15 min for HPU (~ 3.2) and HPH (~ 4.0). Over time, the m of HPU was ~ 3.1 from 20 min to 8 h, whereas a reduction of m was found in HPH, suggesting a loosening of the aggregate microstructure as digestion proceeds.

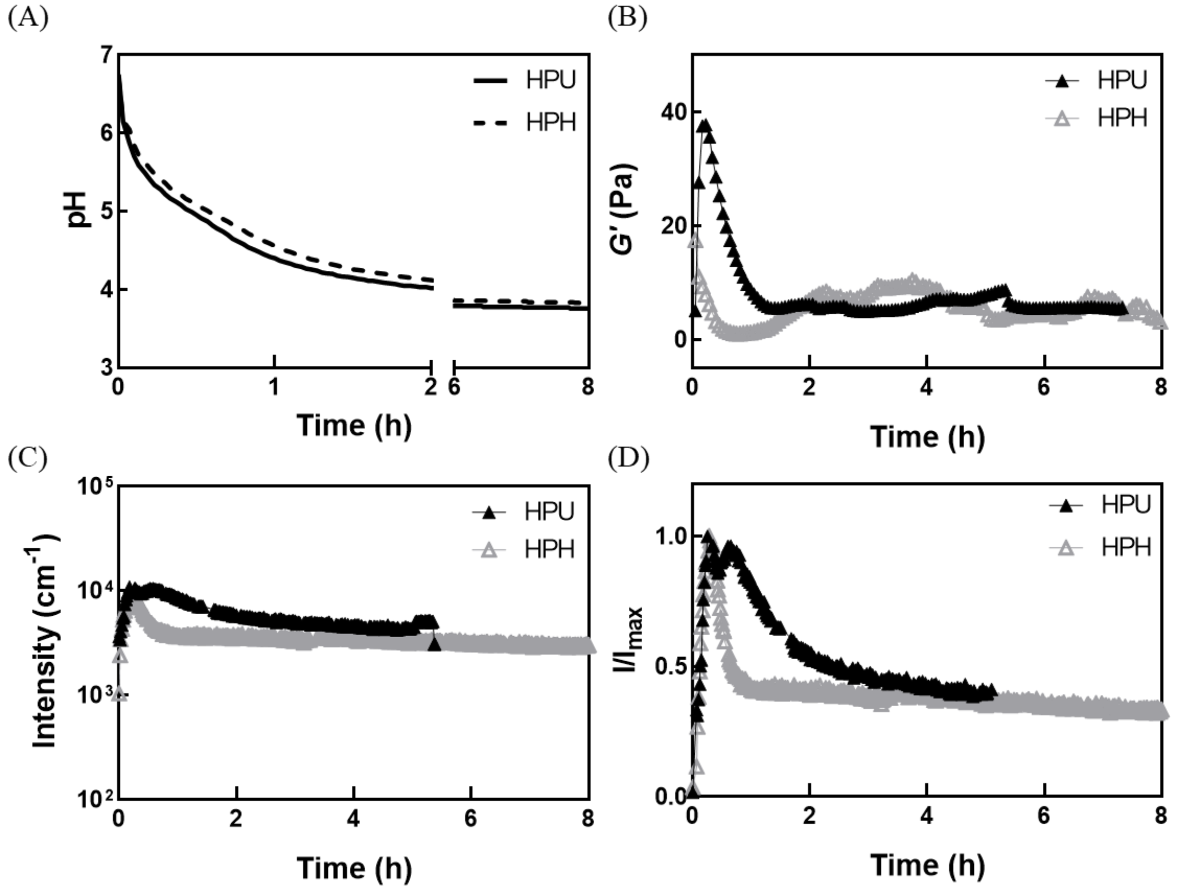

Figure 8-9 Evolution of pH (A), *G′* (B), absolute USANS intensity at $q = 1 \times 10^{-4}$ Å^{-1} (C), normalized USANS intensities (I_t/I_{max}) (D) for unheated milk (HPU) and heated milk (HPH) after the addition of 2% GDL and 2000 U/mL pepsin.

The decrease in pH in the presence of 2000 U/g pepsin in the HP sample simulates the gastric digestion environment where milk is ingested. For the first 5 min, the pH of samples was > 6, higher than the pI of casein proteins. At this pH (> 6), coagulation occurs due to the pepsin-induced specific hydrolysis of κ-casein (Yang et al., 2022a), as shown by the increases in *G′* (**Figure 8-9 B**) and absolute intensity (**Figure 8-9 C**); whereas the activity of pepsin for general hydrolysis is relatively low (Kageyama, 2002). Besides, at pH > 6, the associated denatured whey proteins perform a barrier role in the network of heated milk proteins (Yang et al., 2023b). Decreases were observed in G′ and absolute intensity. The decrease in the low-*q* power law exponent (*m*) (**Figure 8-10 C**) suggests that the aggregated micelles in the protein curd network (on a ~ 100 nm scale) were degraded due to hydrolysis of the curd into smaller protein fragments, peptides or amino acids, which is in agreement with the results from Bayrak et al. (2023). Compared to HPU, the relatively faster decrease in firmness (**Figure 8-9 B**) and I_t/I_{max} (**Figure 8-9 D**) of HPH indicates a faster rate of curd degradation (due to the general hydrolysis of proteins by pepsin) of HPH. These might be attributed to the higher diffusion co-efficient of pepsin in the looser curd structure of HPH than that of HPU. Over time, the pH gradually decreased further to pH < 5, and the general hydrolysis activity of pepsin increased. This is in agreement with the effect of heat treatment on the digestibility of milk proteins in the simulated gastric environment (Ye et al., 2017). The microstructure of HPU and HPH after 8 h of incubation observed by CLSM is shown in **Figure 8-10 D**. This figure shows a large pore area (serum) in both HPU and HPH, whereas a relatively larger protein area was found for the HPU. Photographs of HPU and HPH are shown in **Figure S 8-1** that demonstrate low turbidity (transparency); as the reaction progressed, the curd was gradually destroyed as most of the proteins become hydrolyzed by pepsin.

Overall, the coagulation time could be estimated from the rheological results. Information about the level of structural complexity, or fractal-like nature of the milk aggregates, was complemented by CLSM micrographs, and a power law exponent was obtained from SANS analysis. At $q = 1 \times 10^{-4}$ Å^{-1}, the change in absolute intensity was positively related to the change in G' when plotted as a function of time. In agreement with previous studies (Bayrak et al., 2023; Huang, Terech, Raghavan, & Weiss, 2005), plotting scattering intensities at a single q value as a function of time is an efficient method for assessing the aggregation kinetics of protein network samples. Although a finite time elapses for significant increases in G' to be observed by rheometry, the changes in the absolute intensity at this fixed q value in USANS was observed from the very beginning, in agreement with Yang et al. (2023d). This study highlights the importance of using complementary measurement techniques, including

USANS and SANS, that are sensitive to multiple length and time scales; in doing so, a comprehensive understanding of the milk aggregate structure and properties can be gained.

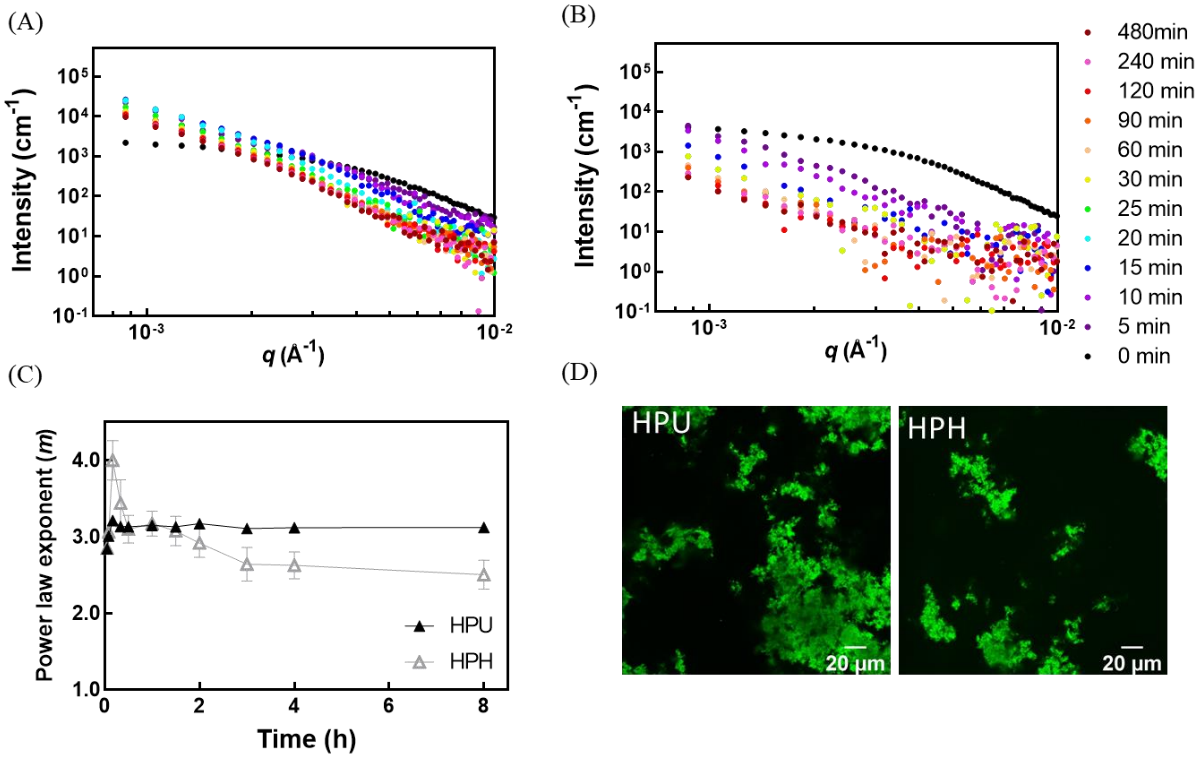

Figure 8-10 (A) Evolution of time-resolved SANS intensity data at 0.007 Å^{-1} < q < 0.0022 Å^{-1}of unheated milk (HPU). (B) Evolution of time-resolved SANS intensity data at 0.007 Å^{-1} < q < 0.0022 Å^{-1}of heated milk (HPH). (C) Power law exponent (m) obtained from the power law fitting of SANS results (0.007 Å^{-1} < q < 0.003 Å^{-1}) [associated with (A) and (B)]. (D) Confocal scanning laser micrographs of unheated milk (HPU) and heated milk (HPH) after the addition of 2% GDL and 2000 U/mL pepsin for 8 h. The stained protein networks are in green. The scale bars are 20 µm for all micrographs.

When the milk is preheated, denatured whey proteins associate with casein micelles. According to the SANS results, regardless of the acid/pepsin/combination-induced coagulation, the surface roughness of the aggregates in the heated milk was higher than those in the unheated samples. However, heat treatment has different effects on aggregate formation in acid-induced, pepsin-induced and combination coagulated milk gels. Heat treatment facilitated acid-induced and combination coagulation, but retarded pepsin-induced coagulation. In general, pH plays an important role in milk coagulation and significantly affects the impact of heat treatment on this process. When milk is preheated, denatured whey proteins associate with casein micelles and the impact on gel network varies with pH conditions. At pH > 6, which is significantly higher than the pI of whey proteins, the associated whey proteins act as a barrier layer between casein micelles. However, closer to the pI of whey proteins, the associated whey proteins initiate isoelectric precipitation/aggregation. According to Yang et al. (2023b), pepsin-induced coagulation of heated milk was retarded at pH 6.3 but facilitated at pH 6.0. Besides, with the continuous decrease in pH (addition of GDL), regardless of the presence of pepsin, the coagulation of heated milk takes place at higher pH (between pH 5.0–6.0) than for unheated milk, with higher G'. This could be due to increased cross-linking or bridging by denatured whey proteins within gels made from heated milk (Lucey et al., 1997). In the simulated gastric environment, pepsin-induced coagulation occurred at pH > 6. At this pH, the associated whey proteins produced a barrier effect for the coagulation of para-casein micelles, resulting in a looser protein curd. The pepsin diffused effectively in this network. As the pH decreased to < 4, the disintegration and hydrolysis of proteins by pepsin was therefore faster in the looser curd formed in the heated milk.

8.4. Conclusions

The nature of the coagulation process of milk affects the diverse properties of the milk curd. Rheological methods are valuable for characterizing the texture of milk curd and have enabled the differentiation of properties of acid-induced, pepsin-induced and combination samples. Using USANS, SANS, and CLSM methods, it has been possible to demonstrate that milk curd with distinct rheological properties exhibits different structures at multiscale levels. It has been observed that during aggregation, the sizes and volume fractions of aggregates increase over time. Heat treatment leads to the association of denatured whey proteins onto the surface of casein micelles, which has different effects on the curds induced by acid, pepsin, and the combination. The aggregation of whey proteins induced by heat treatment could facilitate acid-induced and combination coagulation of milk but hinders pepsin-induced coagulation, where pH plays an important role. In the presence of a high pepsin concentration, faster coagulation and degradation of milk curd were observed in heated milk samples. This study provides further understanding on the effect of heat treatment on milk coagulation and the curd degradation process.

8.5. Acknowledgments

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8.6. Supplementary material

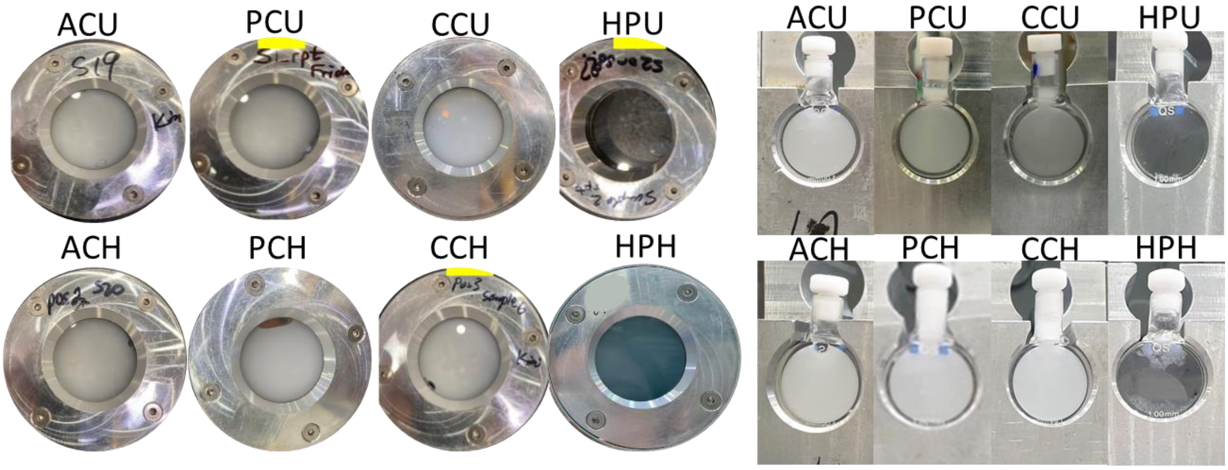

Figure S 8-1 Photograph of samples in the cells after 2-hour reaction. ACU, PCU, CCU, HPU, samples arising from the acid-induced coagulation of unheated milk, pepsin-induced coagulation of unheated milk, combination coagulation of unheated milk, unheated milk treated with acid and high concentration of pepsin. ACH, PCH, CCH, HPH, samples arising from the acid-induced coagulation of heated milk, pepsin-induced coagulation of heated milk, combination coagulation of heated milk, heated milk treated with acid and high concentration of pepsin.

Table S 8-1 Fitting parameters obtained for the data associated with time-resolved SANS results when using the power law model for AC, PC, CC and HP systems.

	ACU				**ACH**			
	Chi2	**scale**	**background**	**power law exponent, *m***	**Chi2**	**scale**	**background**	**power law exponent, *m***
5	2.19	1.52(0.798)	0.001	1.14(0.08)	0.82	2.93E-01(1.19E-01)	0.001	1.48(0.06)
10	0.96	5.37E-01(2.06E-01)	0.001	1.40(0.06)	0.48	5.38E-03(1.92E-03)	0.001	2.15(0.05)
15	2.61	4.57E-01(1.53E-01)	0.001	1.47(0.05)	0.72	1.13E-03(4.26E-04)	0.001	2.41(0.06)
20	6.18	5.44E-02(1.72E-02)	0.001	1.85(0.05)	1.99	3.91E-05(1.48E-05)	0.001	2.94(0.06)
25	12.06	1.40E-05(4.98E-06)	0.001	3.09(0.05)	4.04	1.95E-05(7.28E-06)	0.001	3.05(0.06)
30	6.79	9.18E-08(3.96E-08)	0.001	3.81(0.06)	3.70	1.73E-05(6.74E-06)	0.001	3.07(0.06)
60	2.74	1.07E-08(3.88E-09)	0.001	4*	5.59	9.78E-06(2.82E-06)	0.001	3.16(0.04)
90	3.69	9.47E-09(3.37E-09)	0.001	4*	6.16	8.20E-06(2.30E-06)	0.001	3.18(0.04)
120	3.26	7.48E-09(2.67E-09)	0.001	4*	5.06	8.72E-06(2.47E-06)	0.001	3.17(0.04)
	PCU				**PCH**			
	Chi2	**scale**	**background**	**power law exponent, *m***	**Chi2**	**scale**	**background**	**power law exponent, *m***
5	0.49	1.68E-03(7.78E-04)	0.001	2.27(0.07)	1.36	4.90E-01(1.97E-01)	0.001	1.41(0.06)
10	0.70	1.24E-03(5.23E-04)	0.001	2.34(0.06)	1.40	5.65E-02(1.89E-02)	0.001	1.77(0.05)
15	0.64	7.80E-04(3.37E-04)	0.001	2.42(0.07)	0.83	1.84E-02(6.58E-03)	0.001	1.96(0.05)
20	0.14	2.64E-04(1.14E-04)	0.001	2.59(0.06)	1.06	1.37E-02(4.95E-03)	0.001	2.01(0.05)
25	0.18	1.86E-04(8.17E-05)	0.001	2.65(0.07)	1.29	8.81E-03(2.93E-03)	0.001	2.09(0.05)
30	1.04	6.62E-05(2.90E-05)	0.001	2.81(0.07)	1.84	6.07E-03(2.22E-03)	0.001	2.15(0.06)
60	0.37	1.96E-06(9.19E-07)	0.001	3.34(0.07)	2.18	2.21E-03(7.30E-04)	0.001	2.32(0.05)
90	0.41	1.14E-07(5.91E-08)	0.001	3.74(0.08)	2.38	4.80E-04(1.61E-04)	0.001	2.56(0.05)
120	0.93	9.15E-09(6.54E-09)	0.001	4*	3.31	1.62E-04(5.77E-05)	0.001	2.72(0.05)
	CCU				**CCH**			
	Chi2	**scale**	**background**	**power law exponent, *m***	**Chi2**	**scale**	**background**	**power law exponent, *m***
5	0.99	3.58E-01(1.42E-01)	0.001	1.42(0.06)	0.88	1.18E+00(5.32E-01)	0.001	1.26(0.07)
10	2.06	1.59E-01(5.55E-02)	0.001	1.62(0.05)	0.29	1.06E-02(4.02E-03)	0.001	2.03(0.06)
15	6.20	1.39E-03(4.65E-04)	0.001	2.39(0.05)	0.45	1.68E-03(6.73E-04)	0.001	2.35(0.06)
20	5.72	1.13E-07(4.86E-08)	0.001	3.76(0.06)	0.84	2.42E-04(9.13E-05)	0.001	2.66(0.06)
25	1.88	6.87E-09(3.55E-09)	0.001	4*	2.57	2.36E-05(9.04E-06)	0.001	3.01(0.06)
30	1.50	5.91E-09(2.23E-09)	0.001	4*	4.22	9.53E-06(2.68E-06)	0.001	3.15(0.04)
60	0.66	7.58E-09(3.04E-09)	0.001	4*	4.37	9.21E-06(2.72E-06)	0.001	3.17(0.04)
90	0.82	5.06E-09(1.92E-09)	0.001	4*	4.57	8.76E-06(2.53E-06)	0.001	3.17(0.04)
120	0.44	6.90E-09(2.81E-09)	0.001	4*	5.01	9.89E-06(2.87E-06)	0.001	3.15(0.04)
	HPU				**HPH**			
	Chi2	**scale**	**background**	**power law exponent, *m***	**Chi2**	**scale**	**background**	**power law exponent, *m***
5	5.39	2.27E-04(2.70E-05)	0.001	2.55(0.02)	1.95	3.09E-06(1.03E-06)	0.001	2.99(0.05)
10	10.76	2.24E-05(2.65E-06)	0.001	2.96(0.02)	1.29	3.85E-07(2.17E-07)	0.001	3.23(0.09)
15	9.53	6.76E-06(8.83E-07)	0.001	3.14(0.02)	1.04	5.35E-09(8.29E-09)	0.001	3.71(0.23)
20	7.61	1.05E-06(1.67E-07)	0.001	3.41(0.02)	0.95	1.70E-07(3.04E-07)	0.001	3.11(0.27)
25	3.58	9.45E-07(1.88E-07)	0.001	3.36(0.03)	1.35	6.62E-09(2.08E-08)	0.001	3.53(0.46)
30	4.01	8.33E-07(1.87E-07)	0.001	3.34(0.03)	1.11	1.87E-07(2.48E-07)	0.001	3.02(0.20)
60	6.93	1.06E-06(1.28E-07)	0.001	3.30(0.02)	1.10	6.65E-08(8.91E-08)	0.001	3.18(0.20)
90	7.30	1.30E-06(1.56E-07)	0.001	3.26(0.02)	1.03	1.40E-07(1.94E-07)	0.001	3.05(0.21)
120	7.13	8.61E-07(1.07E-07)	0.001	3.32(0.02)	0.87	3.82E-07(5.23E-07)	0.001	2.89(0.21)
180	6.37	1.18E-06(1.49E-07)	0.001	3.27(0.02)	0.88	3.95E-06(5.60E-06)	0.001	2.49(0.22)
240	5.95	1.15E-06(1.51E-07)	0.001	3.26(0.02)	0.68	3.76E-06(4.38E-06)	0.001	2.54(0.18)
480	5.63	9.92E-07(1.34E-07)	0.001	3.28(0.02)	0.73	8.45E-06(1.04E-05)	0.001	2.39(0.20)

*, the power law exponent was fitted at 4.

Chapter 9 Overall discussion, conclusions, and future recommendations

9.1 Overall discussion and conclusions

The digestion of milk protein in the stomach is a dynamic and intricate process, affected by several factors including temperature, pH, pepsin concentration, and gastrointestinal motility. Although *in vivo* or *in vitro* digestion studies primarily provide infomation about milk digestion behavior, this thesis focuses on investigating the underlying mechanisms by studying the enzymatic reaction between casein micelles and pepsin under controlled environmental conditions. It is worth noting that the enzymatic reaction is similar to the reaction between chymosin and casein micelles. The environmental conditions in this thesis were set to mimic the *in vivo* gastric environment more closely.

The kinetics of the enzymatic hydrolysis of κ-casein and subsequent protein aggregation can be measured separately. RP-HPLC is a successful method for separation, identification, and quantification of milk proteins and the products resulting from the hydrolysis of κ-casein with pepsin (CMP and para-κ-casein). A series of experiments were carried out to characterize the coagulation process and coagulum structure. The evolution of mechanical properties and microstructures of the proteins during the pepsin-induced reaction was studied by small oscillatory deformation rheology, confocal laser scanning microscopy, and small and ultra-small angle neutron scattering (SANS and USANS). In addition, large oscillatory deformation rheology and water holding capacity were carried out to determine the coagulum properties during the reaction. Furthermore, a human gastric simulator was employed to explore the coagulation and digestion behavior of selected milk samples.

The pepsin-induced hydrolysis of κ-casein followed a combined kinetic model of first-order hydrolysis and pepsin denaturation, $ln\left(1-\frac{H_t}{100}\right)=\frac{K_{enz}\cdot C}{K_{den}}\cdot[exp(-K_{den}\cdot t)-1]$, where H_t is the percent hydrolysis of κ-casein at time t, K_{den} (min^{-1}) is the reaction rate constant for the denaturation reaction, C (U/mL) is the pepsin concentration, K_{enz} [min^{-1}/(U/mL)] is the reaction rate constant for the enzymatic reaction per unit quantity of enzyme solution. $K_{\mathrm{enz}} \cdot C$ is defined as the overall reaction rate constant K (min^{-1}), which is an indication of the hydrolysis rate. This equation describes all the experimental data well, in which proteolysis and denaturation take place simultaneously.

In **Chapter 3**, at 37 °C, the hydrolysis of κ-casein was reported to depend highly on the pepsin concentration (0.275–5.50 U/ml milk) and milk pH (5.3–6.7). The enzymatic reaction rate constant K increased as pepsin concentration increased with a positive linear correlation at all pH values. At a fixed pepsin concentration, the highest reaction rate constant K was found at pH 6.0, which is the optimal pH for pepsin-induced hydrolysis of κ-casein. The effective interaction between pepsin and κ-casein at pH 6.0 is related to the balance between ionic strength, electrostatic repulsion and protrusion of caseinomacropeptide at this pH value. By adding the same concentration of pepsin into milk samples at pH 6.0, the optimum temperature for pepsin to specifically hydrolyze κ-casein was found to be around 37−40 °C, with a temperature coefficient of ~ 1.95, as reported in **Chapter 4**.

At the optimum pH and temperature, **Chapter 5** and **Chapter 6** demonstrated that pepsin-induced hydrolysis of κ-casein was independent of whey protein concentration (in the range of casein:whey protein ratios of 4:0, 4:0.6, 4:1, 4:2 wt/vol), calcium concentration (in the range of added $CaCl_2$ from 0 to 17.5 m*M*) and ionic strength (up to 52.5 m*M*). However, the reaction rate constant K for heated samples was lower than unheated samples, indicating that heat treatment impairs the hydrolysis rate for the samples containing whey proteins. It seems likely that the small reduction in enzymatic hydrolysis after heat treatment was caused by the denaturation of whey proteins and subsequent interaction with casein micelles. On the other hand, the increase in casein micelle size in heated milk (due to the interaction of denatured whey proteins with the casein micelles and/or the heat-induced whey protein aggregation) might affect the diffusivity of the enzyme significantly, leading to an appreciable change in the rate of κ-casein hydrolysis.

The content of κ-casein is different in sheep, cow and goat milk. In **Chapter 7**, when controlled at the same pepsin-to-κ-casein ratio, the hydrolysis rate of κ-casein was found to be most pronounced in sheep milk, followed by cow milk and goat milk. These differences are probably relate to the sequence around the Phe-Met bond, the glycosylation degree of κ-casein, conformation of κ-casein, and differences in the casein micelle sizes among different milk species.

Coagulation of the casein micelles occurred when a critical amount of κ-casein had been hydrolyzed. At a given pH, the critical degree of hydrolysis was independent of the pepsin concentration. At a constant pepsin concentration, it depends on the pH: the degree of κ-casein hydrolysis required for coagulation decreased markedly from ~ 73 to ~ 33% when the pH decreased from 6.3 to 5.3, likely due to the lower pH promoting aggregation of casein micelles through neutralization of surface charge; the decrease in milk pH could also result in the release of Ca^{2+} by CCP dissolution. According to **Chapter 6**, Ca^{2+} plays a critical role in the coagulation process. Higher concentrations of $CaCl_2$ led to a decrease in pH. Therefore, the investigation examining the impact of various concentrations of $CaCl_2$ on the coagulation of casein micelles was carried out at a fixed pH of 6.0. It was found that even though the hydrolysis of κ-casein was extensive (> 80%), there was a critical calcium concentration (~ 4.8 m*M* [Ca^{2+}] at pH 6.0) required for coagulation of casein micelles. The addition of Ca^{2+} promoted the coagulation of the casein micelles and enhanced the strength and structure of the curd, with formation of inter-micellar calcium bridging. The degree of κ-casein hydrolysis required for coagulation decreased markedly from 79% to 15% when [Ca^{2+}] was increased from 7.50 to 22.5 m*M*. The highest maximum storage modulus of the curd occurred at 17.5 m*M* $CaCl_2$ and decreased when concentration increased to 22.5 m*M* $CaCl_2$.

Coagulation of milk in the gastric environment normally occurs at 37 °C, i.e., body temperature. However, the coagulation behavior and the structure of the curd was found to depend markedly on the set temperature. **Chapter 4** provides a better understanding of the effect of temperature. Under the same conditions, a faster coagulation but lower firming rate and G'_{max}, with larger aggregates and voids, were observed at higher temperatures.

Chapter 5 demonstrates that whey proteins impair the rate of aggregation of the para-casein micelles over a wide range of casein:whey protein ratios. In the presence of whey proteins, heat treatment impacts coagulation behavior of casein micelles but this highly depended on the pH. Denatured whey proteins retard aggregation of casein micelles at the higher pH (6.3); however, when the pH decreases to 6.0 (closer to the isoelectric point of whey proteins), aggregation was enhanced, mainly due to the incorporation of whey proteins into the curd. Since the ratio of casein:whey protein is around 4:1 in milk, **Chapter 8** extended the research on the effect of heat treatment on the structure of the milk proteins during coagulation process, either induced by acid, pepsin, or a combination. The combination of rheological properties, small-angle scattering (USANS, SANS), and confocal microscopy enhanced our understanding of milk coagulation. The whey proteins associated with the casein micelles promoted acid-induced and acid-pepsin-induced coagulation of milk, while hindered the pepsin-induced coagulation. Additionally, the influence of heat treatment on milk coagulation and curd degradation was monitored by decreasing the pH (2% GDL as an acidulant) and addition of pepsin (2000 U/mL). Faster aggregation and degradation of the milk curd was found in heated milk samples. Heat treatment resulted in a looser structure of the milk curd, accelerating the diffusion of pepsin and consequently faster protein degradation.

The coagulation behavior of cow, goat and sheep milk are different as well (**Chapter 7)**. Sheep milk coagulated at an earlier time when 64% κ-casein was hydrolyzed at pH 6.3, and when 59% κ-casein was hydrolyzed at pH 6.0, with the highest curd firmness and the densest structure. The coagulation of goat milk required the highest hydrolysis extent of κ-casein: 90% at pH 6.3 and 86% at pH 6.0. The curd formed from goat milk was softer (lower storage modulus) and the microstructure contained more large pores with fewer crosslinks between the protein aggregates compared to cow and sheep milk curds. Even with a similar protein content, the coagulation process for pepsin-induced curds on a nanoscale varies for different species of milk, according to the SANS results. Therefore, the different coagulation processes of the milk are not only due to the casein content, but also related to other physicochemical properties of different milk, e.g., genetic difference of κ-casein, size difference in casein micelles, as well as variations in mineralization in milk systems.

Overall, the fundamental insights generated from this study provide an improved understanding of the interactions between milk protein and the pepsin digestive enzyme. This includes hydrolysis of κ-casein, and the consequent coagulation of casein micelles under different conditions, including different environments (pepsin concentration, pH, temperature), pre-treatment on the milk (heat treatment), and the milk system (milk species). The effects of these variables on pepsin-induced hydrolysis of κ-casein and coagulation of milk proteins are summarized in **Table 9-1**. The results from this thesis expands our knowledge of the digestion of milk and potentially offer new possibilities for developing dairy products with particular digestion requirements for specific consumer groups. e.g., when considering the same quantity of milk, enhanced satiety through slower emptying could be a useful outcome. Alternatively, for athletes seeking accelerated nutrient release, expedited digestion could be beneficial.

Although, the hydrolysis of κ-casein and the consequent coagulation was carried out at a fixed pH and pepsin concentration, the results obtained from these experiments provides further understanding on the coagulation behavior of milk during the initial gastric stage of digestion. Factors including pepsin concentration, change of pH, and equilibration of temperature varies from person to person, and is impacted by the ingested volume and rate of the milk consumption. The HGS has been widely recognized as a useful tool to study the physical and chemical properties of milk in a simulated gastric environment. Using this model, the simulated gastric secretion (dynamic pH and enzyme concentration) can be precisely controlled. Digestion of milk proteins and the composition of chyme delivery into the small intestine highly depends on the curd structure in the gastric phase. In general, slower coagulation and a loose curd structure results in efficient diffusion of pepsin, and lead to a faster degradation and emptying rate of the milk protein.

Compared to milk at 37 and 50 °C, a slower coagulation rate was found at lower initial ingestion temperatures of milk (4 °C). Protein digestion was relatively faster for 4 °C milk, which was attributed to a softer structure during the early stages of digestion (**Chapter 4**). The pH plays a crucial role in influencing milk protein digestion. It not only affects the hydrolysis of κ-casein but also impacts upon coagulation properties. In the presence of pepsin, κ-casein hydrolysis occurs within the pH range of 5.3–6.7, with optimum activity at pH 6.0. At lower pH, aggregation occurs at a lower extent of hydrolysis of κ-casein. Moreover, the change in pH alters the distribution of calcium. The decrease in pH increases the ionic calcium and decreases calcium in the colloidal state (dissolution of CCP). As a result, a dense protein network with lower water holding capacity was found for the low pH samples. The effect of initial pH on the *in vitro* digestibility of milk proteins has not been previously explored. Lower pH during the initial stage may promote casein micelle aggregation. The resulting dense structure could hinder pepsin diffusion, thus slowing down the emptying rate of milk proteins from the stomach. However, rapid pH decreases could accelerate general pepsin hydrolysis activity. Therefore, achieving a balanced milk pH could facilitate milk protein emptying. Introducing lactic acid bacteria or other acidulants before ingestion could achieve a lower pH. Below a certain amount of calcium, the casein micelles only coagulated following induction by acid, at pH ~ 5 in the HGS. The addition of calcium salts facilitate the formation of curds at pH > 6. The curds formed from samples with higher [Ca^{2+}] had a much denser and firmer structure, with a slower release of proteins (**Chapter 6**). At a fixed amount of milk protein, a greater whey protein ratio has been reported to facilitate the emptying rate of milk proteins. As discussed in **Chapter 5**, adding whey proteins to casein micelles can impair the coagulation of unheated milk. Although, the associated whey proteins after heat treatment may facilitate the aggregation of the protein network at pH ≤ 6, the heat treatment impacts upon the pepsin-induced milk coagulation at relatively higher pH (> 6) (**Chapter 5)**. The relatively slower coagulation and looser curd structure in the heated sample could result in a greater rate of pepsin diffusion and faster degradation of the milk proteins (**Chapter 5** and **8)**. Consequently, the addition of whey proteins following heat treatment is one of the tools to facilitate the emptying rate of the casein proteins. The digestion of different species of milk varies and depends on the physicochemical properties of the milk. Consuming the same amount of skim milk, the emptying rate greatly depends on the structure of the milk protein curd. Fresh goat milk is considered to have faster digestibility, whereas sheep milk is associated with slower digestibility.

Table 9-1 Summary of the effects of some conditions and milk processing on pepsin-induced hydrolysis of κ-casein, coagulation of milk proteins and digestion of proteins.

Conditions		**Hydrolysis of κ-casein**	**Coagulation**		
			Coagulation time	**$H_{\mathrm{ct}}{}^{*}$ (%)**	**Curd properties**
Temperature (4-48 °C) (Chapter 4)	Cow skim milk pH 6.0, 1.10 U/mL pepsin	Faster hydrolysis rate of κ-casein at higher temperature. Extensive pepsin denaturation occurred at temperature > 40 °C. Therefore, optimum temperature is ~ 37−40 °C.	Shorter coagulation time at higher temperature. At very low temperatures (e.g., <25°C), no coagulation of casein occurs even if κ-casein is totally hydrolyzed.	Relatively lower degree of hydrolysis of κ-casein was required for coagulation at higher temperatures.	Larger aggregates and voids were observed in the curd formed at higher temperatures.
pH (5.3-6.7) (Chapter 3)	Cow skim milk at 37 °C, 1.10 U/mL pepsin	Denaturation of pepsin occurs at pH 6.7 and hydrolysis rate of κ-casein was optimal at pH ~ 6.0.	Coagulation time decreased as pH decreased from 6.7 to 5.3.	H_{ct} decreased markedly from ~ 73% to ~ 33% when pH decreased from 6.3 to 5.3.	Larger pores were observed within the sample at pH 5.3. At lower pH (< 6.0), rearrangement of the casein micelle network appears more extensive with an increased likelihood of whey separation than at higher pH.
Pepsin concentration (0.275-5.50 U/mL) (Chapter 3)	Cow skim milk at pH 6.0, 37 °C	Positive effect: greater hydrolysis rate of κ-casein at higher pepsin concentration.	Coagulation time decreased with increases in pepsin concentration. No coagulation occurs at very low pepsin activity.	The extent of κ-casein hydrolysis required for coagulation was independent of the pepsin concentration at a given pH.	The storge modulus was independent of pepsin concentration once the pepsin concentration is enough to induce coagulation.
Whey protein concentration (CN:WP ratios at 4:0, 4:0.6, 4:1, 4:2) ** (Chapter 5)	MC solution, pH 6.0, 37 °C, 1.10 U/mL pepsin	Hydrolysis of κ-casein was independent of whey protein concentration.	Coagulation time increased with increases in whey protein concentration.	The extent of κ-casein hydrolysis required for coagulation was independent of the whey protein concentration.	The microstructure became more heterogenous and less crosslinked for the whey protein enhanced sample. Higher whey protein concentration resulted in sparse and weak structures.

Heat-treatment (90°C for 5 min) (Chapter 5)	pH 6.0, 37 °C, 1.10 U/mL pepsin, WP-free; WP-containing samples	Heat treatment had little impact on hydrolysis in WP-free samples, but impaired the hydrolysis rate of κ-casein in WP-containing samples.	Heat treatment had little impact on coagulation in WP-free samples. Compared with unheated WP-containing sample at same pH, the coagulation process of the heated sample was retarded at pH 6.3 but enhanced at pH 6.0.	Lower H_{ct} was found in heated WP-containing samples.	The curd in heated WP-containing samples with smaller pores had higher water holding capacity.
Ca^{2+} (micellar casein system with addition of $CaCl_2$ from 0 to 22.5 m*M*) (Chapter 6)	pH 6.0, 37 °C, 1.10 U/mL pepsin	Hydrolysis of κ-casein was independent of $[Ca^{2+}]$.	Coagulation time decreased with increases in $[Ca^{2+}]$.	Lower H_{ct} for samples with higher $[Ca^{2+}]$.	Addition of $CaCl_2$ up to 17.5 m*M* resulted in stronger curd, maximum storage modulus (G'_{max}); further addition of $CaCl_2$ (22.5 mM) resulted in a lower G'_{max}.
Ionic strength (micellar casein system with addition of NaCl from 0 to 30mM) (Chapter 6)	pH 6.0, 37 °C, 1.10 U/mL pepsin	Hydrolysis of κ-casein was independent of ionic strength.	Coagulation time increased with increases in ionic strength.	Higher H_{ct} for samples with higher ionic strength.	The increased ionic strength up to 52.5 m*M* by adding NaCl retarded the coagulation process and resulted in a looser curd structure.
Milk species (cow, sheep, goat skim milk) (Chapter 7)	pH 6.3; pH 6.0, 37 °C, at the same pepsin to κ-casein ratio (0.1 U/mg)	Hydrolysis rate of κ-casein was found to be most pronounced in sheep milk, followed by cow milk and goat milk.	Sheep milk coagulated most rapidly, followed by cow milk and goat milk.	Sheep milk coagulated at lowest κ-casein hydrolysis extent; goat milk coagulation required the highest extent of κ-casein hydrolysis.	Sheep milk formed a curd with denser microstructure; the curds formed from goat milk exhibited a more porous microstructure.

[*] Critical hydrolysis degree for coagulation.

[**] CN:WP ratios at 4:0, 4:0.6, 4:1, 4:2 wt/vol while keeping the casein concentration constant.

9.2. Recommendations for future work

9.2.1. Investigation of the coagulation and digestion behavior of heat-treated milk

The samples used in **Chapter 3**, **Chapter 4,** and **Chapter 7** were raw fresh milk collected from a dairy farm. The results provide some fundamental knowledge about the raw milk coagulation and digestion. The milk pH, pepsin concentration, reaction temperature had a significant impact the coagulation behavior of raw milk and therefore the consequent digestion behavior. However, for safety reasons, pasteurized or UHT milk is more commonly consumed. Heat treatment involved in these processes leads to whey protein denaturation and association with casein micelles. Therefore, the effect of milk pH, pepsin concentration and temperature on heated milk is recommended for the future studies.

9.2.2. Effect of various cations on the pepsin-induced milk coagulation and digestion behavior

Several minerals including calcium, iron, magnesium, and zinc, are often included in milk, infant formula, and dairy products to enhance their nutritional value. **Chapter 7** explored the role of Ca^{2+} in pepsin-induced milk coagulation and on the *in vitro* digestion behavior of milk. It was found that the divalent nature of calcium can strengthen the connection between casein micelles through calcium bridges. Considering these findings, it would be valuable to investigate the impact of other divalent ions (such as Fe, Mg, Zn) on the coagulation and digestion behavior of milk and infant formulae. By gaining insights into how divalent cations affect the coagulation and digestion processes, an enhanced understanding of the behavior of fortified dairy products can be obtained. This knowledge has the potential to inform more effective fortification strategies and improve the nutritional outcomes.

9.2.3. Investigation of the coagulation and digestion behavior of animal-free milk and plant hybrid milk

With a growing awareness towards sustainability, so-called alternative proteins, such as insect proteins, plant proteins, cultured proteins, algal proteins, etc., have become increasingly desirable. The consumption of these alternative protein sources is considered as a future trend and a viable strategy that could potentially contribute to global food security. This highlights the importance of understanding the digestibility, structural changes, and macronutrient accessibility of these alternative proteins. For the enhanced development of food based on such proteins, more investigation on the interaction between alternative proteins and pepsin, and associated digestion behavior, should be conducted.

As dairy products are a source of high quality protein and many micronutrients, synthesizing nature-identical milk proteins has been considered as a possible solution to replace milk proteins (Hettinga & Bijl, 2022). A previous study reported that using only (human) β-casein and (bovine) κ-casein was sufficient to produce reassembled casein micelles (Sood, Erickson, & Slattery, 2002). However, there is currently no literature available that investigates the interaction between pepsin and casein/whey proteins derived from non-animal sources. Exploring such interactions would be of interest and could provide valuable insights.

Another increased interest lies in food products that combine animal and plant proteins, as provide potential nutritional, sensory, and sustainability benefits, e.g., hybrid milk (e.g., novel blended products containing oat and bovine milk). Partial replacement of dairy proteins by plant proteins in formulated dairy products appears as a viable alternative. Alves & Tavares (2019) have suggested that mixed animal and plant protein systems are very efficient in modulating the texture of protein gels, the formation of low-cost edible films and the stabilization of emulsions and foams. It has been reported that the coagulation of oat milk–bovine skim milk blend slowed the gastric emptying of macronutrients, leading to a delay in the release of protein and lipids into the small intestine compared with oat milk (Wang et al., 2022). However, limited information on the coagulation and digestion of such mixed protein systems has been reported. Further research in this area could have significant implications for the development of alternative protein sources and the applications in various food products.

9.2.4. The effect of fat and homogenization on pepsin-induced milk coagulation

Fresh whole milk contains fat globules distributed in a range of diameters from 0.1 to 10 μm. Native fat globules are known to weakly interact with the casein proteins structure. However, the effect of the presence of fat globules on pepsin catalysis of κ-casein has not yet been explored. It would be worthwhile to study the effect of varying fat levels while maintaining constant casein protein content on pepsin-induced hydrolysis and coagulation of casein micelles.

Two-stage high pressure homogenization is commonly carried out to reduce the size of fat globules in milk. During homogenization, casein micelles are adsorbed to the interface of fat globules, which increases the effective casein surface area in milk. The greater surface area onto which the casein micelle can adsorb will change the susceptibility of κ-casein to enzymatic hydrolysis. In addition, homogenization alters the size and interfacial composition of fat globules, therefore the contribution to the milk clots are expected to be different. In *in vivo* and *in vitro* digestion studies, homogenization has been shown to also lead to softer and smaller curds and improved digestibility compared to unhomogenized milk (Ye et al., 2017). Therefore, more information of the presence of fat globules and homogenization on milk coagulation and digestion behavior is required.

9.2.5. Investigation of the role of whey proteins on pepsin-induced milk coagulation using small angle neutron scattering techniques

Chapter 5 reported that the whey protein concentration and heat-treatment significantly impact casein coagulation. A lower casein:whey protein (CN:WP) ratio resulted in an increased coagulation time and decreased firming rate on pepsin-induced milk curd. It has hypothesized that, when κ-casein is hydrolyzed on the casein micelle surface, the whey proteins are sufficiently small to occupy gaps, providing a physical barrier between para-casein micelles. When the milk is heated, it is generally accepted that the aggregation phase is retarded due to steric hindrance arising from the binding of β-lactoglobulin to κ-casein. Therefore, the ratio of CN:WP and heat-treatment plays an important role in the digestion of dairy products.

SANS, USANS and SAXS techniques were employed to access a broad range of length scales to study this hierarchical system, i.e., USANS for employed to examine protein aggregates in a milk clot structure; the structure of casein micelles after the addition of pepsin in cow skim milk and the colloidal calcium phosphate nanocluster (CCP) features were highlighted using SANS; and SAXS was used to study the casein micelle substructure during digestion, including protein inhomogeneities (2–3 nm). It would be valuable to further apply these three techniques to monitor the structural formation, aggregation, and disintegration of the milk proteins and probe the role of native and denatured whey protein on the curd structure. The pertinent research areas of such a study should be: (i) the structure of casein micelles after the addition of pepsin in a WP-free micellar casein system; (ii) the effect of CN:WP ratio on the structure of the clots; (iii) the role of denatured whey proteins on the structure of the clots at pH 6.3 (relatively high) and 5.7 (closer to PI of whey proteins).

9.2.6. Future potential use of neutron scattering techniques

More work should be pursued utilizing neutron scattering techniques (SANS and USANS) with tandem methods. Accompanied by real-time monitoring of parameters, such as turbidity or in-situ rheometry (i.e., rheo-SANS), these parallel measurements could yield a comprehensive understanding of the structural transformations and interactions within milk proteins during digestion. Furthermore, to advance our comprehension of milk protein digestion, it is advisable to establish a link between an *in vitro* digestion unit or pH-stat and neutron scattering techniques. This integration should offer deeper insights into the dynamic changes unfolding during the digestion process of milk proteins.

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