



## Kinetics of pepsin-induced hydrolysis and the coagulation of milk proteins

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### 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  $\kappa$ -casein and the coagulation of the casein micelles in bovine skim milk was investigated at 37°C using reverse-phase HPLC, oscillatory rheology, and confocal laser scanning microscopy. The hydrolysis of  $\kappa$ -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  $\kappa$ -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 with high pH and low pepsin concentration.

**Key words:** pepsin, milk coagulation, rheology, microstructure, enzymatic hydrolysis kinetics

### 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, 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 ~2,000 U/mL (Gao et al., 2002; Minekus et al., 2014; Ye et al., 2016, 2017). Both in vitro and in vivo studies on the digestion of milk have found that the milk proteins coagulate at an 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; Ye et al., 2016; Huppertz and Chia, 2021). 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 and Pelissier, 1981; Ye et al., 2017; van Lieshout et al., 2020).

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

As the first 2 overlapping phases broadly resemble the mechanisms occurring in cheese manufacture,

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the coagulation of milk induced by pepsin could be similar to that induced by chymosin. Over the years, chymosin-initiated hydrolysis of  $\kappa$ -CN and the aggregation of the casein micelles in milk have been extensively studied (Dalglish, 1980, 1988; van Hooydonk, 1987). Chaplin and Green (1980) demonstrated that chymosin hydrolyzes the Phe<sup>105</sup>–Met<sup>106</sup> 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  $\kappa$ -CN hydrolysis catalyzed by pepsin in milk systems have not been fully elucidated. Carlson et al. (1987a) briefly reported on hydrolysis of  $\kappa$ -CN by pepsin with that by rennet, but the effects of the concentration of pepsin and the pH on  $\kappa$ -CN hydrolysis have not been studied in detail.

After a minimum amount of  $\kappa$ -CN has been hydrolyzed, the casein micelles begin to aggregate (Dalglish, 1979), leading to changes in the physicochemical properties of the milk which can be monitored using various techniques (Everett and Auty, 2008), such as confocal laser scanning microscopy (Auty et al., 1999), 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- $\delta$ -lactone (**GDL**, used to obtain a gradual decrease in pH) and porcine pepsin (Roy et al., 2020). 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 of milk) and pH (5.3–6.7) on protein hydrolysis and the aggregation characteristics of skim milk. The amount of para- $\kappa$ -CN released by hydrolysis was determined by reverse-phase HPLC, and the rheological properties and microstructural characteristics of coagulum were examined using oscillatory rheology and confocal laser scanning microscopy, respectively. Using the above approach, an enhanced understanding was gained of the action of pepsin on  $\kappa$ -CN in fresh bovine skim milk.

## MATERIALS AND METHODS

### 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) at  $3,000 \times g$  and 4°C for 15 min. The skim milk contains 0.12% fat, 4.31% protein, and 4.67% lactose as determined by a Milkoscan FT120 (Foss Electric). 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 of protein was obtained from Sigma-Aldrich. 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 5 pepsin concentrations: 2,750, 1,100, 550, 275, and 110 U/mL. The diluted pepsin solutions were added to the skim milk samples at a ratio of 1  $\mu$ L 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 of 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.

### Measurement of $\kappa$ -CN Hydrolysis

Reverse-phase HPLC was used to quantify para- $\kappa$ -CN. A 2- $\mu$ L 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. A HPLC buffer solution (0.8 mL: 6 M guanidinium hydrochloride, 0.1 M Bis-Tris, 19.5 mM DL-dithiothreitol, and 5.37 mM 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.

Reverse-phase HPLC analysis, based on previous methods established by Bonfatti et al. (2013) and Day

et al. (2015) with some modifications, was carried out using a Nexera-X2 ultra-HPLC instrument equipped with an SPD-M20A diode array detector (Shimadzu). Separation was carried out using a Phenomenex Aeris Widepore XB-C18 column (100 mm × 4.6 mm, 3.6- $\mu$ m 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  $\mu$ L 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 to 2.5 min, isocratic conditions, 10% B; 2.5 to 22 min, 10% to 49% B; 22 to 23 min, 49% to 10% B; 23 to 30 min, isocratic conditions, 10% B. The amount of para- $\kappa$ -CN was quantified using LabSolutions Main (Shimadzu Corp.) software based on peak areas.

### Measurement of Protein Coagulation

The coagulation process was carried out in a stress-controlled rheometer (MCR301 Anton Paar) 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  $\mu$ L) 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.

### Microstructural Characterization of Coagulum

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

### Statistical Analysis

Samples were prepared in triplicate and rheological measurements of each sample were carried out in triplicate. Ten time points were examined by reverse-phase HPLC and 4 of them (1, 10, 50, and 120 min) were examined in triplicate. The ANOVA tests were carried out using Prism 8 (GraphPad Software Inc.) to determine the significance of the differences. Differences were significant at  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Kinetics of $\kappa$ -CN Hydrolysis by Pepsin

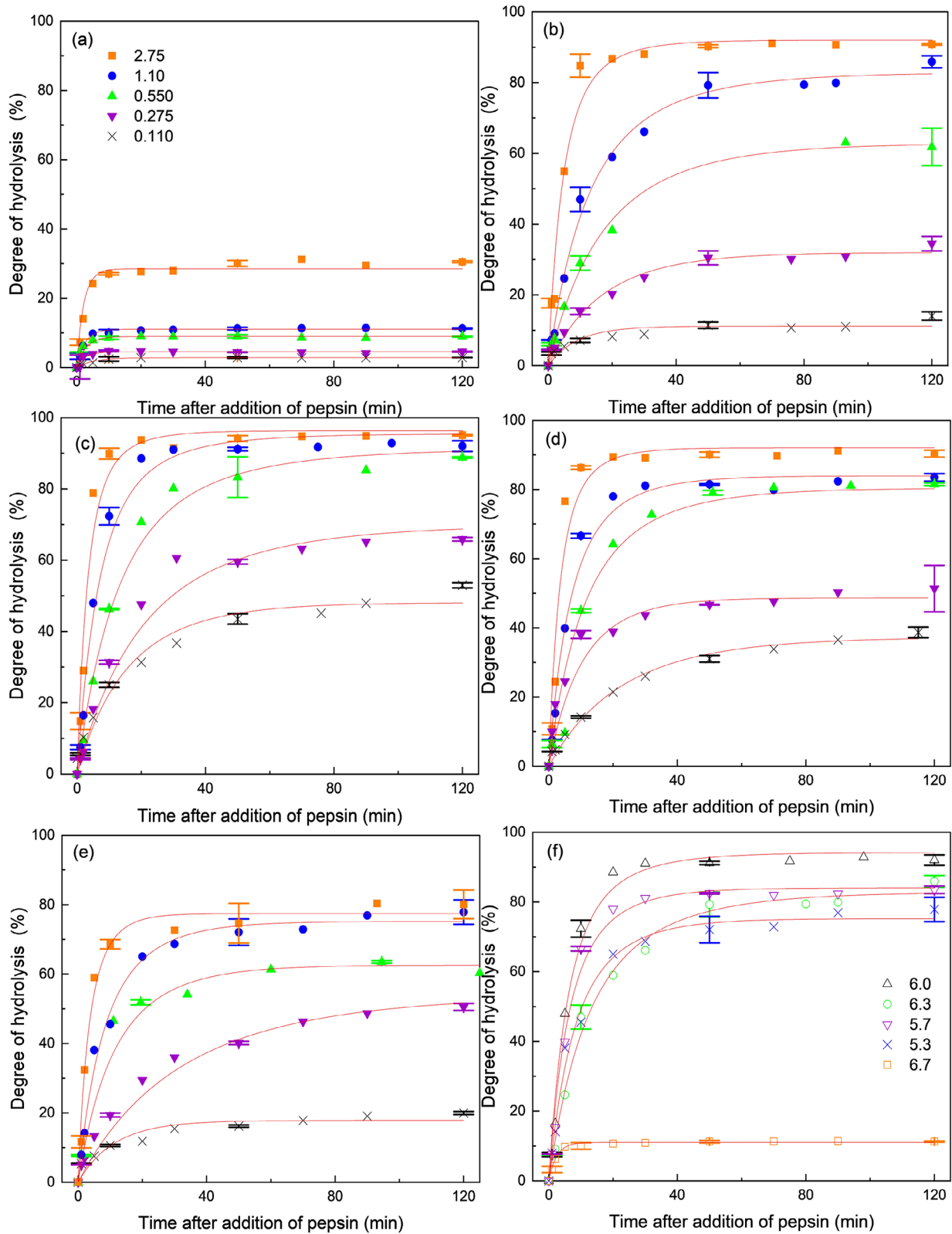
The degree of hydrolysis of the  $\kappa$ -CN was determined by comparing the peak area of para- $\kappa$ -CN released at each time point with the peak area of the completely hydrolyzed milk (a sample treated with 2.75 U/mL pepsin at pH 6.0 for 8 h; Gastaldi et al., 2003; Liu et al., 2014). This is expressed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

$$H_t = 100 \times P_t/P_{tot}, \quad [1]$$

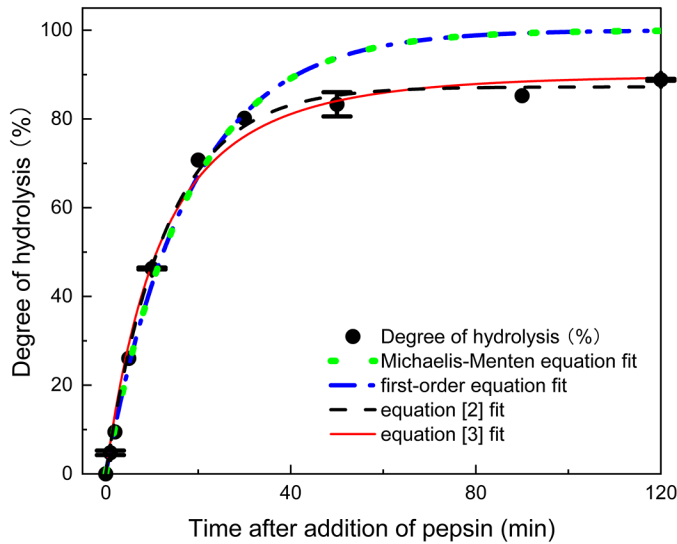
where  $H_t$  is the percent hydrolysis of  $\kappa$ -CN at time  $t$ ,  $P_t$  is the peak area of para- $\kappa$ -CN released at time  $t$ , and  $P_{tot}$  is the peak area of para- $\kappa$ -CN for a completely hydrolyzed sample. The  $\kappa$ -CN peaks almost completely disappeared after holding for a period of 8 h at pH 6.0 in the presence of 2.75 U/mL pepsin (Supplemental Figure S1, [https://figshare.com/articles/figure/supplemental\\_figures\\_pdf/17009372/1](https://figshare.com/articles/figure/supplemental_figures_pdf/17009372/1); Yang, 2021), and this was designated as a sample where complete hydrolysis had taken place. In the presence of pepsin at concentrations of 0.110 to 2.75 U/mL, the degree of hydrolysis of  $\kappa$ -CN in skim milk at pH 6.7 to 5.3 increased with increasing reaction times, as plotted in Figure 1(a)–(e).

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

$$H_t = H_{120} \cdot [1 - \exp(-K_2 \cdot t)], \quad [2]$$



**Figure 1.** Degree of hydrolysis of  $\kappa$ -CN 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 of milk) at 37°C and (a) pH 6.7, (b) pH 6.3, (c) pH 6.0, (d) pH 5.7, and (e) pH 5.3, and (f) degree of hydrolysis of  $\kappa$ -CN in skim milk with time after the addition of pepsin at 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 solid curves are the fits of equation [3]. Error bars represent SD from triplicate.



**Figure 2.** Degree of hydrolysis of  $\kappa$ -CN in skim milk at pH 6.0 in 120 min with a pepsin concentration of 0.550 U/mL (black dots). The green dotted curve is the Michaelis-Menten equation fit, the blue dashed-dotted curve is according to the first-order equation, the black dashed curve is according to equation [2], and the red solid curve is according to equation [3]. Error bars represent SD from triplicate.

where  $K_2$  ( $\text{min}^{-1}$ ) is the reaction rate constant and  $H_{120}$  is the degree of hydrolysis of  $\kappa$ -CN at 120 min. With a calculated  $K_2$  of  $0.08 \text{ min}^{-1}$  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 2.  $H_{120}$  was lower than 100%, which means that  $\kappa$ -CN 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  $\kappa$ -CN due to a pH greater than 5, and denaturation could be complete before there is complete hydrolysis of the  $\kappa$ -CN. This enzyme denaturation also occurs in chymosin-catalyzed proteolysis of  $\kappa$ -CN according to van Hooydonk (1987), who proposed an overall reaction that was derived from a combination of first-order proteolysis and first-order enzyme denaturation:

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

where  $H_t$  is the percent hydrolysis of  $\kappa$ -CN at time  $t$ ,  $K_{den}$  ( $\text{min}^{-1}$ ) is the reaction rate constant for the dena-

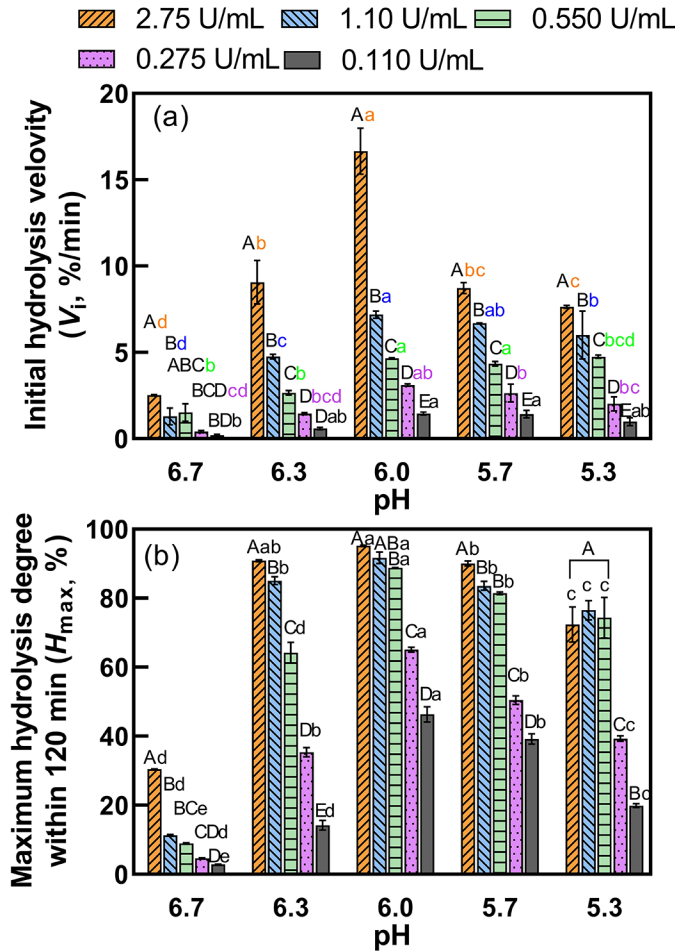
**Table 1.** Hydrolysis kinetics of  $\kappa$ -casein in bovine skim milk at different pH values and pepsin concentrations<sup>1</sup>

Pepsin concentration (U/mL)	Milk pH	According to equation [2]			According to equation [3]		
		Enzymatic reaction rate constant $K_2$ ( $\text{min}^{-1}$ )	Hydrolysis degree at 120 min ( $H_{120}$ ) (%)	$R^2$	Enzymatic reaction rate constant $K$ ( $K_{enz} \cdot C$ ) ( $\text{min}^{-1}$ )	Denaturation rate constant ( $K_{den}$ ) ( $\text{min}^{-1}$ )	$R^2$
2.75	6.7	$0.292 \pm 0.028^{A,b}$	$29.32 \pm 0.28^{C,a}$	0.989	$0.104 \pm 0.032^{C,a}$	$0.230 \pm 0.194^{A,cd}$	0.964
	6.3	$0.228 \pm 0.036^{A,ab}$	$91.16 \pm 0.71^{A,a}$	0.987	$0.218 \pm 0.046^{B,a}$	$0.083 \pm 0.014^{AB,a}$	0.980
	6	$0.239 \pm 0.017^{A,a}$	$95.29 \pm 0.63^{A,a}$	0.982	$0.258 \pm 0.017^{A,a}$	$0.074 \pm 0.013^{B,a}$	0.973
	5.7	$0.225 \pm 0.019^{A,a}$	$91.44 \pm 0.19^{A,a}$	0.972	$0.237 \pm 0.015^{A,a}$	$0.092 \pm 0.007^{B,a}$	0.961
	5.3	$0.212 \pm 0.049^{A,a}$	$78.29 \pm 3.53^{B,a}$	0.987	$0.190 \pm 0.034^{B,a}$	$0.123 \pm 0.033^{B,a}$	0.985
1.10	6.7	$0.403 \pm 0.045^{A,a}$	$11.41 \pm 0.37^{D,b}$	0.996	$0.046 \pm 0.005^{C,b}$	$0.377 \pm 0.040^{A,ab}$	0.991
	6.3	$0.324 \pm 0.420^{AB,a}$	$82.50 \pm 1.30^{B,b}$	0.990	$0.072 \pm 0.012^{C,b}$	$0.038 \pm 0.008^{B,a}$	0.989
	6	$0.138 \pm 0.001^{B,a}$	$92.58 \pm 0.37^{A,a}$	0.995	$0.146 \pm 0.001^{A,b}$	$0.053 \pm 0.002^{B,a}$	0.990
	5.7	$0.145 \pm 0.005^{B,a}$	$82.76 \pm 0.73^{A,b}$	0.994	$0.131 \pm 0.019^{AB,b}$	$0.072 \pm 0.012^{B,a}$	0.983
	5.3	$0.122 \pm 0.014^{B,a}$	$74.05 \pm 3.56^{C,a}$	0.989	$0.104 \pm 0.010^{B,b}$	$0.076 \pm 0.013^{B,a}$	0.992
0.550	6.7	$0.418 \pm 0.170^{A,a}$	$8.26 \pm 0.22^{D,bc}$	0.993	$0.029 \pm 0.005^{B,bc}$	$0.318 \pm 0.055^{A,abc}$	0.932
	6.3	$0.056 \pm 0.005^{B,b}$	$68.72 \pm 5.82^{C,c}$	0.993	$0.060 \pm 0.011^{AB,bc}$	$0.037 \pm 0.003^{B,a}$	0.995
	6	$0.075 \pm 0.003^{B,a}$	$86.51 \pm 2.89^{A,b}$	0.997	$0.072 \pm 0.003^{A,c}$	$0.033 \pm 0.003^{B,a}$	0.993
	5.7	$0.079 \pm 0.004^{B,a}$	$80.64 \pm 0.56^{B,b}$	0.976	$0.059 \pm 0.019^{AB,c}$	$0.054 \pm 0.023^{B,a}$	0.967
	5.3	$0.088 \pm 0.016^{B,a}$	$64.87 \pm 1.94^{C,b}$	0.988	$0.067 \pm 0.008^{A,c}$	$0.056 \pm 0.016^{B,a}$	0.975
0.275	6.7	$0.606 \pm 0.007^{A,a}$	$4.20 \pm 0.19^{E,cd}$	0.977	$0.013 \pm 0.005^{A,bc}$	$0.298 \pm 0.119^{A,bcd}$	0.750
	6.3	$0.060 \pm 0.002^{B,b}$	$33.89 \pm 1.67^{C,d}$	0.982	$0.020 \pm 0.001^{A,cd}$	$0.051 \pm 0.001^{B,a}$	0.985
	6	$0.056 \pm 0.012^{B,a}$	$65.87 \pm 0.45^{A,c}$	0.976	$0.035 \pm 0.003^{A,d}$	$0.031 \pm 0.004^{B,a}$	0.986
	5.7	$0.049 \pm 0.014^{B,a}$	$54.30 \pm 6.71^{B,c}$	0.963	$0.022 \pm 0.003^{A,d}$	$0.026 \pm 0.007^{B,a}$	0.968
	5.3	$0.047 \pm 0.009^{B,a}$	$40.09 \pm 2.69^{D,c}$	0.979	$0.018 \pm 0.002^{A,d}$	$0.032 \pm 0.006^{B,a}$	0.969
0.110	6.7	$0.400 \pm 0.229^{A,a}$	$2.88 \pm 0.11^{D,cd}$	0.979	$0.012 \pm 0.005^{A,c}$	$0.438 \pm 0.170^{A,a}$	0.964
	6.3	$0.074 \pm 0.025^{B,b}$	$13.18 \pm 0.70^{C,e}$	0.729	$0.012 \pm 0.001^{A,d}$	$0.093 \pm 0.020^{B,a}$	0.863
	6	$0.046 \pm 0.016^{B,a}$	$45.88 \pm 2.36^{A,d}$	0.960	$0.019 \pm 0.003^{A,d}$	$0.030 \pm 0.007^{B,a}$	0.993
	5.7	$0.042 \pm 0.007^{B,a}$	$38.12 \pm 1.95^{B,d}$	0.987	$0.017 \pm 0.002^{A,d}$	$0.035 \pm 0.006^{B,a}$	0.990
	5.3	$0.092 \pm 0.011^{B,a}$	$17.98 \pm 0.54^{C,d}$	0.897	$0.017 \pm 0.002^{A,d}$	$0.086 \pm 0.011^{B,a}$	0.901

<sup>A-E</sup>Mean values between samples at same pepsin concentration in the same column with different superscripts are significantly different ( $P < 0.05$ ).

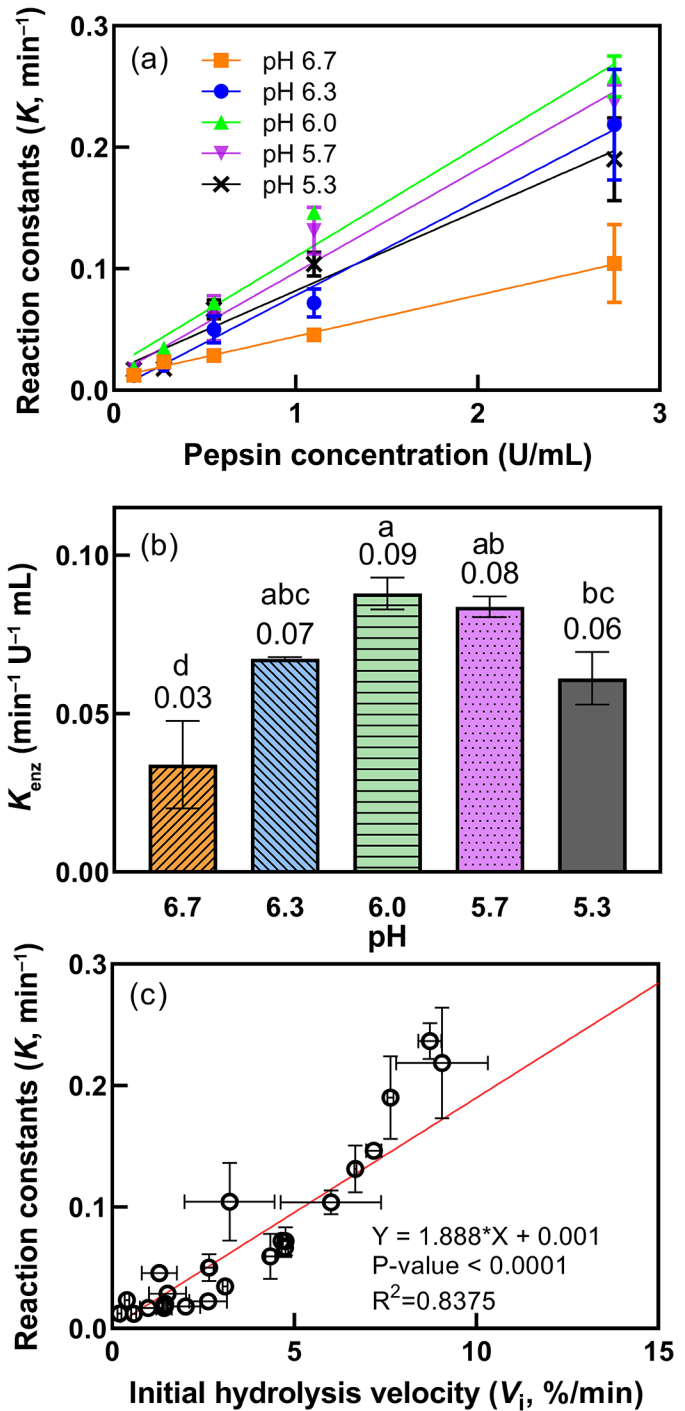
<sup>a-e</sup>Mean values between samples at same pH in the same column with different superscripts are significantly different ( $P < 0.05$ ).

<sup>1</sup>Results are expressed as the mean  $\pm$  SD of the mean ( $n = 3$ ).



**Figure 3.** (a) Initial hydrolysis velocity ( $V_i$ , %/min) and (b) maximum hydrolysis percent within 120 min for samples at 6.7 to 5.3 when the concentrations of pepsin were 0.110 to 2.75 U/mL (orange, 2.75 U/mL; blue, 1.10 U/mL; green, 0.550 U/mL; purple, 0.275 U/mL; and gray, 0.110 U/mL). Mean values between samples at same pH with different uppercase letters (A–E) are significantly different ( $P < 0.05$ ). Mean values between samples at same pepsin concentration with different lowercase letters (a–e) are significantly different ( $P < 0.05$ ). Error bars represent SD from triplicate.

turation reaction,  $C$  (U/mL) is the pepsin concentration,  $K_{enz}$  ( $\text{min}^{-1} \text{U}^{-1} \text{mL}$ ) is the reaction rate constant for the enzymatic reaction per unit quantity of enzyme solution, and  $K_{enz} \cdot C$  is defined as the overall reaction rate constant  $K$  ( $\text{min}^{-1}$ ). This equation describes the experimental data well, in which proteolysis and denaturation take place simultaneously, as shown in Figure 2 (red solid curve). Both equations [2] and [3] were used for fitting the data in Figure 1, yielding the parameters shown in Table 1. However, the  $K_2$  values cannot reflect the initial hydrolysis velocities [ $V_i$  (%/min)] as determined by linear regression of the first 4 time points of the degree of hydrolysis [Figure 3(a)] as, for example,



**Figure 4.** (a) Enzymatic reaction rate constant  $K$  ( $K_{enz} \cdot C$ ,  $\text{min}^{-1}$ ) as a function of pepsin concentration (0.110–2.75 U/mL) at  $\blacksquare$  pH 6.7,  $\bullet$  pH 6.3,  $\blacktriangle$  pH 6.0,  $\blacktriangledown$  pH 5.7, and  $\times$  pH 5.3. (b)  $K_{enz}$  ( $\text{min}^{-1} \text{U}^{-1} \text{mL}$ ), calculated from the slopes of the curves in (a) at (orange) pH 6.7, (blue) pH 6.3, (green) pH 6.0, (purple) pH 5.7, and (gray) pH 5.3. (c) Reaction constants ( $K$ ,  $\text{min}^{-1}$ ) as a function of the initial hydrolysis velocity ( $V_i$ , %/min). Mean values with different lowercase letters (a–d) are significantly different ( $P < 0.05$ ). Error bars represent SD from triplicate.

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  $0.292 \pm 0.028$ , lower than  $0.403 \pm 0.045$  for 1.10 U/mL. Therefore, equation [3] was selected in this study as the best method to calculate the degree of hydrolysis of  $\kappa$ -CN at specific times during coagulation. For this reason, the degrees of hydrolysis are reported by the red solid curves in Figure 1.

**Effect of Pepsin Concentration on the Hydrolysis of  $\kappa$ -CN.** According to Figure 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  $K$  ( $K_{enz} \cdot C$ ,  $\text{min}^{-1}$ ; Table 1) increased as pepsin concentration increased, and there was a positive linear correlation between  $K$  and the pepsin concentration [Figure 4(a)], which was found at all pH values. The slope of the curve, equal to  $K_{enz}$ , is shown in Figure 4(b). By plotting the reaction constants ( $K$ ) as a function of the initial velocities ( $V_i$ ) [Figure 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  $V_i$ .

The pepsin denaturation rate constants ( $K_{den}$ ; Table 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 3b). 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.

**Effect of pH on the Hydrolysis of  $\kappa$ -CN.** As shown in Figure 3(b),  $H_{max}$  values over 120 min were below 100%. According to van Hooydonk (1987),  $\kappa$ -CN cannot be totally hydrolyzed by chymosin because of the denaturation of this enzyme at  $\text{pH} > 7.0$ ; however,  $K_{den} > 0$  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 (Andr n, 2011), the milk-clotting activity of porcine pepsin is more pH dependent than chymosin. Andr n (2011) reported that the porcine pepsin is sensitive to inactivation that denatures rapidly at  $\text{pH} \sim 6.5$  and temperature  $\sim 30^\circ\text{C}$  (when used for cheesemaking), and only 50% of its milk-clotting activity remains after 1 h. Consequently, denaturation of pepsin could have occurred in the pH range of 6.7 to 5.3 in this study.

The hydrolysis processes at pH 6.7 to 5.3 in the presence of pepsin at a concentration of 1.10 U/mL

are shown in Figure 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 with other pH values (Table 1). In addition, the sample at pH 6.7 had lower  $K$  values [Table 1, Figure 4(a)],  $V_i$  values [Figure 3(a)] and  $K_{enz}$  values [Figure 4(b)]. As both the casein micelles (pI 4.6) and pepsin (pI 2.7) are negatively charged in the pH range 6.7 to 5.3 (Herriott et al., 1940; McMahon and Brown, 1984), a decrease in pH increases the ionic strength (due to the dissolution of colloidal calcium phosphate (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 4(b)], which indicates that the optimal pH for pepsin-induced hydrolysis of  $\kappa$ -CN 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  $\kappa$ -CN Phe<sup>105</sup>–Met<sup>106</sup> bond for chymosin might be a function of the protrusion of the caseinomacropptide part of  $\kappa$ -CN into the serum, which may also be the case for pepsin accessibility. The protrusion of caseinomacropptide 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  $\kappa$ -CN (van Hooydonk, 1987). 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 2 opposite effects on  $K_{enz}$  could also explain an optimal pH for pepsin-induced hydrolysis of  $\kappa$ -CN at pH 6.0.

### Pepsin-Induced Milk Coagulation

The increase in the storage modulus ( $G'$ ) of skim milk after the addition of pepsin is shown in Figure 5(a)–(e), representing the samples at pH values 6.7 to 5.3 (the loss modulus,  $G''$ , was also recorded, and Supplemental Figure S2 ([https://figshare.com/articles/figure/supplemental\\_figures\\_pdf/17009372/1](https://figshare.com/articles/figure/supplemental_figures_pdf/17009372/1); Yang, 2021) 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 for samples

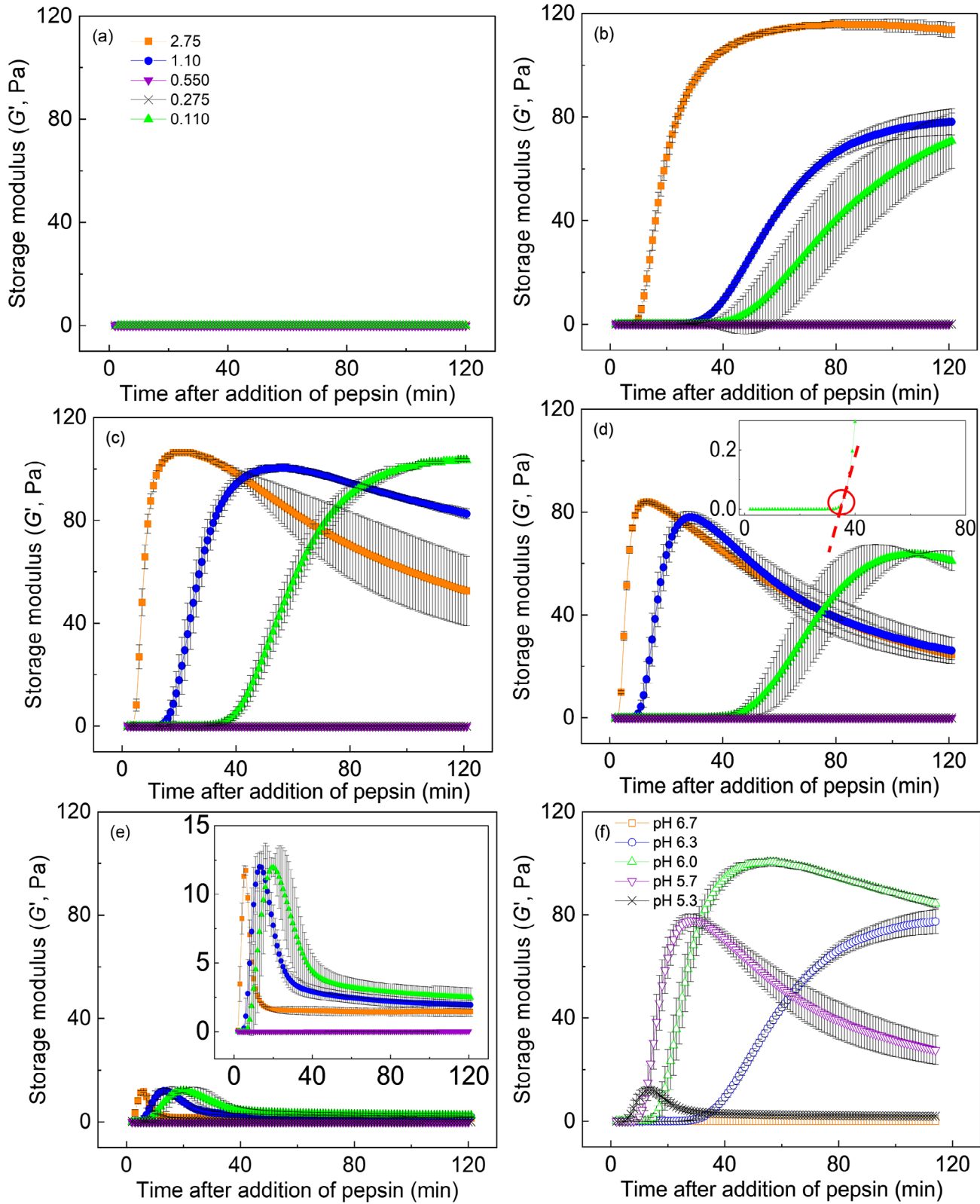
at pH 6.7, the  $G'$  versus time curves showed 3 stages, depending upon the concentration of pepsin and the pH: (1) in the early stages of coagulation, the sample was still in liquid phase that  $G'$  showed a lag phase; (2) as time progressed,  $G'$  increased significantly with increasing hydrolysis time; (3) in the later stages,  $G'$  reached a plateau for the samples at pH 6.3 [Figure 5(b)] or decreased for the samples at pH values 6.0 to 5.3 [Figure 5(c)–(e)]. For the second stage, after hydrolysis of  $\kappa$ -CN, steric repulsion due  $\kappa$ -CN is reduced and the destabilized para-CN 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. In addition, the protein network density between para-CN 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 and Dalgleish, 2006) or the point at which  $G'$  is greater than 1 Pa (Meletharayil et al., 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 5(d), a linear regression can be applied to the first 4 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 ( $dG'/dt$ ) is an indication of the curd firming rate (Guinee et al., 1997; Panthi et al., 2019).

**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 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 6(a)]. The degree of hydrolysis of  $\kappa$ -CN 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  $\kappa$ -CN 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 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  $\kappa$ -CN that

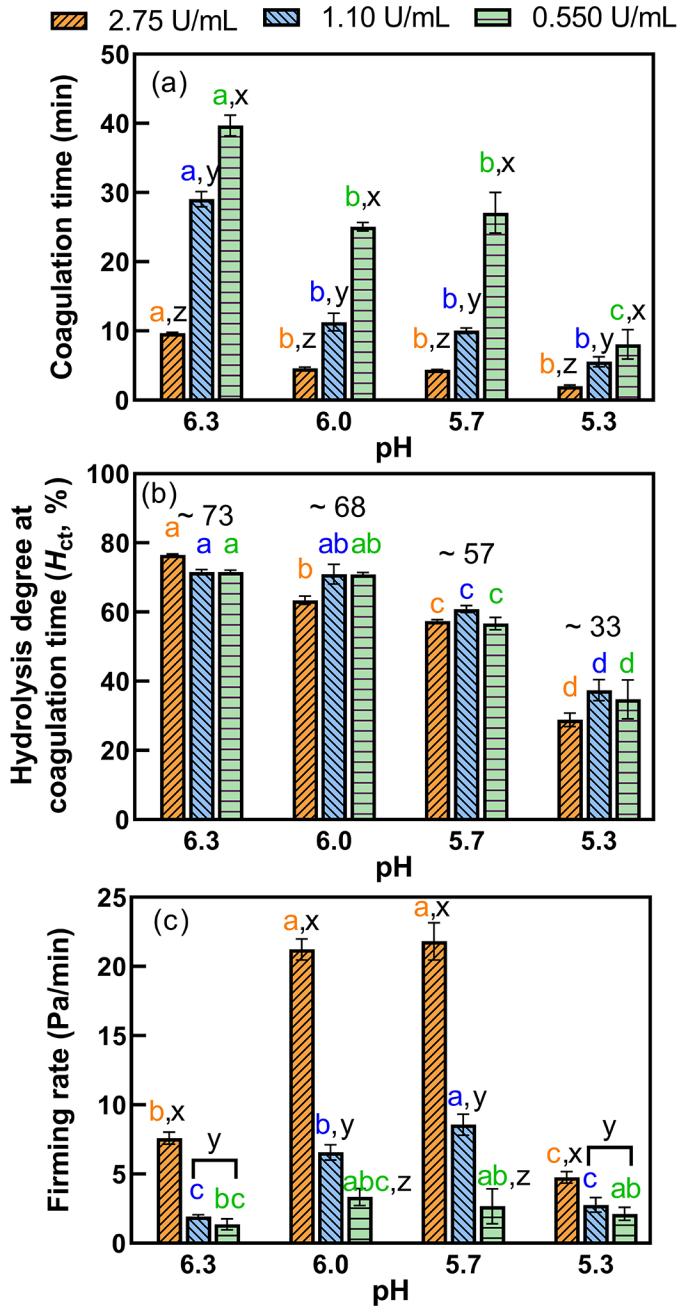
is required for coagulation seems to be independent of the pepsin concentration for a fixed pH. In the presence of a higher pepsin concentration (2.75 U/mL), with a higher reaction rate constant  $K$ , it took less time to reach the critical degree of hydrolysis (~68% at pH 6.0), which explains the shorter 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  $\kappa$ -CN within 120 min.

As shown in Figure 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 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 10° from 20°C to 30°C) of the hydrolysis phase compared with the aggregation phase (Dalgleish, 1979; van Hooydonk, 1987). However, according to Bringe and Kinsella (1986), the curd firming rate is determined not only by para-CN micelle aggregation but also by the initial rate of hydrolysis reaction because of the influence of the enzymatic reaction on aggregation; for example, for the samples at pH 6.0, coagulation occurred when 68% of the para- $\kappa$ -CN had been released ( $H_{ct}$  was ~68%), and further increase in  $G'$  requires additional unstable para-CN micelles (the limiting step of  $dG'/dt$  could shift to the further hydrolysis of  $\kappa$ -CN). The higher hydrolysis reaction constant ( $K$ ) at higher pepsin concentrations led to more unstable para-CN micelles over time, and this is shown by the increase in the firming rate (Carlson et al., 1987b).

**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 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 6. As shown in Figure 6(a), the coagulation time decreased as pH decreased. When the coagulation time was substituted into equation [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 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,c; van Hooydonk,

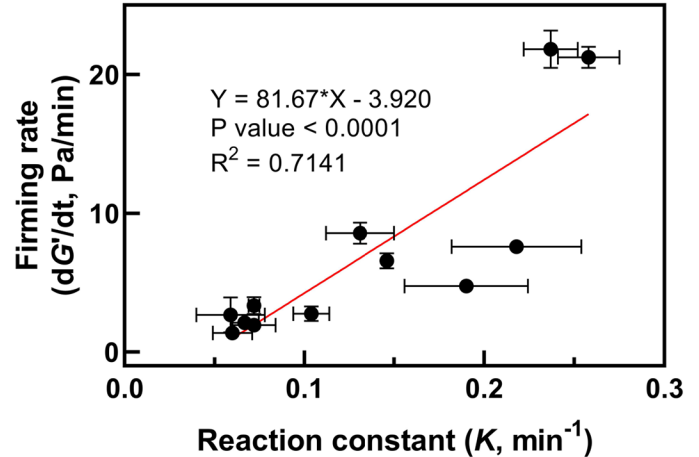


**Figure 5.** Storage modulus ( $G'$ ) of samples as a function of pepsin concentration (■ 2.75 U/mL, ● 1.10 U/mL, ▲ 0.550 U/mL, ▼ 0.275 U/mL, and × 0.110 U/mL) at 37°C: (a) pH 6.7, (b) pH 6.3, (c) pH 6.0, (d) pH 5.7, with an inset to show coagulation time (details are described in text); and (e) pH 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). Error bars represent SD from triplicate.



**Figure 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 to 5.3: (orange: 2.75 U/mL, blue: 1.10 U/mL, and green: 0.550 U/mL). Mean values between samples at same pH with different uppercase letters (X–Z) are significantly different ( $P < 0.05$ ). Mean values between samples at same pepsin concentration with different lowercase letters (a–d) are significantly different ( $P < 0.05$ ). Error bars represent SD from triplicate.

1987); this was due to partial charge neutralization of the negatively charged para-CN micelles. The decrease in the magnitude of repulsion forces and increased hy-

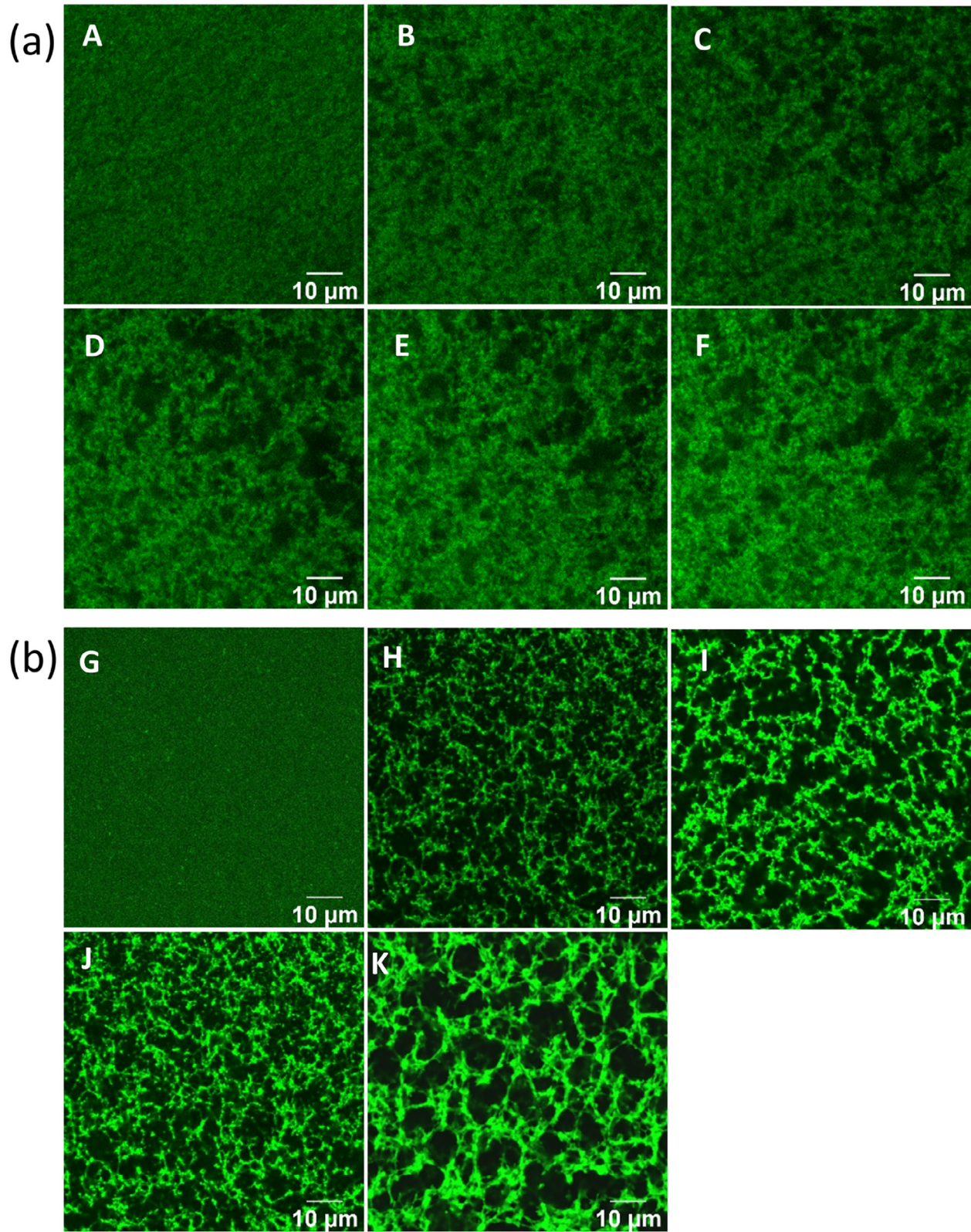


**Figure 7.** Firming rate ( $dG'/dt$ , Pa/min) of samples as a function of reaction rate constant  $K$  (min<sup>-1</sup>). Error bars represent SD from triplicate.

drophobic interactions allowed a closer approach of the casein micelles, leading to faster onset of aggregation (van Hooydonk, 1987; Zoon et al., 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 and Fox, 1993), the onset of casein aggregation (as measured by coagulation time) occurred more rapidly, even when the degree of hydrolysis of  $\kappa$ -CN was low. The coagulation time was therefore shorter for milk samples at lower pH.

As shown in Figure 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 2 highest pepsin concentrations. As previously mentioned, the firming rate is positively correlated with the reaction rate constant ( $K$ ; Figure 7); therefore, higher firming rates were found for the samples at pH 6.0 and 5.7, which had higher  $K$  values (Table 1).

The  $G'$  reached a plateau for the sample at pH 6.3 [Figure 5(b)] and decreased for the other samples at pH < 6.3 [Figure 5(c)–(e)]. Because 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 microsineresis (Roefs et al., 1990), a shrinkage of the casein curd over time, due to small oscillations of the sample (Zoon et al., 1988), thus eliminating the required adhesion of the coagulum to the rheometer



**Figure 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, and (F) 80 min (skim milk pH 6.0 with the addition of pepsin at a concentration of 0.550 U/mL); the micrographs were captured 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, and (K) pH 5.3. The scale bars are 10 µm for all micrographs.

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).

### 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 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  $\mu\text{m}$ ) and the network became closely knitted with an increase in the reaction time.

The microstructures of the coagulum after a prolonged incubation of 120 min at 37°C are shown in Figure 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. (2013). 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).

### CONCLUSIONS

The pepsin-induced hydrolysis of  $\kappa$ -CN 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  $\kappa$ -CN had been hydrolyzed. At a given pH, the critical degree of hydrolysis was independent of the pepsin concentration. The degree of  $\kappa$ -CN 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 releas-

ing  $\text{Ca}^{2+}$  by CCP 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  $\kappa$ -CN, the milk coagulation behavior, and the coagulum properties. The knowledge obtained from this study provides further understanding of 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.

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