Purification, characterization, and milk coagulating properties of ginger proteases

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ABSTRACT

Ginger proteases are used as milk coagulants in making a Chinese traditional milk product (Jiangzhinai or Jiangzhuangnai), suggesting their potential as a source of rennet substitute that might be applicable in the modern dairy industry. In this study, ginger proteases were extracted from fresh ginger rhizome by using phosphate buffer and subsequently purified by ion exchange chromatography. Ginger proteases, all with a molecular weight around 31 kDa, were found to exist in 3 forms with isoelectric point values around 5.58, 5.40, and 5.22, respectively. These enzymes had very similar biochemical behavior, exhibiting optimal proteolytic activity from 40 to 60°C and maximum milk clotting activity at 70°C. They were capable of hydrolyzing isolated α_{S1} -, β -, and κ -casein, of which α_{S1} -casein was most susceptible to the enzyme; κ -casein was hydrolyzed with a higher specificity than α_{S1} - and β -casein. In addition, the ginger proteases exhibited a similar affinity for κ -casein and higher specificity with increasing temperature. Gel electrophoresis and mass spectra indicated that Ala90-Glu91 and His102-Leu103 of κ -casein were the preferred target bonds of ginger proteases. The milk clotting activity, affinity, and specificity toward κ -casein showed that ginger protease is a promising rennet-like protease that could be used in manufacturing cheese and oriental-style dairy foods.

Key words: ginger protease, milk coagulation, specificity, κ -casein

INTRODUCTION

Jiangzhinai, also called Jiangzhuangnai, is a kind of Chinese traditional dairy product originating in southern China. It is made of hot milk mixed with ginger juice and forms a gel product with a smooth texture. Like Chinese Royal cheese, Jiangzhinai is a typical

oriental-style cheese with characteristic texture and taste, and it is particularly favored by the local people. The main factor in manufacturing Jiangzhinai is the milk coagulating activity of ginger protease, indicating its potential as a source of a novel rennet-like enzyme available for dairy production.

Ginger protease is a cysteine protease, belonging to peptidase family C1 (papain family), which is characterized by a cysteine residual at the active center of the enzyme (Choi et al., 1999). Until now, extensive studies have been focused on the proteolytic activity (PA), purification, structure analysis, and meat tenderization properties of ginger protease (Ichikawa et al., 1973; Thompson et al., 1973; Ohtsuki et al., 1995; Choi et al., 1999; Choi and Laursen, 2000; Naveena et al., 2004; Kim et al., 2007). Thompson et al. (1973) named ginger protease “Zingibain” according to conventional nomenclature. At the same year, Ichikawa et al. (1973) separated ginger protease into 2 fractions (GPI and GPII) by ion exchange chromatography. Later, Choi et al. (1999) and Choi and Laursen (2000) determined the primary and tertiary structure of GPII. It has been found that ginger proteases are capable of hydrolyzing native collagen, showing preference for the peptide bonds where Pro is in the P2 position (Kim et al., 2007). Ascorbic acid protects the activity of ginger proteases (Adulyatham and Owusu-Apenten, 2005; Su et al., 2009) and Cu^{2+} , Fe^{2+} , Cd^{2+} , and Hg^{2+} inhibit their activity, whereas Ca^{2+} , EDTA, and dithiothreitol activate the enzymes (Ohtsuki et al., 1995). Few works have studied the caseinolytic activity and milk clotting activity (MCA) of ginger proteases, although their milk coagulating properties have been used in producing Jiangzhinai for more than 100 yr (Su et al., 2009).

Coagulation of milk is the basic process in the manufacture of cheese, including oriental-style cheese. During the primary phase of milk curdling, rennet hydrolyzes κ -casein at the Phe105-Met106 bond and destabilizes the casein micelles, resulting in milk gelation (Horne and Banks, 2004). Proteases from *Cynara* species are shown to hydrolyze the same bond as chymosin (Faro

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et al., 1992). Besides the Phe105-Met106 bond, other peptide bonds are cleaved by fungal proteases, including that in para- κ -casein (Shammet et al., 1992). Nevertheless, nonspecific hydrolysis of κ -casein affects the curd-firming rate, which in turn influences the texture and yield of cheese (Roseiro et al., 2003). Proteases of different origin exhibit varied specificity for κ -casein and other casein fractions. Moreover, vegetable proteases usually have a high PA. The objectives of the present study were to investigate the PA, milk clotting characteristics, and specificity of ginger proteases on bovine caseins, especially the main products and cleavage sites on κ -casein, and to illustrate the mechanism of milk coagulation initiated by ginger juice.

MATERIALS AND METHODS

Materials

Fresh ginger roots were purchased from a local market. Low-heat skim milk powder was supplied by Westland (Hokitika, New Zealand). Ion exchange resin was purchased from GE Healthcare (Uppsala, Sweden). Whole casein, α_S -casein, β -casein, κ -casein, BSA, and 12-kDa molecular weight cut-off dialysis membrane were from Sigma (St. Louis, MO). Coomassie Brilliant Blue R-250 was obtained from Bio-Rad Laboratories (Hercules, CA). Ultrafiltration membrane module (Omega Centramate-C) was supplied by Pall (Ann Arbor, MI). A centrifugal filter unit with a molecular weight cut-off of 3,000 Da was supplied by Millipore (Billerica, MA). All the other chemicals were of analytical grade.

Extraction and Purification of Ginger Protease and Protein Determination

Ginger proteases were prepared according to the method of Choi et al. (1999) and Kim et al. (2007) with some modifications. Fresh ginger roots were peeled, cut into small pieces, and homogenized using a blender with 0.1 M phosphate buffer (pH 6.5), containing 10 mM cysteine and 5 mM EDTA. The resulting slurry was diluted with phosphate buffer and adjusted to 2% NaCl, stirred for 1 h, and then filtered through 4 layers of cheesecloth. The residual was re-extracted with phosphate buffer. The combined filtrates were filtered through a 0.22- μ m membrane, and the microfiltrate was concentrated by ultrafiltration with a 10-kDa molecular weight cut-off membrane. The retentate was then applied to a column (2.6 $\times$ 10 cm) packed with DEAE-Sephacel resin, which had been equilibrated with 20 mM phosphate buffer, pH 6.5. After the column was washed with binding buffer, a 1,000-mL linear gradient of 0 to 0.3 M NaCl (in binding buffer) was loaded at a

flow rate of 30 mL/h, and 8-mL fractions were collected. The eluant was monitored at 280 nm with a UV detector to detect the protein. Individual fractions showing MCA were pooled and dialyzed with a 12-kDa cut-off dialysis membrane against 4 L of deionized water twice at 6-h intervals. The desalted fractions were lyophilized by using a vacuum freeze dryer (Martin Christ, Osterode, Germany) and stored at -20°C . The extraction and purification processes were carried out below 10°C to protect the enzyme. The protein concentration was measured according to Lowry et al. (1951), with BSA as the standard.

Hydrolysis of Bovine Caseins

Bovine α_S -, β -, and κ -caseins were dissolved at 5 mg/mL in 0.1 M phosphate buffer, pH 6.5. Ginger proteases were added to the substrate at a ratio of 1 to 20 (vol/vol) and digested at different temperatures from 30 to 80°C at 5°C intervals for 15 min. For the timescale proteolysis investigation, substrates were incubated at 60°C , and aliquots were taken at 5, 15, 30, 60, 120, and 180 min. The reactions were terminated by mixing with an equal volume of urea SDS-PAGE sample buffer containing urea and β -mercaptoethanol and then boiled at 100°C for 5 min. After cooling to the room temperature, the samples were centrifuged at $5,000 \times g$ for 1 min and analyzed by urea SDS-PAGE.

Gel Electrophoresis

The purity and molecular weight of purified enzymes were checked by SDS and native PAGE with 12% gels using a Mini Protein system (Bio-Rad, Watford, UK). The molecular weights of the purified enzymes were determined by the method based on Laemmli (1970) using a 12% gel. The native PAGE was performed according to the method described by García-Carreño et al. (1993). The isoelectric points (pI) of ginger proteases were determined by isoelectric focusing. Urea SDS-PAGE analysis of the casein hydrolysate was based on a modification of the method by Andrews (1983) using a 15% gel. The stacking gel, separating gel, and sample buffer contained 6 M urea and 0.4% SDS.

Caseinolytic Activity Assay

The caseinolytic activity of purified enzymes was determined with a modified method previously reported by Mohanty et al. (2003), which involves digestion of bovine whole casein. The substrate solution was prepared by dissolving 2% (wt/vol) of casein in 0.1 M phosphate buffer, pH 6.5. The assay was performed by incubating 1 mL of substrate with 0.1 mL of purified enzyme

solution, and the mixture was incubated at 40°C for 1 h, followed by the addition of an equal volume of 0.4 M TCA to stop the reaction. A blank was prepared by adding TCA to the casein solution before addition of enzyme. After 20 min standing at room temperature, the precipitated proteins were removed by centrifugation at $12,000 \times g$ for 10 min, and the protein content in the supernatant was determined by the method of Lowry et al. (1951). One unit of PA was defined as the amount of enzyme required to release 100 μg of protein in 1 min.

Milk Clotting Activity Assay

Milk clotting activity was determined as described by Nouani et al. (2009). The substrate was composed of 12% milk powder (low-heat skim milk powder) reconstituted with 10 mM CaCl_2 . It involved adding 0.2 mL of enzyme to 2 mL of substrate at 35 and 70°C, and the time necessary for the formation of curd fragments was recorded. The MCA was expressed in Soxhlet units (SU). One unit of MCA was defined as the volume of milk coagulated per volume of the enzyme in 40 min.

Proteolytic Properties: Effects of pH and Temperature

Whole bovine casein with a concentration of 2% was used to investigate the effect of pH and temperature on the hydrolysis activity of ginger protease A (GPA) and ginger protease B (GPB). The substrate prepared in 0.1 M phosphate buffer of required pH (6.2 to 7.0) was incubated at 60°C, and the PA was measured. Temperature-dependent PA was assessed with 2% whole casein prepared in 0.1 M phosphate buffer (pH 6.5) at the indicated temperatures (30 to 75°C), and the PA was determined.

Kinetics Analysis

Purified GPA and GPB were dissolved in 20 mM phosphate buffer (pH 6.5) at 0.065 and 0.057 mg/mL (corresponding to 2.10 μM and 1.84 μM when molecular weight is taken to be 31,000 Da), respectively. κ -Casein (Sigma) stock solutions were dissolved in 0.1 M phosphate buffer, pH 6.5.

The reaction was initiated by adding enzymes (at a ratio of 1 to 20, vol/vol) into κ -casein solutions diluted at concentrations from 0 to 25 mM in 0.1 M phosphate buffer (pH 6.5). The reaction mixture was incubated at required temperatures for 120 s and the reaction terminated immediately by mixing with TCA, resulting in a final concentration of 3% TCA. After being left in room temperature for 30 min, 3% TCA-insoluble protein was

removed by centrifugation at $12,000 \times g$ for 10 min, and the supernatant was subjected to protein test in triplicate. Initial velocity as derived from protein assay was used to construct a double-reciprocal plot of $1/v$ against $1/[S]$ (Lineweaver and Burk, 1934). The kinetic parameters Michaelis constant (K_m), catalytic turnover number (k_{cat}), and proteolytic coefficient (k_{cat}/K_m) were calculated from the Lineweaver-Burk plot.

Milk Clotting Characteristics

Effects of pH, Temperature, and Substrate Concentration. Reconstituted skim milk was used to estimate the effect of pH, temperature, and substrate concentration on MCA of GPA and GPB. The skim milk (12%) prepared in demineralized water was adjusted to pH from 6.0 to 7.5 with 1 M HCl or 2 M NaOH, and the MCA was assayed at 60°C. The substrate with a concentration of 12% (pH 6.5) was incubated at different temperatures from 30 to 80°C, and the MCA was determined. The substrate reconstituted to different concentrations (2 to 16%) at pH 6.5 was incubated at 60°C, and the MCA was measured.

Effect of Metal Ions. Reconstituted skim milk (12%) with a pH value of 6.5 was subjected to changes in calcium ion (0 to 20 mM) and sodium ion (0 to 12%), and their effects on MCA of purified enzymes were determined at 60°C.

Mass Spectrometry Analysis of Peptides and Protein Identification

κ -Casein was prepared in 0.1 M phosphate buffer (pH 6.5) at a concentration of 5 mg/mL. Enzymes were added to the substrate at a ratio of 1 to 20 (vol/vol) and incubated at 60°C for 1 h. The digests were split into 2 parts. Sample for mass analysis was subjected to a 95°C water bath to inactivate the enzymes, and centrifuged using a 3,000-Da centrifugal filter unit at $7,500 \times g$ for 30 min to remove the salt. The retentate was analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF/MS; Bruker, Billerica, MA). At the same time, the other one was mixed with sample buffer and was subsequently separated with urea SDS-PAGE. The product band was excised from the gel and digested by trypsin overnight at 37°C. The obtained digests were lyophilized and analyzed using quadrupole time-of-flight mass spectrometry (Q-TOF 2; Micromass, Manchester, UK).

RESULTS AND DISCUSSION

Purification of Ginger Protease

Concentrated ginger protease extracts derived by ultrafiltration were subjected to weak anion exchange

Figure 1. Separation of ginger proteases A, B, and C by ion exchange chromatography with a linear gradient of 0 to 0.3 M NaCl in binding buffer.

column (2.6×10 cm). The chromatogram of the purification process is shown in Figure 1. Ginger proteases were separated into 3 peaks with proteolytic activity, namely peaks A, B, and C, using a linear gradient elution mode of 0 to 0.3 M NaCl ion strength. Sodium dodecyl sulfate-PAGE showed that peaks A and B appeared as a single band with the same molecular weight of around 31 Da (Figure 2a), but they were not the same molecule, as indicated by their different mobility in the native PAGE profiles shown in Figure 2b. The pI values for peaks A, B, and C, and crude ginger proteases determined by isoelectric focusing supported this observation, as the bands with pI values of 5.58, 5.40, and 5.22 corresponded to the bands in peaks A, B, and C. Peaks B and C contained the same 2 bands, implying that peak B was a mixture of 2 similar proteins that could not be resolved by ion exchange elution, whereas the protein in peak C was the same as that in peak B (Figure 2c). It has been reported that ginger proteases are separated into 2 fractions by Ichikawa et al. (1973), in which the first fraction is composed of 2 closely related proteins with pI 5.05 and 5.16, and the second fraction has a protein with pI of 4.8 (Choi et al., 1999). These 2 fractions share a homology of 82% (Choi et al., 1999). The multiplicity of ginger proteases

Figure 2. (a) Sodium dodecyl sulfate-PAGE, (b) native PAGE, and (c) isoelectric focusing profiles of ginger proteases from ginger root. (a) Lane M = protein standard, lane 1 = peak A fraction, lane 2 = peak B fraction; (b) lane n1 = peak A fraction, lane n2 = peak B fraction; (c) lane 0 = crude ginger protease, lane 1 = peak A fraction, lane 2 = peak B fraction, lane 3 = peak C fraction. Black arrow indicates proteins concomitant with ginger proteases.

is not rare, because rennet is heterogeneous in nature, containing 3 isozymes, including chymosin A, B, and C (Crabbe, 2004). Therefore, it is suggested that the ginger proteases with different isoelectric points are isozymes. Hence, ginger protease fractions obtained by purification on ion exchange column here were GPA and GPB, corresponding to peak A and peak B, respectively. Their MCA and PA are shown in Table 1; GPA had higher MCA and PA than GPB, but the MCA:PA ratio differed to a lesser extent.

Proteolytic Properties

The pH and temperature dependence curves of the PA for GPA and GPB exhibited a similar pattern (Figure 3). The PA of the enzymes increased as pH decreased from 7.0 to 6.2 and activity was maintained under neutral and alkaline conditions. The characteristic of maintaining PA in an acidic pH range fits one of the basic requirements for rennet-like coagulants because milk

Table 1. Milk coagulating activity (MCA) and proteolytic activity (PA) of ginger proteases (GP) A and B¹

Protease	MCA (SU/mg of protein ²)	PA (U/mg of protein)	MCA:PA
GPA	442.2 ± 1.9	9.44 ± 0.21	46.8
GPB	288.3 ± 3.7	6.58 ± 0.32	43.8

¹Means ± SD.

SU = Soxhlet unit.

Figure 3. Effect of pH and temperature on proteolytic activity of ginger protease A (■) and ginger protease B (◆).

has a natural pH of 6.5 to 6.7 and milk coagulation is usually displayed at pH 6.3 to 6.6 (Horne and Banks, 2004). Ginger proteases A and B had a broad optimum temperature between 40 and 60°C on hydrolysis of whole casein, but enzyme activity decreased rapidly at temperatures <40°C and >60°C. The purified enzymes underwent dramatic heat denaturation as implied by a rapid reduction of activity at temperatures >60°C. However, about 70% residual activity was retained when the temperature was increased to 65°C, indicating that ginger proteases were highly thermal resistant.

Milk Clotting Characteristics

Different proteases have very different MCA as well as PA depending on origin. Milk clotting was

Figure 4. Effect of pH, temperature, and substrate concentration on milk coagulating activity of ginger protease A (■) and ginger protease B (◆).

Figure 5. Effect of CaCl_2 and NaCl concentration on milk coagulating activity of ginger protease A (■) and ginger protease B (◆).

affected by various factors, such as pH, temperature, ionic strength, enzyme concentration, and metal ions. Both GPA and GPB presented similar milk clotting patterns with variations in pH, temperature, substrate concentration, ionic strength, and metal ion (Figures 4 and 5). As the pH of reconstituted skim milk increased from 6.0 to 7.5, the MCA of both enzymes decreased dramatically (Figure 4a). Both enzymes retained MCA under neutral and weak alkaline conditions, but the loss of activity was very significant, with no more than 10% remaining. The loss of activity under alkaline conditions may due to the irreversible conformation changes (Horne and Banks, 2004). The MCA increased with increasing temperature up to 70°C. Increasing the temperature above 70°C caused a rapid loss of MCA;

Figure 6. Lineweaver-Burk plots (double-reciprocal plot) for the hydrolysis of κ -casein by ginger protease A (■) and ginger protease B (▲) at 40 (panel a) and 70°C (panel b).

at 75°C, the relative activity was around 10% of the maximum. When the temperature was <60°C, MCA was relatively low, although a high milk coagulating rate was observed at higher temperatures. In the case of rennet, the optimal temperature is 42 to 45°C (Fox, 1969), and milk clotting is usually performed at temperatures <40°C. At higher temperatures, a rapid loss of activity is observed when the temperature increased to 55°C (Kawaguchi et al., 1987) and the activity would be completely lost at the higher temperature at which ginger protease reached its maximum (Shindo and Arima, 1979). Hence, ginger protease would not be suitable for producing traditional cheese (30–40°C), but the advantage is that the pasteurization process is

Table 2. Kinetic parameters¹ of ginger proteases (GP) A and B on κ -casein at different temperatures

Enzyme	40°C			70°C		
	K_m (mg·L ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mg ⁻¹ ·L·s ⁻¹)	K_m (mg·L ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mg ⁻¹ ·L·s ⁻¹)
GPA	359 ± 2	153 ± 3	0.424 ± 0.007	1,128 ± 123	332 ± 18	0.295 ± 0.017
GPB	237 ± 14	108 ± 4	0.457 ± 0.014	1,057 ± 28	315 ± 9	0.298 ± 0.005

¹ K_m = Michaelis constant; k_{cat} = catalytic turnover number; and k_{cat}/K_m = proteolytic coefficient.

accomplished during milk coagulation. Obviously, high temperature imparts a unique quality assurance to the product, which is unavailable by other milk clotting enzymes.

Substrate concentration had a great effect on MCA because the ratio changed between enzyme and substrate. The MCA declined dramatically with milk concentration from 2 to 10% and decreased more slowly with higher concentrations up to 16%.

Calcium chloride may be added to milk for cheese-making to reduce the milk clotting time and the amount of rennet needed. We have shown that calcium ion accelerated the MCA of purified enzymes at concentrations from 0 to 20 mM (Figure 6a). A similar trend was observed for *Silybum marianum* flower extract, whose MCA increased with the addition of CaCl₂ up to 30 mM (Vairo-Cavalli et al., 2005). An exception is the lettuce protease investigated by Lo Piero et al. (2002), in which CaCl₂ had no effect on its milk clotting. In cheese manufacture, salt is usually added for preservative, functional, and sensory effects (Schroeder et al., 1988). Sometimes, salt is also directly added to the raw milk before curdling; for example, in Domiati-type cheese 2 to 15% salt is applied (Mehaia, 1993). As shown in Figure 6b, a dramatic reduction in MCA of GPA and GPB was observed with increasing salt concentration. When the salt concentrations reached 2, activity was inhibited by 50%. The inhibitory effect of salt was more dramatic on GPA than on GPB. This is because sodium chloride affects degradation of caseinate by modifying the state of substrate aggregation or folding, which makes it less susceptible to enzymatic attack (Sousa and Malcata, 1998, 2002).

Kinetic Parameters

Among all of the casein fractions, κ -casein plays a crucial role in stabilizing casein micelles and is involved in milk curdling: the hydrophilic moiety is cleaved off by renneting enzymes, whereas the hydrophobic N-terminal moiety remains on the micelles (Horne and Banks, 2004). The more hydrophobic the generated N-terminal moiety of κ -casein is, the faster the aggregation rate of casein micelles (Shammet et al., 1992).

Therefore, kinetic parameters of ginger proteases on κ -casein were investigated to provide a better understanding of their milk clotting behaviors. Our experiments involved sedimentation of unhydrolyzed κ -casein and generated hydrophobic N-terminal moiety by TCA and measurement of TCA-soluble peptides of κ -casein digests. The concentration of TCA used here was 3%, because previous research suggests that this concentration is sufficient to precipitate proteins completely while keeping the carbon terminal hydrophilic peptides in solution (Vreeman et al., 1986).

The 1/v against 1/[S] plot for hydrolysis of whole κ -casein at different temperatures is shown in Figure 6. The catalytic center activities (k_{cat}) and proteolytic coefficient (k_{cat}/K_m) of both enzymes differed only to a lesser extent compared with those at 40 °C (Table 2). The K_m observed for GPA was higher than that of GPB at 40°C, but the K_m values for the 2 enzymes were similar at 70°C. However, the K_m value at 70°C was more than 3 times that at 40°C. These results indicate that GPA and GPB have similar affinity for whole κ -casein under the same conditions but a substantially lower affinity at high temperatures. On the other hand, the hypothesis that ginger proteases are isozymes was supported by the observed behavior of GPA and GPB, which exhibited very similar PA, milk clotting behavior, and affinity for the same substrate.

Analysis of Casein Hydrolysate

Comparison of the specificity of ginger proteases on hydrolysis of isolated α_s -, β -, and κ -caseins in solution at different temperatures was analyzed using urea SDS-PAGE (Figure 7). Ginger proteases exhibited activity on all 3 casein components and were most active on α_s -casein within a broad temperature range, showing complete proteolysis even when the temperature increased to 70°C. However, β - and κ -casein were only partially hydrolyzed, and when the temperature was >60°C, the activity on β - and κ -casein declined, which might be attributed to the thermal denaturation of the enzymes. In addition, we show in Figure 7a that α_{s1} -casein was readily degraded into 3 peptides, α -CN 1, α -CN 2, and α -CN 3, and degradation of α -CN 1

Figure 7. Urea SDS-PAGE patterns of isolated bovine (a) α-casein, (b) β-casein, and (c) κ-casein by ginger proteases at 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80°C, corresponding to lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11, respectively. Isolated bovine α-, β-, and κ-casein were included as controls in lane M of each panel, respectively.

Figure 8. Urea SDS-PAGE electrophoretograms of bovine casein fractions by ginger proteases at 5, 15, 30, 60, 120, and 180 min, corresponding to lanes 1, 2, 3, 4, 5, and 6 (ginger protease A), and lanes 7, 8, 9, 10, 11, and 12 (ginger protease B), respectively; lane M represents (a) α-casein, (b) β-casein, and (c) κ-casein, respectively.

increased with increasing temperature from 30 to 70°C. The main products formed from the digestion of κ-casein were κ-CN 2 and κ-CN 3, along with a low concentration of κ-CN 1 at all tested temperatures (Figure 7). Low temperatures resulted in some peptides

Figure 9. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF-MS) spectra of κ -casein digests.

between κ -casein and κ -CN 1, gradually disappearing with increasing temperature. However, the concentration of κ -CN 1, κ -CN 2, and κ -CN 3 decreased with increasing temperature above 65°C. These results revealed that ginger proteases had a higher specificity for κ -casein as temperature increased.

Degradation of caseins by ginger proteases as a function of time at 60°C was analyzed by urea SDS-PAGE (Figure 8). α_{S1} -Casein was completely hydrolyzed after 1 h of incubation, and β -casein was the most resistant, whereas κ -casein was only partially hydrolyzed even after 3 h of degradation. The peptide α -CN 1 band from α_{S1} -casein underwent further degradation with extended time, and generated the diffuse band with a low molecular weight at the bottom of the gel. Proteolysis of κ -casein showed that it experienced more extensive hydrolysis than β -casein. Most of κ -casein was hydrolyzed after 30 min digestion, but no evident proteolysis happened during subsequent incubation. The concentration of the 3 breakdown products that were observed

in Figure 7c was constant after 15 min of hydrolysis, indicating that they underwent no further degradation and were resistant to the enzyme action. It is known that bovine glycomacropeptide is poorly stained with a pervading electrophoretic pattern due to its high acidic polyhydroxylic attribute induced interaction with the polyacrylamide gel matrix and is very difficult to observe (Coolbear et al., 1996). Therefore, the breakdown products from κ -casein might correspond to the N-terminal part of κ -casein because they showed clear bands in urea SDS-PAGE. Because of a small amount of α_{S2} -casein occurring in these 3 isolated caseins, the urea SDS-PAGE pattern of α_S -, β -, and κ -caseins seems to be somewhat complicated. However, α_{S2} -casein appears to be very resistant to the proteolysis by ginger proteases, demonstrated by the unhydrolyzed α_{S2} -casein in all 3 isolated caseins, especially at temperatures >60°C, as shown in Figure 8a.

To assay the peptide mass of main breakdown products of κ -casein by ginger proteases, κ -casein was

Table 3. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of the main products of κ -casein hydrolysate digested by ginger protease

Main product		Molecular weight (Da)		
		Observed	Calculated	Peptide ¹
Para- κ -casein-like peptide	κ -CN 1	10,556	10,545	κ -CN (f 1–90)
	κ -CN 2	11,941	11,934	κ -CN (f 1–102)
	κ -CN 3	14,083	14,077	κ -CN (f 1–121)
Glycomacropeptide-like peptide		7,069	7,054	κ -CN A (f 103–169)
		7,197	7,149	κ -CN A-1P (f 103–169)

¹Nomenclature of the peptides is according to Farrell et al. (2004); f = fragment.

Table 4. Identification of peptide fragments (f) of in-gel digests of peptide segment from κ -casein using quadrupole time-of-flight mass spectrometry

Observed fragment ion ($[M+2H]^+$) ¹	Peptide sequence	Molecular weight (Da)		Peptide origin
		Expected	Calculated	
478.26	FFSDKIAK	954.51	954.52	κ -CN (f 17–24)
626.35	YIPIQYVLSR	1,250.69	1,250.70	κ -CN (f 25–34)
990.52	SPAQLQWQVLSNTVPAK	1,979.03	1,979.08	κ -CN (f 69–86)
812.91	HPHPHLSFMAIPPK	1,623.81	1,623.83	κ -CN (f 97–111)

¹ $[M+2H]^+$ represents mass and charge of molecule ion.

proteolyzed by ginger proteases at 60°C for 1 h, and the digests were subjected to MALDI-TOF/MS analysis. Figure 9 shows the MALDI-TOF mass spectra of κ -casein digests, and a series of peptides signals were obtained. The intense signals with mass-to-charge ratios (m/z) of 7,069 and 7,197 Da corresponded to the C-terminal peptide 103 to 169 residuals of κ -casein (Table 3), which had a calculated molecular weight of 7,054 Da, whereas the signal with m/z of 7,197 Da was κ -CN (f103–169) with a phosphate group (the molecular weight of phosphate group is 95). This peptide has a molecular weight close to GMP generated by rennet cleavage of κ -casein between Phe105–Met106 with m/z of 6,787.4 Da observed by Egito et al. (2007). Three peptides with $m/z > 10,000$ Da were detected, including peptide segments with observed molecular weights of 10,556, 11,941, and 14,083 Da. These 3 signals corresponded to the band with molecular weight > 10 kDa in the gel, which were recognized as κ -CN (f1–121), κ -CN (f1–102), and κ -CN (f1–90), respectively, according to the urea SDS-PAGE pattern of κ -casein digests in Figure 8c. However, these 3 signals had low response due to the sulfhydryl cross-linking between κ -casein and released N-terminal peptides caused by heat denaturation of the proteins (Farrell et al., 2004). In addition, several signals with m/z between 4 and 10 kDa had lower signal intensity than κ -CN (f103–169), implying that the C-terminal moiety of κ -casein was hydrolyzed at multiple sites by ginger protease and formed peptides with a lower molecular weight. Moreover, a small amount of other proteins occurred in κ -casein (e.g., α_{S2} -casein); their digest may have an interference effect on the signals of κ -casein breakdown products.

To confirm whether these 3 peptides were located at the N-terminal of κ -casein, the band corresponding to κ -CN 1 was digested by trypsin and the digests were sequenced directly using Q-TOF/MS. Table 4 shows that the obtained sequence information of digested peptide segments and the number of peptides matched to κ -casein was 4 with sequence coverage of 30%. The Molecular Weight Searching (MOWSE) score was 210 (Matrix Science accession no.: gi 162811, <http://www.matrixscience.com/>; Pappa et al., 2008), indicating a significant identification for κ -casein ($P < 0.05$). Thus, peptides with $m/z > 10$ kDa were confirmed as being from the N-terminal parts of κ -casein. The results described above shows that the primary cleavage sites of ginger proteases on κ -casein were His102–Leu103 and Ala90–Glu91.

matrixscience.com/; Pappa et al., 2008), indicating a significant identification for κ -casein ($P < 0.05$). Thus, peptides with $m/z > 10$ kDa were confirmed as being from the N-terminal parts of κ -casein. The results described above shows that the primary cleavage sites of ginger proteases on κ -casein were His102–Leu103 and Ala90–Glu91.

CONCLUSIONS

Our studies showed that ginger protease coagulated milk in a pattern distinctive from that of rennet, and revealed that the most susceptible bonds of κ -casein were His102–Leu103 and Ala90–Glu91, which are close to the bond hydrolyzed by chymosin. Moreover, ginger protease possessed high MCA and PA, and exhibited a higher ratio of MCA to caseinolytic activity at temperatures above 60°C, proving its potential use as a rennet substitute and accelerating agent in the dairy industry; for example, in cheese maturation and manufacture of oriental-style cheese.

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