JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Proteomic Profiling of the Coagulation of Milk Proteins Induced by Chymosin

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ABSTRACT: Chymosin-induced coagulation of individual milk proteins during incubation at 30 °C was investigated using a proteomic approach. The addition of chymosin (0.006 units/mL) caused the milk proteins to coagulate after a 3 h incubation period. Approximately 88% of the milk proteins were coagulated into the milk pellet fraction, and the protein concentration of the milk supernatant fraction (MSF) decreased from 29.88 ± 0.12 to 3.74 ± 0.13 mg/mL. SDS−PAGE analysis showed that α_S-, β- and κ-caseins in the MSF were almost depleted and that the total intensity of the protein bands corresponding to α_S-caseins (α_{S1} and α_{S2}), β-casein, and κ-casein decreased from 1088.0, 901.5, and 617.0 area units to 6.9, 6.1, and 5.2 area units, respectively. Two-dimensional electrophoresis analysis indicated that α_{S1}-, α_{S2}-, β-, and κ-casein and a fraction of the β-lactoglobulin and serum albumin were found in the MSF following incubation with chymosin.

KEYWORDS: *milk, chymosin, proteomic, two-dimensional electrophoresis*

INTRODUCTION

Milk, which contains 3.3% protein, is a valuable nutrient source for humans. Approximately 80% of milk protein is composed of caseins, and 20% is composed of whey proteins, including the well-known β-lactoglobulin (β-LG), α-lactalbumin, and milk serum proteins.[1] The caseins in milk are α_{S1}-casein (α_{S1}-CN), α_{S2}-casein (α_{S2}-CN), β-casein (β-CN), and κ-casein (κ-CN) in a 4:1:4:1 ratio.[2] The majority of the caseins in milk are incorporated into large colloidal structures known as casein micelles. Caseins are linked together with colloidal calcium phosphate, and κ-CN-rich caseins are concentrated at the surface.[3] These casein micelles can be processed into many types of food products, such as cheese.[4] During the cheesemaking process, an important step is the enzymatic coagulation of milk proteins, during which the casein micelles are enzymatically destabilized.[5] Traditionally, chymosin is the major enzyme responsible for the coagulation of milk proteins.[6]

Chymosin (E.C. 3.4.23.4) is the principal protease used for cheesemaking because it has highly specific milk-clotting activity relative to its proteolytic activity.[7] The chymosin-mediated coagulation reaction includes two steps, the first of which is the enzymatic hydrolysis of the caseins.[8] Chymosin hydrolyzes κ-CN to form two peptides as reaction products. This protease is responsible for the specific cleavage of the Phe_{105}-Met_{106} bond of κ-CN, resulting in the destabilization of casein micelles.[9] Chymosin also readily hydrolyzes certain peptide bonds in α_{S1}-CN, α_{S2}-CN and β-CN.[10] The second step of the chymosin-mediated coagulation reaction involves the aggregation of casein micelles induced by calcium ions (Ca^{2+}), which occurs after a sufficient amount of κ-CN has been hydrolyzed.[11]

Recently, proteomics approaches have been used to investigate milk proteins.[12,13] Proteomics is a powerful method that can simultaneously analyze several hundred proteins in complex mixtures through the use of high-resolution two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry.[14] On the basis of 2 independent biochemical characteristics of proteins, 2-DE combines isoelectric focusing, which separates proteins according to their isoelectric points, and SDS−PAGE, which separates them further according to their molecular masses.[15] The 2-DE approach to protein profiling has been successful because it is an accessible, inexpensive, and powerful tool for analyzing patterns of protein expression.[16] All protein spots that are resolved and detected can be studied qualitatively and quantitatively in relation to each other.[17] Therefore, 2-DE combined with protein identification by mass spectrometry has been employed to identify individual milk proteins. However, the proteomic analysis of chymosin-induced coagulation of individual milk proteins during incubation has never been reported. In this study, we used SDS−PAGE and 2-DE coupled with mass spectrometry to investigate the effects of chymosin on the coagulation of milk proteins. The objective of this study was to analyze chymosin-induced coagulation of milk proteins using a proteomic approach.

MATERIALS AND METHODS

Preparation of Milk Samples. Fresh raw milk from a healthy Holstein−Friesian cow was obtained from a local farm in Taipei in northern Taiwan. The milk was skimmed at 5000*g* for 20 min, and the skim milk was collected and stored at 4 °C. Chymosin (20 units/mg protein) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). One unit of coagulation activity was defined as the amount of chymosin that coagulated 10 mL of milk per min at 30 °C. Chymosin was dissolved in phosphate buffer (50 mM, pH 7.0), and the milk samples with or without chymosin (0.006 units/mL) were incubated at 30 °C for 0, 1, 2, and 3 h. After incubation, the milk samples were fractionated into the milk supernatant fraction (MSF) and the milk pellet fraction (MPF) by centrifugation for 20 min (5,000*g*). The MSF samples (1 mL) were collected, and the MPF samples were redissolved

Received: November 9, 2011
Revised: January 30, 2012
Accepted: February 1, 2012
Published: February 4, 2012

dx.doi.org/10.1021/jf204582g | J. Agric. Food Chem. 2012, 60, 2039−2045

in an equal volume (1 mL) of lysis solution containing 7 M urea, 2 M thiourea, and 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate prior to use.

Determination of Protein Concentrations. The protein concentrations of the milk samples were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). The Bio-Rad protein assay dye was diluted with 4 volumes of water and then mixed with the standards or milk samples. The absorbance at 595 nm was measured using a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA), and bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) was used as the standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Milk samples with or without chymosin were analyzed by reducing SDS–PAGE according to Hsieh and Chen.[18] SDS–PAGE analysis of MSF and MPF samples was performed with a 12.5% separating gel and a 5% stacking gel, and each sample was performed in triplicate. For each sample, 0.1 mL was mixed with 0.9 mL of reducing buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, and 70 mM Tris-HCl, pH 6.8) and heated at 95 °C for 5 min. A protein ladder and the samples (6 μL) were loaded into separate wells. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. The stained gels were then digitized using an Epson perfection 1270 image scanner and analyzed using the Quantity One 1-D analysis software (version 4.6.3, Bio-Rad) and Gel-Pro Analyzer software (version 4.0, Media Cybernetics, Inc.). Changes in the reducing electrophoretic profile were used to evaluate protein coagulation caused by chymosin.

Two-Dimensional Electrophoresis (2-DE). Milk samples were analyzed by 2-DE, and each sample was performed in triplicate. For the first separation, 100 μg of total milk protein was immobilized and loaded into pH gradient (IPG) gel strips (pH 4–7, 18 cm, GE Healthcare), which had been rehydrated for 12 h in a solution containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 40 mM Tris-base, 2% IPG ampholyte, 65 mM 1,4-dithioerythritol (DTE), and 0.0002% bromophenol blue prior to use. Isoelectric focusing of the strips was performed using the IPGphor 3 IEF system (GE Healthcare) at 20 °C at 6000 V for a total of 60 kVh. The strips were then equilibrated for 15 min in equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 2% DTE) and added to the top of a vertical 12.5% SDS–PAGE gel with 0.5% agarose. The second electrophoresis step was performed using the Protean II xi Cell system (Bio-Rad) at 10 mA per gel for 1 h and then 45 mA per gel for 5 h until the bromophenol blue reached the bottom of the gel. After electrophoresis, the gels were immersed in 10% methanol and 7% acetic acid for 30 min and then stained in 350 mL of Sypro Ruby protein gel stain solution overnight.[19] The developed gels were digitally scanned as 2-D images with a Typhoon 9200 imaging system (Amersham Pharmacia Biotech) and analyzed using the PDQuest software package (version 7.3, Bio-Rad).

Protein Digestion and Mass Spectrometry Analysis. Fifteen selected milk protein spots were excised and destained by two 15 min washes in a solution containing 250 μL of acetonitrile/50 mM ammonium bicarbonate (1:1 v/v). The gels were dried using a centrifugal vacuum concentrator. Cysteine residues in the samples were reduced and alkylated using DTE and iodoacetamide, respectively. For tryptic digestion, the gel was rehydrated in trypsin solution (12.5 ng/mL) and incubated at 37 °C for 16 h. Peptide fragments were then extracted with an equal volume of 100% acetonitrile/2% trifluoroacetic acid and sonicated in a bath for 10 min. The extracted peptides were concentrated in a vacuum centrifuge. For matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) analysis, the extracted peptides were mixed 1:1 with a matrix solution (5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% v/v trifluoroacetic acid, and 2% w/v ammonium citrate) and spotted onto a 96-well format MALDI sample stage. Data were obtained directly using a quadrupole time-of-flight (Q-TOF) Ultima MALDI instrument (MALDI, Micromass, UK).

Protein Identification. Peptide mass fingerprint data from MALDI-Q-TOF were used to identify the protein candidates in the Swiss-Prot protein databases using the Mascot (http://www.matrixscience.com) search program.[20] Search parameters allowed for were methionine oxidation, cysteine carbamidomethylation, one missed cleavage site, and a peptide mass tolerance of 0.25 Da.[21] The product ion spectra generated by Q-TOF MS/MS were then compared to the Swiss-Prot protein databases, and exact matches were found with the Mascot search program.

Statistical Analysis. Data were expressed as the mean ± standard deviation. The data were analyzed using the Statistical Package for the Social Sciences software (SPSS for Windows, version 10.0.7C, SPSS Inc., Chicago, IL, USA). Statistical significance among the treatments was determined by a one-way ANOVA followed by a Duncan's multiple range test. Three determinations for each treatment were made, and the significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Effects of Chymosin on the Coagulation of Milk Proteins. Milk samples with or without chymosin (0.006 units/mL) were incubated at 30 °C for 1, 2, or 3 h, and the amounts of protein concentration in the MSF and MPF were determined. The protein concentration in the chymosin-treated MSF decreased significantly after 3 h of incubation ($p < 0.05$) from 29.88 ± 0.12 (control) to 3.74 ± 0.13 mg/mL (Figure 1).

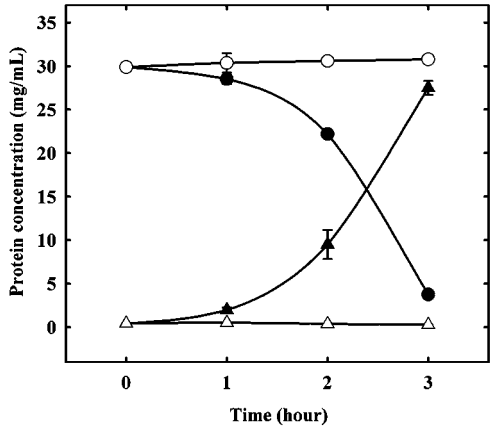

Figure 1. Effect of incubation time on the coagulation of milk proteins by chymosin. ○/●, milk supernatant fraction (MSF); Δ/▲, milk pellet fraction (MPF). Solid symbols, milk with chymosin (0.006 units/mL); open symbols, milk without chymosin. Vertical bars represent the standard deviation.

According to these results, approximately 88% of the milk proteins were coagulated by chymosin after the 3 h incubation period. As mentioned previously, about 80% of milk protein is composed of caseins, and 20% is composed of whey proteins.[1] Therefore, the results suggested that the coagulated proteins may consist of 80% caseins. Moreover, the protein concentration in the chymosin-containing MPF increased markedly with incubation times up to 3 h. Chymosin is the major enzyme responsible for the coagulation of milk proteins.[22] We also found that there were no significant changes in the protein concentration in the MSF or MPF without chymosin treatment after a 3 h incubation period, indicating that protein coagulation did not occur in milk samples in the absence of chymosin. However, proteolysis in milk may occur by native proteases, such as plasmin (EC 3.4.21.7), during 3 h of incubation. Plasmin-induced proteolysis can have either beneficial or detrimental effects on the texture and flavor of dairy products, depending on the extent of hydrolysis and type of dairy product.[23]

SDS−PAGE Analysis of the Effects of Chymosin on Caseins and β-Lactoglobulin. SDS−PAGE analysis revealed that the amounts of α_S-CN (α_{S1}- and α_{S2}-), β-CN, and κ-CN in the chymosin-treated MSF decreased with increasing incubation time (Figure 2A). The disappearance of the caseins (α_{S1}-, α_{S2}-, β-, and κ-CN) suggested the occurrence of coagulation because chymosin possesses a highly specific milk-clotting activity. We noticed that the β-LG levels decreased in the SDS−PAGE analysis in an incubation time-dependent manner. β-LG is the major component of whey protein in milk. Hallén et al. reported that during chymosin-mediated coagulation, curd containing whey proteins is formed.[24] Therefore, β-LG coagulation is thought to occur in the curd. We also noticed that the intensity of α_S-CN (α_{S1}- and α_{S2}-), β-CN, and β-LG in the chymosin-containing MPF increased with incubation time (Figure 2B). The total intensity of the protein bands corresponding to α_S-CN (α_{S1}- and α_{S2}-), β-CN, and κ-CN in the milk were 1088.01, 901.59, and 617.08 area units, respectively. Morris et al. reported that α_S-CN (α_{S1}- and α_{S2}-), β-CN, and κ-CN are present in milk in a ratio of approximately 5:4:1.[2] After incubation of chymosin-containing milk for 3 h, the total intensity of α_S-CN (α_{S1}- and α_{S2}-CN), β-CN, κ-CN, and β-LG in the MSF decreased from 1088.0, 901.5, 617.0, and 486.9 area units to 6.9, 6.1, 5.2, and 86.2 area units, respectively.

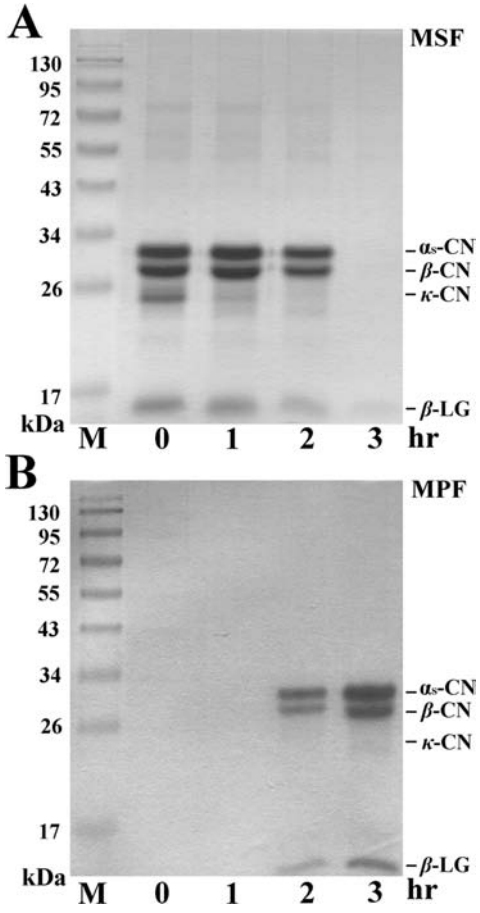

Figure 2. Changes in SDS−PAGE profiles of milk proteins incubated with chymosin (0.006 units/mL) at 30 °C for various amounts of time. A, milk supernatant fraction (MSF); B, milk pellet fraction (MPF); M, protein marker.

Identification of Milk Proteins by 2D-PAGE Analysis. The 2-DE image of the milk proteins is shown in Figure 3.

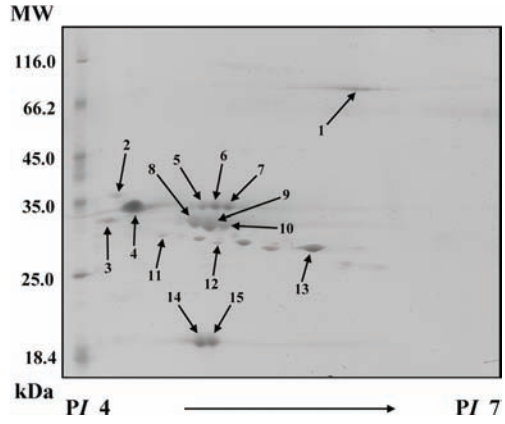

Figure 3. Two-dimensional gel electrophoresis analysis of milk proteins. Milk proteins were separated by SDS−PAGE on a 12.5% gel using a pH 4−7 IPG strip. The arrows indicate the protein spots identified in this study. MW, molecular weight; p*I*, isoelectric point.

Fifteen spots selected from the 2D gel were digested with trypsin, and the resultant peptides were analyzed by mass spectrometry. These proteins were identified by comparison to mammalian protein sequences in the Swiss-Prot databases. In total, 15 identified proteins were assigned a number and cataloged according to their molecular weight (MW) and isoelectric point (p*I*) (Table 1). Spot 1 was serum albumin, while spots 14 and 15 were isomers of β-LG. Serum albumin and β-LG are major components of whey protein isolate.[25] Moreover, spots 2−4, 5−7, 8−10, and 11−13 were isomers of α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN, respectively. The 2D-PAGE analysis of milk samples was performed using a 12.5% separating gel. Therefore, we did not identify α-lactalbumin. We found that there were multiple spots for α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, and β-LG on the 2D gel. Multiple spots on a 2D gel could be isoforms with different signal or targeting sequences, which could produce shifts in the p*I* and molecular weight. The proteins could also be post-translationally modified by the addition of side chains, phosphate groups, methyl groups, among others, which could also affect the p*I* and molecular weight.[26] For example, the κ-CN (spots 11−13) is the glycoprotein, and the O-glycosylation sites are in the C-terminal portion of the molecule. Finally, protein degradation could be responsible for multiple spots of the same protein. Chevalier et al.[27] reported that casein is present in various forms that mainly depend on different glycosylation patterns and levels of degradation. In addition, the phosphorylation of bovine caseins has been well characterized, and the vast majority of these proteins exist in a phosphorylated form.[28]

2D-PAGE Analysis of the Effect of Chymosin on Caseins and Whey Proteins. Milk samples with or without chymosin (0.006 units/mL) were incubated at 30 °C for 1, 2, or 3 h, and the MSF and MPF samples were electrophoretically separated in 2D gels. According to the 2-DE results, a portion of the casein isomers in the chymosin-treated MSF, including α_{S1}-CN (spots 2−4), α_{S2}-CN (spots 5−7), and β-CN (spots 8−10), was depleted after 2 h of incubation. However, κ-CN isomers (spots 11−13) almost completely disappeared under the same conditions. After incubation of chymosin-containing milk for 3 h, the casein isomers (α_{S1}-, α_{S2}-, β-, and κ-) in the

Table 1. Milk Proteins Identified and Cataloged from 2-DE[a] Analysis

spot no.[b]	protein name	MW[c] (Da)/p*I*[d] apparent	MW[c] (Da)/p*I*[d] theoretical	Mowse score	MS[e] method	% coverage	Species
1	serum albumin	72000/5.9	69248/5.82	169	MS/MS	8%	bovine
2	α_{s1}-casein	36000/4.3	24570/4.98	163	MS/MS	20%	bovine
3	α_{s1}-casein	30000/4.2	24570/4.98	268	MS/MS	17%	bovine
4	α_{s1}-casein	32000/4.4	24570/4.98	229	MS/MS	20%	bovine
5	α_{s2}-casein	32000/4.8	26002/8.54	80	MS/MS	9%	bovine
6	α_{s2}-casein	32000/4.9	26002/8.54	99	MS/MS	13%	bovine
7	α_{s2}-casein	32000/5.1	26002/8.54	40	MS/MS	8%	bovine
8	β-casein	30000/4.7	25148/5.26	80	MS/MS	8%	bovine
9	β-casein	30000/4.8	25148/5.26	94	MS/MS	8%	bovine
10	β-casein	30000/5.0	25148/5.26	91	MS/MS	8%	bovine
11	κ-casein	28000/4.6	21370/6.30	49	MS/MS	5%	bovine
12	κ-casein	28000/4.9	21370/6.30	42	MS/MS	5%	bovine
13	κ-casein	28000/5.8	21256/6.30	131	MS/MS	22%	bovine
14	β-lactoglobulin	20000/4.8	20269/4.93	100	MS/MS	14%	bovine
15	β-lactoglobulin	20000/4.9	20269/4.93	125	MS/MS	24%	bovine

[a]2-DE = two-dimensional gel electrophoresis. [b]Spot no. = spot number, corresponds to Figure 3. [c]MW = molecular weight. [d]p*I* = isoelectric point. [e]MS = mass spectrometry.

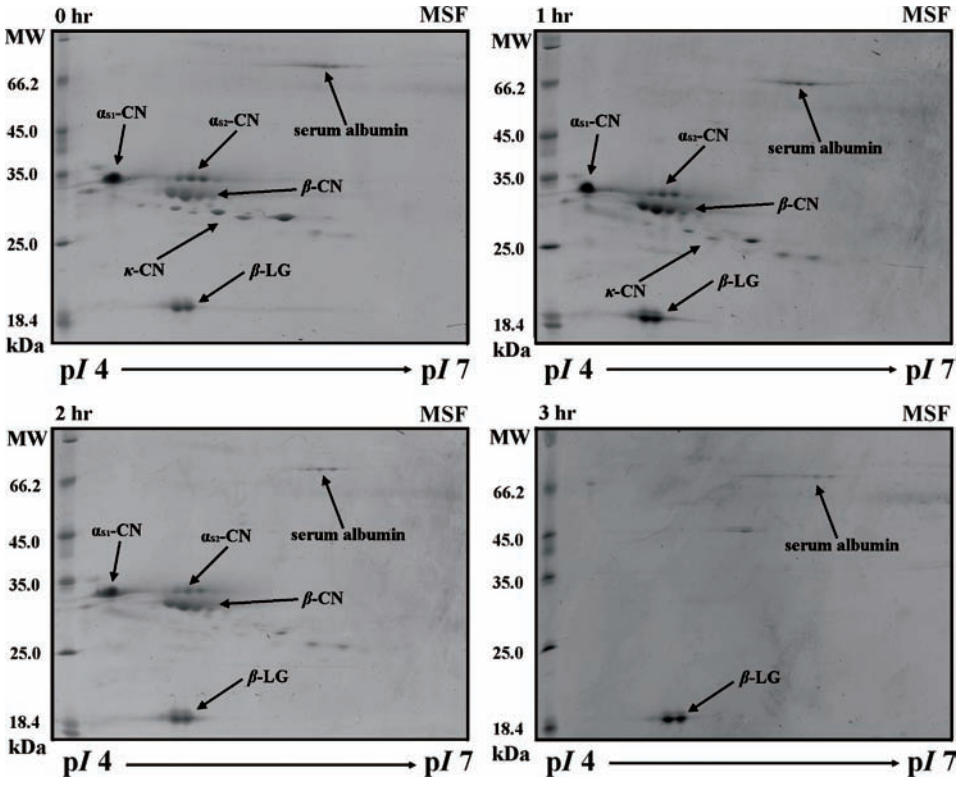

Figure 4. Changes in 2-DE profiles of milk proteins after treatment with chymosin (0.006 units/mL) at 30 °C for various amounts of time. MSF: milk supernatant fraction. MW, molecular weight; p*I*, isoelectric point.

MSF disappeared, but serum albumin (spot 1) and β-LG isomers (spots 14 and 15) were still clearly present (Figure 4). This protease is also known to hydrolyze the Phe_{23}-Phe_{24}, Phe_{88}-Tyr_{89}, and Leu_{192}-Tyr_{193} bonds in α_{S1}-CN, α_{S2}-CN, and β-CN, respectively.[10] We noticed that the intensities of serum albumin, the casein isomers (α_{S1}-, α_{S2}-, β-, and κ-CN), and the β-LG isomer in the chymosin-treated MPF increased with incubation time (Figure 5).

Densitograms corresponding to the 2-DE images of the chymosin-treated MSF and MPF samples were also generated. The fold changes of serum albumin, α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, and β-LG isomers in the chymosin-treated MSF decreased with incubation time. The κ-CN isomers in the chymosin-treated MSF almost disappeared after 2 h of incubation. The fold changes of spot 11 (κ-CN), spot 12 (κ-CN), and spot 13 (κ-CN) in the chymosin-treated MSF were 0.11-, 0.01- and 0.21-fold, respectively. This analysis revealed that 89% of spot 11 (κ-CN), 99% of spot 12 (κ-CN), and 79% of spot 13 (κ-CN) were hydrolyzed by chymosin after 2 h of incubation. We noticed that the profile of spot 11 (κ-CN) was different from that of spot 13 (κ-CN). Similar to the results of Holland et al.,[29] spots 11 and 13 were identified as triglycosylated and

dx.doi.org/10.1021/jf204582g | J. Agric. Food Chem. 2012, 60, 2039−2045

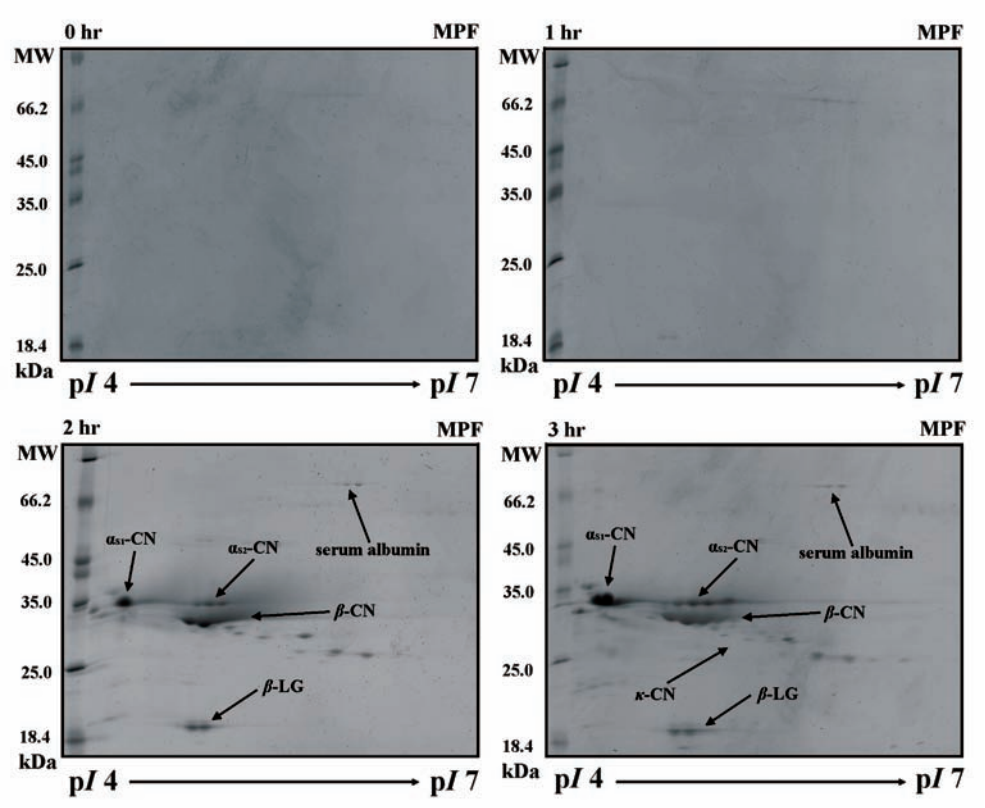

Figure 5. Changes in 2-DE profiles of milk proteins after treatment with chymosin (0.006 units/mL) at 30 °C for various amounts of time. MPF: milk pellet fraction. MW, molecular weight; p*I*, isoelectric point.

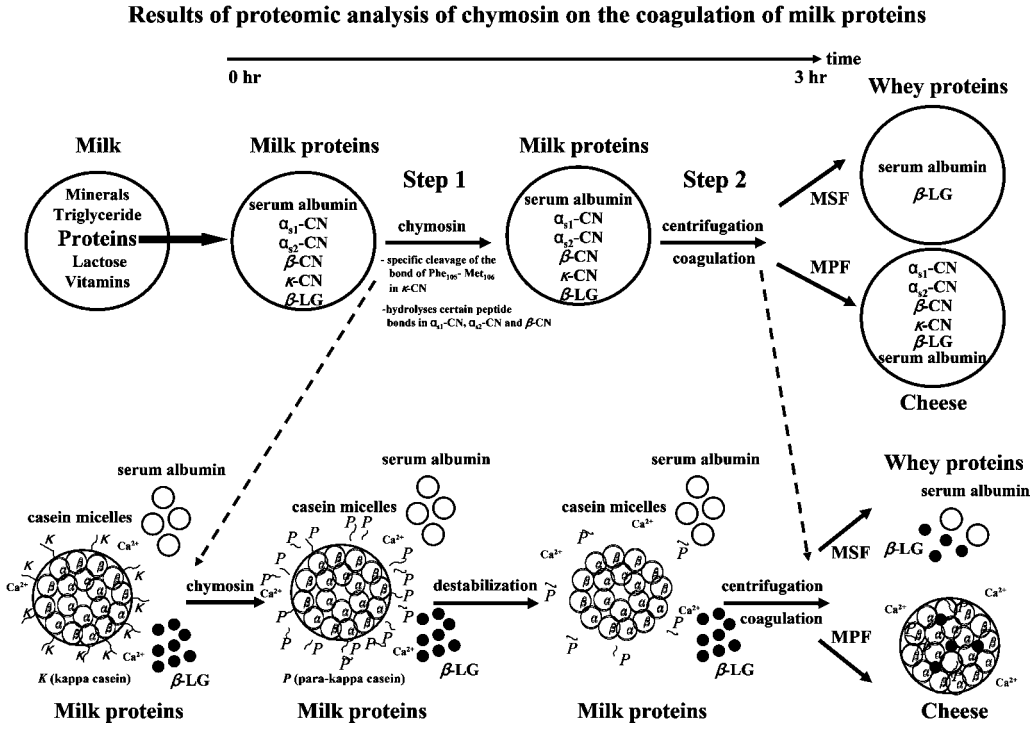

Figure 6. Reaction scheme for the effect of chymosin on the coagulation of milk protein.

monoglycosylated κ-CN, respectively. According to our results, spot 11 had a higher fold change than spot 13, suggesting that triglycosylated κ-CN may be considered as an excellent substrate for chymosin. Furthermore, the fold changes of spot 1 (serum albumin), spot 14 (β-LG), and spot 15 (β-LG) in the chymosin-treated MSF after 3 h of incubation were 0.34-, 0.21-, and 0.32-fold, respectively. From this analysis, 66% of spot 1 (serum albumin), 79% of spot 14 (β-LG), and 68% of spot 15

dx.doi.org/10.1021/jf204582g | J. Agric. Food Chem. 2012, 60, 2039–2045

(β-LG) were considered to be entrapped in the curd. These results suggested that caseins (α_{S1}-, α_{S2}-, β-, and κ-CN) and a portion of serum albumin and β-LG were coagulated to form cheese curds. Moreover, the coagulation of milk proteins was also observed in the chymosin-treated MPF. The serum albumin, α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, and β-LG isomers increased with incubation time.

Reaction Scheme for the Effect of Chymosin on Milk Proteins. Chymosin is the principal protease used for cheesemaking. On the basis of our results, a reaction scheme for the effect of chymosin on the coagulation of individual milk proteins in the cheesemaking process is shown in Figure 6. The chymosin-mediated coagulation reaction includes 2 steps. The first step is an enzymatic process in which chymosin hydrolyzes the caseins. κ-CN is located in the outer region of the casein micelles and is hydrolyzed first by chymosin. Chymosin specifically and rapidly hydrolyzes the Phe_{105}-Met_{106} bond of κ-CN, resulting in the destabilization of the casein micelles. Moreover, the second step of the chymosin-mediated coagulation reaction involves the aggregation of casein micelles. Hydrophobic interactions are an important driving force in coagulation and are enhanced indirectly by Ca^{2+} in milk. Ju and Kilara reported that Ca^{2+}-induced protein aggregation is thought to arise due to 3 effects: (1) electrostatic shielding, (2) ion-specific hydrophobic interactions, and (3) cross-linking of adjacent anionic molecules by forming protein−Ca^{2+}−protein bridges.[30] Therefore, chymosin splits the casein micelles, and then the casein micelles aggregate. These casein micelles contain α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, and a portion of serum albumin and β-LG. The serum albumin and β-LG are thought to entrap in the curd.

In summary, we performed a proteomic analysis of the effects of chymosin on the coagulation of individual milk proteins. SDS−PAGE and 2-DE analyses showed that chymosin hydrolyzed κ-CN and that the coagulation of κ-CN occurred earlier than that of the other caseins (α_{S1}-, α_{S2}- and β-CN). Moreover, a portion of the whey proteins, including serum albumin and β-LG, were entrapped in the curd.

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Funding

We thank the National Science Council (NSC 98-2313-B-030-001) for financial support.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Shui-Tein Chen for technical assistance.

ABBREVIATIONS USED

2-DE, two-dimensional polyacrylamide gel electrophoresis; DTE, 1,4-dithioerythritol; MALDI, matrix-assisted laser desorption ionization; MPF, milk pellet fraction; MS, mass spectrometry; MSF, milk supernatant fraction; MW, molecular weight; p*I*, isoelectric point; Q-TOF, quadrupole time-of-flight; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; α_{S1}-CN, α_{S1}-casein; α_{S2}-CN, α_{S2}-casein; β-CN, β-casein; β-LG, β-lactoglobulin; κ-CN, κ-casein

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