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Proteomic profiling of the coagulation of milk proteins induced by glucono-delta-lactone

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ABSTRACT

This study investigated the glucono-delta-lactone (GDL)-induced coagulation of milk proteins at 30 °C. The addition of 0.5 M GDL caused milk proteins to coagulate following a 1 h incubation period. Approximately 90.7% of milk proteins were coagulated into the milk pellet fraction (MPF), and the protein concentration of the milk supernatant fraction (MSF) decreased from 29.2 ± 1.1 mg mL^{-1} (control) to 2.7 ± 1.1 mg mL^{-1}. The SDS-PAGE analysis demonstrated that the protein bands corresponding to α_S-casein, β-casein and κ-casein in the MSF decreased to 0.2 ± 0.1, 0.5 ± 0.2 and 0.5 ± 0.3% of their original levels, respectively. However, only 29.5% of the β-lactoglobulin was coagulated into the MPF following the treatment with 0.5 M GDL. Two-dimensional electrophoresis analysis indicated that isomers of α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein, as well as a fraction of β-lactoglobulin and α-lactalbumin, were coagulated from the MSF into the MPF following incubation with 0.5 M GDL.

1. Introduction

Milk, a colloidal solution containing lipids, lactose and approximately 3% protein. Milk proteins are fundamental functional constitutes for food manufacturing because of their high nutritional benefits and unique structural and physicochemical properties (Ye & Harte, 2013). Whey proteins, which are composed primarily of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), are major milk proteins and account for 20% of total milk protein. The isoelectric points (p*I*) of β-LG and α-LA are 5.2 and 4.8, respectively (Haginaka, 2000). The other major family of milk proteins is casein, which accounts for 80% of the total milk protein (Morris, Foster, & Harding, 2000). The casein micelles consist of a complex mixture of four common caseins, α_{S1}-casein (α_{S1}-CN), α_{S2}-casein (α_{S2}-CN), β-casein (β-CN) and κ-casein (κ-CN). The p*I* of these proteins range from 4.9 to 5.0 for α_{S1}-CN, from 5.2 to 5.4 for α_{S2}-CN, from 5.1 to 5.4 for β-CN, and from 5.4 to 5.6 for κ-CN (Wal, 2002). Casein micelles can be processed into a wide variety of dairy products, such as cheese and yogurt. The coagulation of milk proteins in the cheese-making process also leads to the destabilization of casein micelles (Singh, Roberts, Munro, & Teo, 1996). In brief, the acidic coagulation of casein micelles commonly involves the fermentation of milk using lactic acid bacteria to produce acid-curd cheese; this is in turn achieved through the conversion of lactose to lactic acid. An alternative approach achieves acidification using an acidulant, such as glucono-delta-lactone (GDL) (Guinee, Feeney, Auty, & Fox, 2002). Fetahagić, Maćej, Djurdjević, and Jovanović (2002) reported that milk acidified under 0.5–3.0% (w/w) GDL at 25–45 °C during cheese preparation. The use of GDL helps reduce a number of difficulties associated with the use of starter lactic bacteria, such as variations related to culture type. Furthermore, GDL does not lead to lactose hydrolysis in milk (Braga, Menossi, & Cunha, 2006).

GDL is a carbohydrate that contains a lactone group. GDL hydrolyzes gradually in water to form gluconic acid, causing a reduction in pH (Lucey, Tamehana, Singh, & Munro, 1998). As the pH of milk is decreased from its natural value of 6.7 by the hydrolysis of GDL, the micellar inorganic calcium phosphate gradually dissolves and becomes fully soluble at a pH of approximately 5.2 (Dalgleish & Law, 1989). However, there is only a slight dissociation of casein from the micelle if the acidification is performed at temperatures greater than 25 °C (Law & Leaver, 1998). As the pH decreases, the surface charges of the casein micelles are balanced, resulting in the collapse of the κ-casein hairy layer. Hence, the steric and electrostatic stabilization are diminished, and at a pH of

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approximately 4.9, the casein micelles coagulate to form a gel (de Kruif, 1997). Therefore, the acidification of milk results in several structural and compositional changes in the casein micelles, which lead to their aggregation at pH 4.9. The addition of GDL promotes the homogeneous acidification of the system. Indeed, in the dairy industry, GDL is used to produce yogurts, cottage cheese and feta cheese because it gives excellent control and reproducibility of the decrease in pH (Martin et al., 2009).

At present, proteomic approaches are commonly used to identify milk proteins using two-dimensional gel electrophoresis (2-DE) coupled to mass spectrometry. 2-DE is commonly employed for proteomic analyses because of the high-resolution and because of the capability for the simultaneous detection and quantification of thousands of protein spots in the same gel. The proteins in the spots isolated from 2-DE gels are digested with trypsin to yield a large collection of peptides. The complex peptide mixture is then analyzed by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Agrawal, Yonekura, Iwahashi, Iwahashi, & Rakwal, 2005). The tandem mass spectra are searched against a protein database to identify the proteins (Hsieh, Yu, Chang, Chen, & Tsai, 2014). In our previously study, we employed 2-DE coupled with mass spectrometry to detect and quantify individual milk proteins (Hsieh & Pan, 2012).

The induced gelation of milk proteins by GDL is of considerable importance in the processing of acidified milk products (Takeuchi, Rosiane, & Cunha, 2008). The use of GDL for the coagulation of casein micelles has previously been reported; however, no previous studies have conducted a proteomic analysis to investigate the coagulation of individual milk proteins. Therefore, our use of proteomic analysis (i.e. by way of SDS-PAGE and 2-DE) to study the effects of GDL on the coagulation of individual caseins and whey proteins is a novel contribution. The objective of this study was to analyze the GDL-induced coagulation of milk proteins using a proteomics-based approach.

2. Material and methods

2.1. Preparation of milk samples containing various concentrations of GDL

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 subsequently pasteurized at 63 °C for 30 min, according to Oeffner et al. (2013). The whey proteins in milk underwent less than 10% denaturation under these pasteurization conditions. The skim milk (30.2 mg mL^{-1}) was collected and stored at 4 °C. The GDL was obtained from Sigma Chemical Co. (St. Louis, MO, USA). To investigate the effects of GDL on the coagulation of milk proteins, milk samples with varying amounts of GDL (0, 0.1, 0.2, 0.3, 0.4 or 0.5 M) were incubated at 30 °C for 1 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 (5000 × g). MSF samples (1 mL) were collected, and MPF samples were resuspended 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.

2.2. Determinations of protein concentrations and pH values

The protein concentrations of the milk samples were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). The Bio-Rad protein-assay dye was diluted with 4 volumes of water and then mixed with individual 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 analyzed as the standard. The pH value was measured with a pH meter (Sartorius Basic Meter PB-10, Germany).

2.3. Sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE)

Milk samples with or without GDL were analyzed by SDS-PAGE. Milk samples were analyzed using a 12.5% separating gel and a 5% stacking gel. For each sample, a 0.1 mL volume of sample was mixed with 0.9 mL of buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, and 70 mM Tris–HCl, pH 6.8) and heated to 95 °C for 5 min. The samples (6 μL) and a protein ladder were loaded into separate wells. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. The stained gels were digitized using an EPSON perfection 1270 image scanner (Epson America Inc., Long Beach, CA, USA) and analyzed using the Gel-Pro Analyzer (version 4.0, Media Cybernetics, Inc.) software programs. The level of protein coagulation induced by GDL was evaluated by the magnitudes of the changes in the electrophoretic profiles.

2.4. Two-dimensional electrophoresis (2-DE)

Milk samples were analyzed by 2-DE according to the method of Hsieh and Pan (2012). For the first separation, 100 μg of total milk protein was immobilized and loaded onto a pH-gradient (IPG) gel strip (pH 4–7, 18 cm; GE Healthcare) that 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. The isoelectric focusing of the strips was performed at 20 °C and 6000 V for a total of 60 kVh using the IPGphor 3 IEF system (GE Healthcare). The strips were equilibrated for 15 min in an equilibration solution (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 2% DTE) and loaded into the top of a vertical 12.5% SDS-PAGE gel with 0.5% agarose. The second electrophoresis step was performed using a Protean II xi Cell System (Bio-Rad) at 10 mA per gel for 1 h, followed by 45 mA per gel for 5 h until the bromophenol blue traveled to the bottom of the gel. After electrophoresis, the gels were immersed in 10% methanol and 7% acetic acid for 30 min before staining overnight in 350 mL of Sypro® Ruby protein gel-stain solution (Lopez et al., 2000). The developed gels were digitally scanned as 2-D images using a Typhoon 9200 imaging system (Amersham Pharmacia Biotech) and analyzed using the Samespots software program (TotalLab Ltd., Newcastle-upon-Tyne, UK).

2.5. Statistical analysis

Data are expressed as the mean values ± standard deviations. The data were analyzed using the Statistical Package for the Social Sciences software program (SPSS for Windows, version 10.0.7C, SPSS Inc., Chicago, IL, USA). The statistical significance of a difference among treatments was determined by one-way ANOVA, followed by a Duncan's multiple-range test to identify the significant differences among means. For the statistical analysis, there were three determinations for each treatment, and the significance level was set at $P < 0.05$.

3. Results and discussion

3.1. Effect of GDL concentration on the coagulation of milk proteins

Milk samples were incubated with varying concentrations of