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Research paper

Rennet-induced coagulation properties of yak casein micelles: A comparison with cow casein micelles

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ABSTRACT

It is essential for yak cheese processing to understand the rennet-induced coagulation properties of gel formation from casein micelles. We have previously discovered that yak milk requires a longer incubation time but forms stronger gels compared with cow milk. In this study, we are aiming to understand the rennet-induced coagulation properties of yak casein micelles comparing with cow casein micelles. Rheological analyses revealed that the gelling times of yak and cow casein micelles were 11.6 ± 0.5 and 8.7 ± 0.4 min ($P < 0.05$) respectively, but yak casein gel had a higher elastic modulus G′ (6.5 ± 0.2 Pa) than cow casein gel (2.5 ± 0.2 Pa; $P < 0.05$). This is consistent with the results obtained by micro-rheology. Confocal laser scanning microscopic images (CLSM) and cryo-scanning electron microscopic images (cryo-SEM) showed that yak casein gel was more homogeneous and had smaller pore size than cow casein gels. Yak casein micelles had higher calcium (26.00 mM), phosphate (19.90 mM) and β-casein (relative 32%) concentrations. In addition, yak casein micelles were larger (Z-average 218.6 nm) than cow casein micelles, and contained lower κ-casein (relative 13%). By comparison with cow casein micelles, yak casein micelle composition corresponding to their micellar calcium phosphate and κ-casein content may greatly contribute to the longer coagulation time and denser gel structure. An initial slower caseinomacropeptide (CMP) release rate and the slower rate of aggregation between *para*-casein micelles contributed to a more homogeneous yak gel network. Higher colloidal calcium phosphate is crucial for yak casein micelle aggregation and gel firmness because sufficient colloidal calcium phosphates can firmly glue sub-micelles and links casein micelles. This study provides valuable information for yak cheese production.

1. Introduction

Yak (*Bos grunniens*) milk is mainly produced in the Chinese Qinghai-Tibet Plateau area at an average altitude of 3000 m above sea level. In China, the annual production of yak milk is > 0.7 million tons. Yak milk contains 16.9–17.7% dry matter, 4.9–5.3% protein, and 5.5–7.2% fat (Li, 2011; Liu et al., 2009) and it is richer in almost all of the main nutritional components compared with cow milk. Traditional yak milk products such as butter and yoghurt are mainly derived from Qula, the raw material used in Tibet for the preparation of buttered tea (Ji, Li, Ma, & Li, 2017). Cheese production has increased worldwide in recent decades (Coetzee, 2014), and yak milk cheese represents a value-added nutritious product that can promote the economic growth of the less developed Qinghai-Tibet Plateau area. Some researchers have been working on yak milk cheese development, Liu et al. (2009) reported that yak milk cheese has adequate yield and good sensory qualities when 3% fermentation starter, 30 μL/100 mL rennet, and 0.3% $CaCl_2$ are used. Li (2011) concluded that the production of yak milk cheese is more time-consuming than the production of cow milk cheese. The difference in milk composition among animal breeds contributes to differences in coagulation properties. However, the rennet-induced coagulation properties of gel formation from yak casein micelles are not fully understood.

The quality of casein gels depends on the physical and chemical properties of casein micelles, the composition and size of which are the most important parameters. The conversion of casein to gel is achieved by the hydrolytic action of chymosin on κ-casein (Phe105-Met106) and the removal of macropeptides from casein micelles. Subsequent

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changes involve the aggregation and fusion of *para*-casein micelles (De Kruif, 2014; Lucey, 2002; S. Sandra & Corredig, 2013). Therefore, casein micelles are essential in the production of cheese. It has been reported that smaller casein micelles form a more compact and firmer gel network than larger micelles, whereas contradictory results have been obtained for rennet clotting time (Glantz et al., 2010). Frederiksen et al. (2011) observed that poorly coagulating and non-coagulating milk samples have a relatively low concentration of κ-casein, because concentration of κ-casein is negatively correlated with casein micellar size. In addition, studies have shown that micelle aggregation is dependent on calcium bridging and hydrophobic interactions (De Kruif, 2012; Zhao & Corredig, 2014). However, the rennet-induced aggregation properties of casein micelles need to be investigated in more details.

The aim of this study is to delineate the rennet-induced coagulation properties of gel formation from yak casein micelles and evaluate the differences and effects of the size, composition of casein micelles on rennet-induced gelation characteristics between yak and cow casein micelles. By determining the concentrations of micellar calcium and phosphorus, casein micelles size distribution and morphology were characterized. Besides, the rate of κ-casein hydrolysis and the coagulation properties of yak casein micelles were measured and compared with those of cow casein micelles. This has allowed us to propose a reason why yak milk requires a longer incubation time and shows stronger gel formation compared with cow milk. Ultimately, we hope to find a way of producing yak cheese more efficiently.

2. Materials and methods

2.1. Preparation of casein micelles

Yak milk was obtained in August of 2016 from Tianzhu grassland on the Qinghai-Tibetan Plateau. After milking, 0.02% (w/v) sodium azide was added. The samples were defatted twice by centrifugation at 4000 × *g* at 20 °C for 30 min. Cow milk collected from a local farm (Gansu Agricultural University, Lanzhou, China) at the same time was subjected to the same treatment.

Casein micelles were obtained by ultracentrifugation at 120,000 × *g* for 40 min at 20 °C according to Wang, Wen, Zhang, Guo, and Ren (2013) in an Optima XPN-100 ultracentrifuge (Beckman, Fullerton, CA, USA) equipped with a type 45 Ti rotor. Following ultracentrifugation, the supernatant was removed, and the pellet was collected after draining by inversion for 5 min and dispersed in simulated milk ultrafiltrate (SMUF) with 0.02% (w/v) sodium azide for 10 h (0.7 g protein/100 mL, calibrated with the Kjeldahl method, ~3.5 g pellet/100 mL). SMUF was prepared according to the method reported by Beliciu and Moraru (2009). The composition of SMUF was 1.58 g/L KH_2PO_4, 1.20 g/L $K_3Citrate{\cdot}H_20$, 1.79 g/L $Na_3Citrate{\cdot}2H_20$, 0.18 g/L K_2SO_4, 1.32 g/L $CaCl_2{\cdot}2H_20$, 0.65 g/L $MgCl_2{\cdot}6H_20$, 0.30 g/L K_2CO_3 and 0.60 g/L KCl, adjusted to pH 6.6 with NaOH.

2.2. Transmission electron microscope analysis of casein micelles

Casein micelles solutions were diluted 1:100 with SMUF to reduce the casein concentration to approximately 350 mg pellet/L. Immediately after dilution, a 10 μL droplet was placed on 300 mesh copper grids for 90 s, and a second droplet was applied for 90 s after blotting the first drop with filter paper. The sample was then wicked off with lens paper, washed with distilled water. After air-drying for 6 h, casein micelles were examined under a Hitachi ht7700 transmission electron microscope (TEM, Hitachi Inc., Tokyo, Japan) operated at 80 kV, according to Karlsson, Ipsen, and Ardö (2007).

2.3. Determination of casein micellar size

Casein micelles solutions were diluted 1:200 with SMUF, and casein micellar size was determined at 25 °C by the dynamic light scattering method (DWS, SZ-100-Z, Horiba Ltd., Kyoto, Japan) according to Giroux, Lanouette, and Britten (2015). The refractive index and viscosity of the solvent were 1.333 and 1.002 mPa × s, respectively. Three replicate measurements were performed for each sample.

2.4. Determination of micellar calcium and phosphorus concentrations

Calcium and phosphorus concentrations were measured by atomic absorption spectrometry (AAS) according to Wang et al. (2013). After the casein micelle pellet had been dispersed in SMUF with 0.02% (w/v) sodium azide for 10 h, the solution was ultracentrifuged at 120,000 × *g* for 40 min at 20 °C and supernatant was collected after draining by inversion for 5 min. The presented results are the averages of three measurements.

Micellar calcium and phosphorus concentrations were determined by the following equation,

Mineral concentration = mineral concentration in solution − mineral concentration in supernatant.

2.5. Determination of casein monomer concentrations

Casein micelles solution (0.5 mL) was diluted with 3 mL of denaturing solution (100 mmol L^{-1} Tris base, 8 mol/L urea, 13 g/L $Na_3Citrate{\cdot}2H_2O$, 0.3%, v/v, β-mercaptoethanol, adjusted to pH 7.0 with HCl) and passed through a 0.45-μm membrane filter (Membrana, Wuppertal, Germany). A 20 μL sample was subjected to reversed-phase high-performance liquid chromatography (HPLC, Shimadzu, LC-20A, Kyoto, Japan) equipped with a Kromasil C4 column (4.6 × 250 mm, 300 Å, AkzoNobel, Bohus, Sweden). Solvent A was water containing 0.1% (v/v) trifluoroacetic acid (TFA) and solvent B was acetonitrile containing 0.1% (v/v) TFA. The flow rate was 0.8 mL/min. A linear gradient from 30.0% to 50.0% solvent B over 50 min was set. The detection wavelength was 220 nm. The relative proportions of individual caseins were calculated from the peak areas as reported by De Kruif and Huppertz (2012). The experiments were performed in triplicate.

2.6. Determination of the degree of κ-casein hydrolysis

Hydrolysis was conducted at 33 °C and stopped by the addition of trichloroacetic acid (20 mg/mL, final concentration) at 0, 1, 2, 3, 5, 8, 12, 20 and 60 min after rennet was added. Each well-stirred sample was centrifuged at 6850 × *g* for 20 min. The supernatants were passed through 0.45 μm membrane filter (Membrana, Wuppertal, Germany). A 100 μL sample was subjected to reversed-phase-HPLC (Shimadzu, LC-20A, Kyoto, Japan) equipped with a PLRP-S 1000 Å 8 μm column (Polymer Laboratories, Shropshire, U.K.). Solvent A was water containing 0.1% (v/v) TFA and solvent B was acetonitrile containing 0.1% (v/v) TFA. The flow rate was 1 mL/min. The gradient for caseinomacropeptide (CMP) started with 10% of solvent B, and this was increased to 20% within 8 min and to 40% within 18 min, and then kept at 90% for 5 min before returning to the initial conditions. The detection wavelength was 226 nm. The relative proportions of individual caseins were calculated from the peak areas as reported by Luo, Wang, Guo, and Ren (2017). Due to the lack of the molecular standard for yak and cow CMP, we assume that the maximum CMP was obtained after 60 min, and this value was set as 100%. The CMP release kinetics was obtained by dividing the CMP area at each time by the area at 60 min for yak and cow casein, respectively. Three replicate measurements were performed for each sample.

2.7. Determination of coagulation properties

Chymosin (Chr-Max Powder Extra, 2235 International Milk Clotting units (IMCU)/g; Chr. Hansen Inc., Hørsholm, Denmark) was added to the casein micelles solutions at a final concentration of 1 mg/mL. The