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The protein interactions and rheological properties of skim milk heated in the presence of low levels of reducing agent

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ABSTRACT

Skim milk with low levels of added β-mercaptoethanol (SM–ME) and untreated skim milk (SM) were heated and then made into acid gels. Acid gels prepared from heated SM–ME had markedly higher firmness and contained more protein connections than acid gels prepared from heated SM. Electrophoretic analyses of the milks showed that the levels of β-lactoglobulin and α-lactalbumin associated with the casein micelles increased with increasing β-ME concentration. The levels of disulphide-linked whey proteins were higher in SM–ME than in SM. This suggested that there may be higher levels of initiators for thiol–disulphide exchange reactions, resulting in an increase in the rate of the reactions and the formation of greater numbers of small aggregates, in SM–ME than in SM. Consequently, acid gels made from SM–ME may have more bonds and more particles participating in the network, resulting in firmer gels, than acid gels made from SM.

1. Introduction

The functional properties of yoghurts and laboratory-replicate yoghurts (acid gels) have been studied extensively as they are important in the commercial market. The firmness of yoghurts is markedly improved when they are made from heated (>70 °C) milk (Lucey, Teo, Munro, & Singh, 1997; Tamime, Marshall, & Robinson, 1995). Heat treatment denatures the whey proteins, resulting in β-lactoglobulin exposing a free thiol group, which can in turn interact with the disulphide bonds of other proteins (e.g. α-lactalbumin, κ-casein and other β-lactoglobulin) via thiol–disulphide exchange reactions to form whey protein/κ-casein aggregates (Jang & Swaisgood, 1990; Lowe et al., 2004). These aggregates can be located in the serum or on the surface of the casein micelles and their distribution is dependent on the pH at which the milk was heated (Anema, 2008; Donato, Guyomarc'h, Amiot, & Dalgleish, 2007; Guyomarc'h, Law, & Dalgleish, 2003). These heat-induced aggregates enhance the firmness of acid gels, with a greater effect when the aggregates are in the serum phase (Anema, Lee, Lowe, & Klostermeyer, 2004). It is considered that the denatured whey proteins that have associated with κ-casein via disulphide bonds provide high levels of cross-linking between the particles in the milk system during acidification and that this contributes to the increase in acid gel firmness (Donato, Alexander, & Dalgleish, 2007; Lucey, Singh, & Munro, 1999). In addition, thiol–disulphide exchange reactions between the proteins may continue during the formation of acid gels and may contribute to their final firmness (Vasbinder, Alting, Visschers, & de Kruif, 2003). Hence, thiol–disulphide exchange reactions that form intermolecular disulphide bonds between proteins play a significant role in determining the functional properties of yoghurts/acid gels.

In skim milk (SM), as β-lactoglobulin is the main source of free thiol groups, the denaturation (unfolding) of β-lactoglobulin can initiate thiol–disulphide exchange reactions (Hoffmann & van Mil, 1997; Roefs & De Kruif, 1994). As reviewed by Morand, Guyomarc'h, and Famelart (2011), increasing intermolecular disulphide bonding between the proteins in milk by increasing the number of free thiol groups in the reactant proteins improves the firmness of acid gels. One approach was to add a reducing agent, such as β-mercaptoethanol (β-ME), to milk to convert disulphide bonds into free thiol groups. Hashizume and Sato (1988) and Goddard (1996) added up to 100 mM β-ME to SM before making gels by heating the treated milks at low pH. The final firmness of the acid-heat-induced gels was increased at low concentrations of β-ME (<10 mM) and was decreased markedly

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at higher concentrations of β-ME. The decrease at high concentrations of β-ME was considered to be due to a complete lack of disulphide bonds because there was a large excess of reducing agent relative to the total number of disulphide bonds in the milk (Goddard, 1996; Hashizume & Sato, 1988).

Other than these studies on acid-heat-induced gels (Goddard, 1996; Hashizume & Sato, 1988), no studies have examined the effect of heating milk in the presence of β-ME on the interactions between the proteins and on the resulting milk products. Therefore, the aim of this study was to investigate the effect of adding low levels of β-ME to SM prior to heating on the functional properties of the acid gels made from the treated milks. The involvement of milk proteins in disulphide interactions and the distributions of the proteins between the colloidal and serum phases were examined to understand how reduction of the disulphide bonds affected the formation of aggregates and, hence, the observed changes in functional properties.

2. Materials and methods

2.1. Preparation of milk samples

SM of 10% w/w total solids was prepared by adding the appropriate quantity of low heat skim milk powder (Fonterra Co-operative Group, New Zealand) to Milli Q water (resistivity $\geqslant$18 MΩ/cm). SM at room temperature had pH ≈6.7. A small quantity of sodium azide (≈0.01% w/v) was added to the milk samples as a preservative. The milk samples were stirred for at least 6 h at room temperature and were kept at ≈5 °C before further use.

2.2. Addition of a disulphide reducing agent to milk samples

The reducing agent β-ME (Sigma–Aldrich, St. Louis, MO, USA) was initially diluted (1:10 (v/v) in Milli Q water) and then added to unheated SM to give β-ME levels from 1.4 to 7.1 mM. The milk was shaken on a vortex mixer for 10 s and then left to react at 20 °C in a thermostatically controlled water bath for 3 h. The milk container was sealed to minimize contact with oxygen in the environment. The SM with added β-ME is referred to as SM−ME.

2.3. Heat treatment of milk samples

Subsamples of milk (6 mL) were transferred to small sealable glass vials and then heated at 80 °C for 30 min in a thermostatically controlled oil bath. After heating, the samples in the vials were rapidly cooled in cold water.

2.4. Centrifugation of milk samples

To separate colloidal protein (pellet) from serum protein (supernatant), milk samples were centrifuged at 20,100*g* and 25 °C for 60 min using a bench centrifuge (Centrifuge 5417R, Eppendorf AG, Hamburg, Germany). Once centrifuged, the supernatant was carefully separated from the pellet. The protein compositions of the original milks and their supernatants were determined by electrophoresis and densitometry. The colloidal protein was determined by subtracting the supernatant protein from the total protein in the milk.

2.5. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA, USA) as described previously (Anema, 2009). The milk samples and the supernatants were diluted 1:40 and 1:20 (v/v), respectively, with SDS sample buffer. Each gel contained at least two fully reduced original milk samples (1:40 dilution). The milk samples and the supernatants were run under both non-reducing conditions and reducing conditions. Under reducing conditions, ≈20 μL of β-ME was added to 1 mL of sample, which was then heated for 10 min at 100 °C; under non-reducing conditions, the samples were analysed without further addition of β-ME.

The gels were run, stained and destained as described previously (Anema, 2009). The destained gels were scanned using an Image Scanner III (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The intensities of the protein bands were integrated using Molecular Dynamics ImageQuant integration software (GE Healthcare Bio-Sciences AB).

The quantity of each protein in the serum phase was obtained from the reduced supernatant samples and was determined as a percentage of the protein in the original milk samples. The quantity of each protein that did not participate in intermolecular disulphide bonds was obtained from the non-reduced milk and supernatant samples and was determined as a percentage of the protein in the original milk samples. The quantification was corrected according to the change in volume induced by dilution differences. As the relation between protein concentration and band intensity is linear, as has been reported previously (Anema, 2009; Hill & Lowe, 1997), the protein concentration effect was eliminated.

2.6. Particle size measurement

The casein micelle size was measured using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, UK), using the techniques described by Anema and Li (2003a).

2.7. Rheological measurement

The rheological properties of the milks during acidification at 30 °C were measured using a US200 rheometer (Paar Physica, Graz, Austria) with a cone (5 cm, 2°) and plate geometry. After the addition of 2% glucono-δ-lactone (GDL) powder, each milk sample was stirred for 30 s and then 1.1 mL was transferred to the rheometer plate. The rheometer cone was lowered until there was a 0.5 mm gap between the cone and the plate. Soya bean oil was added to the edge of the sample to prevent water evaporation.

Gelation was monitored while the sample was oscillated at a frequency of 0.1 Hz and a strain of 0.5. Measurements were made every 0.3 min for 3 h. The pH of the acidified milk was also monitored for 3 h during acidification. The relation between the G' values and the pH values was then established.

2.8. Confocal laser scanning microscopy

Nile blue dye (1% w/v in water) was added to selected milk samples (20 μL of dye per 5 g of milk) before the addition of GDL. The milk sample with added dye and GDL was deposited into the concave region of the glass plate and a coverslip was immediately placed on top, ensuring that no air was trapped. The gel was allowed to form in an incubator set at 30 ± 0.5 °C. After 3 h, the gel sample was examined using a Zeiss LSM510 Meta confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany), using the 63× objective. For imaging of the proteins, the excitation wavelength was 633 nm and the emission at 650–735 nm was collected. The samples were imaged at 5 μm below the surface.