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Acid-induced gelation of enzymatically cross-linked caseinate in different ionic milieus

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ABSTRACT

Acid casein powder was used to prepare caseinate solutions (27 g/kg) with different ionic milieus: sodium caseinate (NaCN, *I* ~0.015 mol/L) and calcium caseinate (CaCN, *I* ~0.03 mol/L) were obtained by neutralisation with NaOH and $Ca(OH)_2$, respectively, and dissolving in phosphate buffer resulted in a high ionic strength caseinate solution (CN-PB, *I* ~0.16 mol/L). Treatment with microbial transglutaminase (mTGase) for defined incubation times lead to different extents of casein cross-linking, which were characterised by size exclusion chromatography and the N-ε-(γ-glutamyl)-lysine isopeptide content (IC). Maximum polymer size was reached at ~90% casein polymerisation, and increased in the order NaCN < CN-PB < CaCN. Further enzyme treatment, however, increased the IC, pointing to cross-links within existing polymers. We suggest that the maximum polymer size is determined by the size of casein particles resulting from self-assembly in solution and that mTGase preferably acts on molecules within the same particle. Enhanced association at higher ionic strength (CN-PB) or in the presence of bivalent cations (CaCN) may therefore result in larger covalently cross-linked casein polymers. Furthermore, oscillation rheometry revealed that the relationship between casein cross-linking and stiffness of gels acidified with glucono-δ-lactone depends on the ionic milieu. While for NaCN G'_{MAX} increased with the time of cross-linking, the presence of ions resulted in the highest G'_{MAX} at moderate cross-linking intensities. This was also observed when NaCl was added to cross-linked NaCN. The results suggest that electrostatic attraction during gel formation are interfered by ions and cannot be compensated by rearrangements in case of extensively cross-linked casein particles.

1. Introduction

Casein is the major protein fraction in milk. As a consequence of weak non-covalent interactions between caseins and strong electrostatic interactions with colloidal calcium phosphate, the molecules are associated to so called casein micelles (de Kruif, Huppertz, Urban, & Petukhov, 2012). These are disintegrated by acidification as charge neutralisation causes the dissociation of colloidal calcium phosphate, and the loss of electrostatic repulsion results in precipitation at pH ~4.6 (O'Regan & Mulvihill, 2011).

Precipitated casein can be redissolved in water by neutralisation with alkali, using, e.g., NaOH that results in sodium caseinate (NaCN), or $Ca(OH)_2$ that results in calcium caseinate (CaCN) (O'Regan & Mulvihill, 2011). In NaCN solutions, molecules were shown to self-assemble to small particles (Chu, Zhou, Wu, & Farrell, 1995), and this association was more pronounced at increasing temperature, decreasing pH, and increasing ionic strength (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008). Huppertz et al. (2017) recently suggested that particles in NaCN solution are similar to the primary casein particles which casein micelles consist of. Self-association was also observed in solutions of pure β- or κ-casein (Cragnell et al., 2017; Dauphas et al., 2005; Moitzi, Portnaya, Glatter, Ramon, & Danino, 2008; O'Connell, Grinberg & de Kruif, 2003a; Ossowski et al., 2012), and this had substantial consequences for the enzymatic cross-linking of casein. Microbial transglutaminase (mTGase; EC 2.3.2.13), which catalyses the formation of isopeptide bonds between protein-bound glutamine and lysine residues (Buchert et al., 2010; Jaros, Partschefeld, Henle, & Rohm, 2006; Romeih & Walker, 2017), was found to act mainly on casein molecules within the same β- or κ-casein particle. Thus, intramolecular isopeptide bonds were formed when β-casein existed as individual molecules at low temperature (around 0 °C), whereas polymerisation occurred when either β- or κ-casein was

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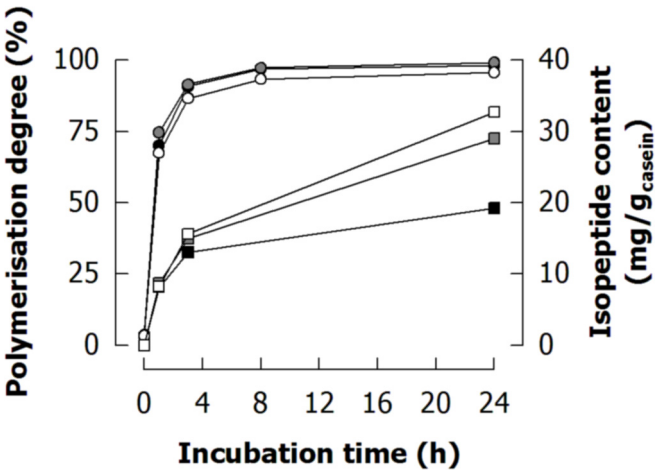

Fig. 1. Polymerisation degree (circles) and isopeptide content (squares) of sodium caseinate (white), casein in 0.1 mol/L phosphate buffer (grey), and calcium caseinate (black) after incubation with 3 U microbial transglutaminase per g casein at 40 °C.

associated to particles at elevated temperature (35–40 °C) (de Kruif, Tuinier, Holt, Timmins, & Rollema, 2002; O'Connell & de Kruif, 2003b).

We suggest that this behaviour applies also for caseinate solutions which comprise of α_{S1}-, α_{S2}-, β-, and κ-casein in a ratio of approx. 3.8: 1: 3.5: 1.5 (O'Mahony & Fox, 2013). This would mean that there is a maximum polymer size with respect to the number of molecules within an individual casein particle. Furthermore, this maximum polymer size might be adjustable via changes in temperature, pH, and ionic strength (HadjSadok et al., 2008).

In previous studies we dissolved acid casein powder in 0.1 mol/L sodium phosphate buffer (pH 6.8), resulting in a system with a relatively high ionic strength. Cross-linking with 3 U mTGase per g protein at 40 °C lead to almost complete polymerisation of casein during incubation for 24 h as was indicated by a disappearing monomer peak in size exclusion chromatography (SEC) (Jaros, Jacob, Otto, & Rohm, 2010; Rohm, Ullrich, Schmidt, Löbner, & Jaros, 2014). A considerable amount of isopeptide bonds was, however, formed even after ~95% of casein was polymerised, but almost no changes in the polymer fraction were observed in SEC (Jaros et al., 2014a, b). This underlines the idea of a maximum polymer size. Interestingly, acidification of the cross-linked casein with glucono-δ-lactone (GDL) resulted in gels with the highest stiffness after 3 h incubation (~80% of casein was polymerised), and further cross-linking lead to lower gel stiffness (Jaros et al., 2014a, 2010; Rohm et al., 2014).

In other studies NaCN was used without addition of ions, however, cross-linking of casein with laccase (EC 1.10.3.2), tyrosinase (EC 1.14.18.1), or mTGase was less extensive and considerable amounts of monomers remained (Ercili Cura et al., 2010, 2009; Myllärinen, Buchert, & Autio, 2007). Therefore, comparability to our gelation experiments is limited.

The aim of this study was to compare different systems reported in literature (i.e., acid casein in phosphate buffer and NaCN solution) to examine the impact of ionic strength on casein polymerisation by mTGase, and on acid-induced gelation of cross-linked casein. Furthermore, CaCN was included as substrate where Ca^{2+} ions cause aggregation of mainly α_{S1}- and α_{S2}-casein and partly β-casein to particles with diameters of up to 430 nm (Cuomo, Ceglie, & Lopez, 2011; Pitkowski, Nicolai, & Durand, 2009; Smialowska, Matia-Merino, Ingham, & Carr, 2017; Thomar, Gonzalez-Jordan, Dittmer, & Nicolai, 2017). To the best of our knowledge, CaCN was not used before for this kind of cross-linking and gelation experiments.

2. Materials and methods

2.1. Materials

Fresh raw milk was obtained from a local farmer (Pulsnitz, Germany), and standards of α_S-, β- and κ-casein were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Microbial transglutaminase (Activa MP from *Streptomyces mobarensis*) received from Ajinomoto Foods Europe SAS (Hamburg, Germany) had an enzyme activity of 92 U/g (determined by the hydroxamate method; Folk & Cole, 1966). Glucono-δ-lactone was obtained from Kampffmeyer Nachf. GmbH (Ratzeburg, Germany).

2.2. Sample preparation

Raw milk was skimmed by centrifugation (3000 g, 6 °C, 20 min; Heraeus BioFuge Stratos, Thermo Electron Corporation, Waltham, MA, USA) and adjusted to pH 4.6 with 6 mol/L HCl at room temperature for casein precipitation. The precipitate was separated from whey by filtration through cellulose filter (Rotilabo type 600P, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), washed with 0.02 mol/L sodium acetate buffer (pH 4.6), and freeze dried (Martin Christ GmbH, Osterode am Harz, Germany). This resulted in acid casein powder with a crude protein content of 889 g/kg as determined by Kjeldahl method (N x 6.38; IDF, 1979).

For preparation of NaCN and CaCN solutions, acid casein powder was dispersed in demineralised water and dissolved at room temperature by neutralising (pH 6.8) with 1 mol/L NaOH or 0.02 mol/L $Ca(OH)_2$, respectively. To obtain a system with high ionic strength, the powder was dissolved in 0.1 mol/L phosphate buffer (pH 6.8) (CN-PB) (see previous studies; Jaros et al., 2010; Raak, Gehrisch, Rohm, & Jaros, 2015). Target protein concentration was 27 g/kg, and 0.3 g/kg sodium azide was added for preservation. Based on ion concentrations required for the preparation step, ionic strengths of ~0.015 mol/L, ~0.16 mol/L, and ~0.03 mol/L were calculated for the (protein-free) serum phases of NaCN, CN-PB, and CaCN, respectively.

Enzymatic cross-linking of casein was achieved by incubation with 3 U mTGase per g casein at 40 °C. The enzyme was inactivated by heat treatment (85 °C, 10 min) after 1, 3, 8, or 24 h, and reference samples (0 h) were treated in the same way without enzyme addition. In an additional sample set, NaCl was added to fresh NaCN solutions after enzyme treatment in concentrations of 0, 0.1, or 0.2 mol/L, and $CaCl_2$ was added in a concentration of 0.015 mol/L, the highest amount not causing casein precipitation, followed by pH readjustment to 6.8 using 1 mol/L NaOH.

2.3. Evaluation of casein cross-linking

The amount of cross-links expressed as isopeptide content (IC, mg N-ε-(γ-glutamyl)-lysine per g casein) was determined by amino acid analysis according to Lauber, Henle, and Klostermeyer (2000) after a three-step enzymatic hydrolysis of the samples according to Henle, Walter, and Klostermeyer (1991). Casein polymerisation was investigated using SEC with an urea containing buffer to suppress non-covalent interactions and after treating the samples with dithiothreitol to cleave disulphide bonds. The polymerisation degree (PD) was calculated from the chromatograms by relating the peak area of polymerised casein to the entire area (Lauber et al., 2000). Details on equipment, buffers, and procedures are given in a previous paper (Raak, Rohm, & Jaros, 2017a). All determinations were carried out as two individual experiments.

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