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Characterization of rennet-induced gels using calf rennet processed by high pressure homogenization: Effects on proteolysis, whey separation, rheological properties and microstructure

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ABSTRACT

This study evaluated the coagulation of milk during 24 h using calf rennet previously subjected to high pressure homogenization (HPH). The enzyme solution was processed at 190 MPa and gel formation was followed by the analysis of proteolysis, syneresis, a rheological assay and confocal microscopy (CSLM). The results showed that HPH reduced the unspecific proteolytic activity of calf rennet and increased the rate of milk clotting. After 24 h, the gels produced with the enzyme processed at 190 MPa formed a more compact network, evidenced by higher syneresis 8.42%, greater values of G′ (23.5%) and G″ (30%) and lower porosity, when compared to gels produced with non-processed enzyme. Therefore, the HPH improved the performance of calf rennet during milk coagulation and the characteristics of the rennet-induced gels.

Industrial relevance: The changes caused by HPH improved the hydrolytic characteristics of calf rennet producing stronger, compact and more consistent milk gels, which possibly results in gels with a higher yield (due to the increment in water binding capacity of the protein) and increased dry matter (due to the reduction in small peptides lost to the whey). Additionally, the expected proteolysis reduction of the gel probably extends the shelf-life of fresh cheese. Therefore, the HPH processing of calf rennet can improve its application in the dairy industry to obtain fresh cheese with high yield, excellent quality and an extended shelf-life.
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1. Introduction

Enzymatic coagulation involves the steps of κ-casein hydrolysis, aggregation of the casein micelles in the presence of Ca^{2+} and development of the coagulated gel. Crosslinks are progressively formed between the chains of flocculated micelles to form the final gel (Bonisch, Heidebach, & Kulozik, 2008; Fox & McSweeney, 1998; Karlsson, Ipsen, & Ardo, 2007; Lagaude, Fernandez, Cuq, & Marchesseau, 2004; Lucey, 2002; Lucey, Tamehana, Singh, & Munro, 2000). After coagulation, the formation of the gel network continues and the characteristics of the enzymatic gel, such as water retention capacity, syneresis and strength are important in the cheese production process, since they affect product parameters such as yield, moisture and texture (Pandey, Ramaswamy, & St-Gelais, 2000).

The kinetics of syneresis is complex and still not well understood (Lodaite, Östergren, Paulsson, & Dejmek, 2000). A deeper understanding of syneresis and its impact on the relevant factors can be investigated using models based on measured material parameters such as porosity, permeability and appropriate rheological coefficients of the casein matrix. Several fundamental studies on the syneresis of rennet-induced casein gels have been reported (Lucey, 2002; Lucey et al., 2000; van Dijk & Walstra, 1986; van Vliet, van Dijk, Zoon, & Walstra, 1991; Walstra, van Dijk, & Geurts, 1985).

The rheology of cheese is a function of its composition, microstructure (i.e., the structural arrangement of its components), the physicochemical state of its components and its macrostructure, which reflects the presence of heterogeneities such as curd granule junctions, cracks and fissures. The physicochemical properties include parameters such as the degree of hydrolysis and hydration of the *para-casein* matrix, and the level of inter-molecular attractions between *para-casein* molecules (O'Callaghan & Guinee, 2004).

The first 24 h after coagulation are the most important for the biochemical profile of cheese during ripening, especially for model cheese systems (which have high proteolytic activity due to the high enzyme concentration, high temperature and moisture content) (Picon, Gaya, Medina, & Nunez, 1995; Silva & Malcata, 2004, 2005). Casein aggregation and syneresis continue taking place throughout the early stages of ripening. The casein becomes compacted inside the curd, water is lost, and fat globules are entrapped and compressed,

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all of which are determinant in the final cheese structure and composition (Choisy, Desmazeaud, Gripon, & Lamberet, 2000; Green & Grandison, 1999; Wilkinson & Kilcawley, 2005). Primary proteolysis is possibly the most important set of events toward the development of flavor and texture in ripened cheeses (Fox, Law, McSweeney, & Wallace, 1999).

It can be seen that various factors can affect cheese production and that the choice of coagulant can change these properties. Thus, the use of new enzymes with greater specificity and different activity can affect the rheological and microstructural properties of the cheese and its proteolysis. Previous results obtained by Leite Júnior, Tribst, and Cristianini (2014) found that the high pressure homogenization (HPH) of calf rennet at 190 MPa caused a reduction in proteolytic activity (52% loss in activity), increased the milk-clotting rate and improved the G′ values obtained for milk coagulation. Considering this previous data, the present work evaluated the impact of calf rennet high pressure homogenized at 190 MPa in the formation of rennet-induced gels during 24 h at 35 °C, aiming to elucidate the effect of the processed enzyme in the casein gel formation, development and stability.

2. Material and methods

2.1. Calf rennet and high pressure homogenization

A commercial calf rennet was used in the experiments (freeze dried powder — Carlina™ Animal Rennet 1650 — Danisco, Vinay, France). This enzyme is composed of 94% chymosin and 6% pepsin.

A Panda Plus high-pressure homogenizer (GEA-Niro-Soavi, Parma, Italy) was used for processing. This equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa and operates at a flow rate of 9 $L \cdot h^{-1}$.

A volume of 1 L of 1.5% (w/v) calf rennet solution prepared in 0.2 M sodium acetate buffer (pH 5.1) was homogenized at 190 MPa using an inlet temperature of 23 °C. After exiting the homogenizing valve, samples (200 mL) were collected and cooled immediately in an ice bath (Leite Júnior et al., 2014). A non-processed sample of calf rennet was evaluated as the control sample.

2.2. Capillary zone electrophoresis of rennet-induced gels

The capillary electrophoresis analysis was determined according to the conditions described by Ortega, Albillos, and Busto (2003) and Otte, Zakora, Kristiansen, and Qvist (1997). The samples were first prepared as follows: an aliquot of 60 mL of skim milk powder reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids) with the addition of 0.01 M $CaCl_2$ and 0.05% (w/v) sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 min. Subsequently, 300 μL of enzyme solution (0.3%, w/v) prepared in 0.1 M acetate buffer (pH 5.1) was added, and the time started. After 40 min, 3 h, 6 h, 18 h and 24 h of coagulation at 35 °C, 20 mg samples were collected and dissolved in 1 mL of 10 mM sodium phosphate buffer solution containing 8 M urea (Merck, Darmstadt, Germany) and 10 mM of dithiothreitol (DTT, Sigma Chemical Co., St. Louis, USA) at pH 8, and left for 1 h at a temperature of 23 °C before filtration (0.22 μm Millex-GV_{13}, Millipore, Molsheim, France).

Capillary zone electrophoresis (CZE) was then carried out using a Beckman P/ACE MDQ system (Beckman Coulter, Santana de Parnaiba, SP, Brazil) controlled by 32 Karat software (Beckman Coulter). The separations were carried out using a 57 cm (50-cm effective length to detector) × 75 μm I.D. fused-silica capillary column (eCap™, Beckman Instruments Inc., San Ramon, CA, USA). The running buffer was prepared with 10 mM sodium phosphate containing 6 M urea and 0.05% hydroxypropyl methylcellulose (HPMC, Sigma Chemical Co., St. Louis, USA); the pH was adjusted to 3.0 with 1 M HCl. All the buffer solutions were filtered through 0.22 μm filters (Millipore, Molsheim, France) before use.

All experiments were carried out in the cationic mode (anode at the inlet and cathode at the outlet). The sample was introduced by pressure injection for 5 s at 0.5 psi. During sample analysis, a constant voltage (18.5 kV, ~35 μA) was applied and the separation temperature maintained at 23 °C by circulating coolant around the capillary column. For all experiments, electrophoresis was carried for 70 min and detection was at 214 nm (data collection rate 5 Hz). After each electrophoretic run the capillaries were conditioned by washing with 0.5 M NaOH for 5 min, then with pure water for 5 min, and finally with running buffer for 5 min.

The first electropherogram in each series was always discarded. The repeatability of peak areas was assessed by replicate injections ($n = 3$). Standard curves were made with a mixture of α_s-casein (C-6780), β-casein (C-6905) and κ-casein (C-0406) at 10 $mg \cdot mL^{-1}$ obtained from Sigma Chemical Co. (St. Louis, MO, USA) in the ratio of 1:1:1. The peaks were assigned based on previous reports by Ortega et al. (2003) and Albillos, Busto, Perez-Mateos, and Ortega (2006).

2.3. Determination of spontaneous syneresis of the rennet-induced gels by the siphon method

The level of spontaneous whey separation in undisturbed rennet-induced gels was determined using the siphon method according to Amatayakul, Sherkat, and Shah (2006). An aliquot of 60 mL of skim milk powder reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids) with the addition of 0.01 M $CaCl_2$ and 0.05% (w/v) sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 min. Subsequently, 300 μL of enzyme solution (0.3%, w/v) prepared in 0.1 M acetate buffer (pH 5.1) was added and the time started. After 40 min, 3 h, 6 h, 18 h and 24 h coagulation at 35 °C, the samples were cooled to 5 °C and then inclined at an angle of 45° and maintained in this position to rest for a period of 1 h. Exuded serum was collected from the surface of the samples (using a syringe) and weighed. Syneresis was expressed as the percent weight of the whey divided by the initial weight of the gel sample, according to Eq. (1).

$$\text{Whey separation}(\%) = (S_F/P_T{-}P_E) \times 100 \quad (1)$$

where, S_F is the weight of the separated whey, P_T is the sample weight plus packaging and P_E is the weight of the empty package.

2.4. Rheological assays of the coagulation process and rennet-induced gels

Milk coagulation was evaluated by monitoring the milk coagulation process using a time sweep in a low deformation oscillatory test in a controlled stress rheometer (AR2000ex, TA Instruments, USA).

The experiments were determined according to Leite Júnior et al. (2014) using 60 mL of skim milk powder reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids) with the addition of 0.01 M $CaCl_2$ and 0.05% (w/v) sodium azide (Merck, Darmstadt, Germany). This mixture was pre-incubated at 35 °C/10 min. Subsequently, 300 μL of enzyme solution (0.3%, w/v) prepared in 0.1 M acetate buffer (pH 5.1) was added, and the sample immediately transferred to the rheometer cup (30 mm diameter and 80 mm height). *Vaned Quarter* geometry (with 28 mm of diameter and 42 mm of length) was used with a 4 mm *gap*. The stress was set at 0.1 Pa and frequency of 0.1 Hz. The parameters G′ (storage modulus) and G″ (loss modulus) were measured at 3 minute intervals (up to the first 40 min) and then at 10 minute intervals (up to 24 h) of the clotting process at 35 °C. The loss tangent (tan δ) was calculated from the G″/G′ ratio. The temperature was controlled using a *Peltier* system.

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