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Comparative study among rheological, near-infrared light backscattering and confocal microscopy methodologies in enzymatic milk coagulation: Impact of different enzyme and protein concentrations

Bruno Ricardo de Castro Leite Júnior [a], Alline Artigiani Lima Tribst [b], Marcelo Cristianini [a, *]

[a] Department of Food Technology (DTA), School of Food Engineering (FEA), University of Campinas (UNICAMP), Monteiro Lobato, 80, 13083–862, Campinas, S.P., Brazil

[b] Center for Food Studies and Research (NEPA), University of Campinas (UNICAMP), Albert Einstein, 291, 13083-852, Campinas, S.P., Brazil

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ABSTRACT

Enzymatic milk coagulation affects parameters such as yield, moisture content and texture and various methodologies have been used to evaluate this process. Thus, the present work comparatively evaluated milk coagulation with different enzyme (10.00, 16.67 and 23.33 IMCU.L^{-1} milk) and protein (1.5, 3.0 and 6.0%) concentrations using rheological methods, near-infrared (NIR) light backscattering and confocal microscopy. The enzymatic hydrolysis (first phase of coagulation) can be explained by the parameters determined in the NIR light backscatter profile. All the methodologies were capable of describing the protein network aggregation process (second phase of coagulation), and using the rheological trials and NIR light backscattering, the gels with greater enzyme or protein concentrations were shown to have more protein aggregation, resulting in more consistent gels. In addition the start of aggregation could be measured and the formation of the gel microstructure visually accompanied by confocal microscopy. Thus, all the methods could be used to make an overall evaluation of the milk coagulation phenomenon, but the method should be chosen according to the parameters one desires to use to compare the different samples.

1. Introduction

The enzymatic milk coagulation step is of great commercial interest in cheese manufacture (Horne & Banks, 2004), since an understanding of this step is fundamental in determining parameters such as yield, moisture content and texture (Pandey, Ramaswamy, & St-Gelais, 2000).

The casein micelles remain suspended in the milk and are stabilized by electrostatic repulsions and impedance of a steric nature due to the presence of negatively charged κ-casein (~−20 mV) on the molecule surface (Fox, Mc Sweeney, Cogan, & Guinee, 2004). Enzymatic milk coagulation involves the phases of enzymatic hydrolysis and micelle aggregation (Dalgleish, 1993; Horne & Banks, 2004; McMahon & Brown, 1984). The first phase is characterized by the enzymatic action of the rennet, which acts on κ-casein in a specific way, cleaving the bond Phe_{105}-Met_{106}, forming para-k-CN and allowing the casein macropeptide (CMP) to go into the aqueous phase (Horne & Banks, 2004; Janhøj & Qvist, 2010; Lucey, 2002). This cleavage results in a reduction in the negative charge (decreasing electrostatic repulsion) and a decrease in the steric impediment of the casein micelle (Horne & Banks, 2004). The start of the second phase of coagulation is characterized by micelle aggregation in the presence of Ca^{2+} at a temperature above 20 °C (Fox et al., 2004). This aggregation starts only when a considerable amount of κ-casein was cleaved by the enzyme (60–80%) for unconcentrated milk (Dalgleish, 1993; Fox et al., 2004; Horne & Banks, 2004; Walstra, Wouters, & Geurts, 2006) or lower percentage (~20%) when coagulation is performed in concentrated milk (Karlsson, Ipsen, & Ardö, 2007). In addition, for unconcentrated milk, significant protein aggregation only occurs when 90% of the

* Corresponding author.
E-mail address: olecram@unicamp.br (M. Cristianini).

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casein has been hydrolyzed (Dalgleish, 1979).

Although the nature of the attractive forces during casein micellar aggregation has still not been completely elucidated (Lucey, 2002), the Ca^{2+} bonds, van der Waals forces and hydrophobic interactions are considered important for this aggregation (Walstra, 1990). After the initial accommodation of the network, the gel develops with an increase in the mechanical strength due to an intensification of the bonds between the gel proteins up to the moment of cutting (Janhøj & Qvist, 2010).

In gels formed by the action of rennet, the percentage of non-hydrolyzed κ-casein and other types of bonding present on the surface of the micelle can represent a barrier (both steric and electrostatic) in order to aggregate the micelles, with a consequent reduction in the firmness of the gel obtained (Lucey, 2002). Thus the enzyme concentration and type, the protein concentration, the amount of soluble calcium and the temperature, amongst other factors, affect the milk coagulation process (Dalgleish, 1993; Horne & Banks, 2004; Lucey, 2002).

Different techniques are available to accompany milk coagulation, and Lucey (2002) carried out a review in which he emphasized various methods capable of accompanying the process. However, a comparison amongst the methods cited in order to determine the advantages and limitations of each one is impossible, since each study was performed using different milk compositions, enzymes (type and concentration), calcium amount and temperature of coagulation. Thus the objective of the present study was to carry out a true comparative evaluation amongst the rheological methods, near-infrared (NIR) light scanning and microscopy, in order to accompany the milk coagulation step and characterize the gels obtained as a function of different protein and enzyme concentrations. The results will permit one to determine if the methods are complementary or substitutes one for the other, and the advantages and limitations of each one.

2. Materials and methods

2.1. Milk and enzyme

Milk was obtained by reconstituting skimmed milk powder (36.9% protein) produced using a low heat process (particle temperature < 55 °C) (Tagará Foods, Brazil). Skimmed milk powder was reconstituted using different concentration of powder - 4.07 (w/v), 8.13% (w/v) and 16.26% (w/v) -, resulting in concentrations of 1.5 (w/v); 3.0 (w/v) and 6.0% (w/v) of protein, respectively. 0.01 M $CaCl_2$ was added to all the milks.

The commercial enzyme used was recombinant chymosin with an activity of 2500 IMCU.g^{-1} (CHY-MAX® M 2500 Power NB, Chr Hansen, Hoersholm, Denmark). For the experiments, enzyme solutions were prepared in a sodium acetate buffer (0.2 M, pH 5.6) at concentrations of 0.03% w/v (10.00 IMCU per liter of milk); 0.05% w/v (16.67 IMCU per liter of milk) and 0.07% (23.33 IMCU per liter of milk).

Using these different enzyme solutions and milk protein concentrations five milk coagulation assays were carried out: (i) using milk with 1.5% of protein and an enzyme solution with a concentration of 16.67 IMCU per liter of milk (EN 0.05%_PTN 1.5%), (ii) using milk with 3.0% of protein and an enzyme solution with a concentration of 10 IMCU per liter of milk (EN 0.03%_PTN 3.0%), (iii) using milk with 3.0% of protein and an enzyme solution with a concentration of 16.67 IMCU per liter of milk (EN 0.05%_PTN 3.0%), (iv) using milk with 3.0% of protein and an enzyme solution with a concentration of 23.33 IMCU per liter of milk (EN 0.07%_PTN 3.0%) and (v) using milk with 6% of protein and an enzyme solution with a concentration of 16.67 IMCU per liter of milk (EN 0.05%_PTN 6.0%).

2.2. Rheological assays of the coagulation process

The milk coagulation process was evaluated by way of a time sweep using a low deformation oscillatory test with a controlled stress rheometer (AR2000ex, TA Instruments, USA).

Two types of small amplitude oscillatory rheological assays were carried out. The first analysis used 60 mL of skimmed milk powder reconstituted (described in item 2.1) and pre-incubated at 35 °C for 10 min. Subsequently, 0.8 mL of enzyme was added and the mixture immediately transferred to a rheometer cup (30 mm diameter and 80 mm height) with a *vaned quarter* geometry (28 mm of diameter and 42 mm of length) and 4 mm gap. The stress was set at 0.1 Pa, frequency at 0.1 Hz and the parameter G′ (storage modulus) read at 1 min intervals for 40 min of the clotting process at 35 °C (Leite Júnior, Tribst, & Cristianini, 2014). The temperature was controlled by a *Peltier* system. Furthermore, the rate of milk-clotting was calculated as the variation of log G′ with log time (dG′/dt) at 3 min intervals, and expressed in Pa.min^{-1}. The storage modulus (G′) describes the elastic (solid) behavior of the product, and consequently, the energy stored and released during each oscillatory cycle.

The second analysis consisted of evaluating the gels after a 40 min period of coagulation at 35 °C, using the same rheometer geometry, but with frequency sweep procedures. Since this analysis preserves the original structure of the gel (Oliveira, Augusto, Cruz, & Cristianini, 2014), it is necessary to choose a strain within the linear viscoelastic region. For this purpose, a strain sweep (0.01–10 Pa) procedure under a fixed frequency of 1.0 Hz was carried out, and the stress within the linear viscoelastic region of 1 Pa determined. Finally, a frequency sweep (0.01–100 Hz) procedure under a controlled strain of 1 Pa was carried out, and the viscoelastic parameter G′ (storage modulus, elastic behavior) attained (Oliveira et al., 2014). For the evaluation of the experimental results, the values obtained before breaking of the gel were used according to the power law model (Eq (1)).

$$G' = k' \cdot \omega^{n'} \quad (1)$$

where G′ is the storage modulus, which represents the elastic behavior of the solid (Pa), k′ is the consistency index (Pa·$s^{n'}$), ω is the oscillation frequency (Hz) and n′ is the behavior index. The parameters of the model were obtained by linear regression after linearization using the software Microsoft Excel 2007, with a significant probability level of 95% (Oliveira et al., 2014).

2.3. Near-infrared (NIR) light backscattering of the gels obtained by the enzyme-induced milk coagulation process

Milk coagulation was monitored using a near-infrared light backscatter at 880 nm (Turbiscan MA2000, Formulaction, Ramonville St. Agne, France). For this, 15 mL of skimmed milk powder reconstituted (described in item 2.1) was pre-incubated at 35 °C for 10 min. Subsequently, 0.2 mL of enzyme was added to the milk. The mixture was immediately transferred to borosilicate glass tubes (12 mm inner diameter and 30 mm high) and the time started. The light source scanned the sample at 1 min intervals from bottom to top with an infrared wavelength (880 nm), and measured the percentage of light backscattered during 40 min at 35 °C. The variation in delta backscattering (ΔBS) was determined from the difference in backscattering between control samples (milk with no added enzyme) and samples with added enzyme. An increase in ΔBS is associated with an increase in particle concentration and size. The data were analyzed using the Turbisoft 2.0 software (Zhao et al., 2014).