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Molecular drivers of structural development in Mozzarella cheese

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ABSTRACT

Mozzarella cheese undergoes significant structural rearrangement during maturation. This evolution in structure is critical to the product's functional performance. Understanding the driving forces responsible for these changes is of significant importance for manufacturers as it allows them to tailor their product and processes to optimise functionality. This investigation took a hierarchical approach to examine structural change and component mobility that occurs following manufacture. This brings together microscopy techniques with both ^{1}H and ^{31}P nuclear magnetic resonance. This enabled the observed structural evolution to be coupled with molecular mobility with regards to both water and phosphorous. Two primary drivers for change were proposed from which the other processes cascaded: changing strength in hydrophobic interactions and proteolytic breakdown. Initially the development of the cheese structure was driven primarily by a relaxation in protein matrix (caused by weakening hydrophobic interactions), resulting in the moisture equilibration processes. Further structural changes occurred as a result of the proteolytic breakdown of the casein and a possible relaxation in the protein structure. These proteolytic mechanisms dominated maturation behaviour after the moisture equilibration processes were substantially completed (typically >20 days).

1. Introduction

Cheese has a bi-continuous structure consisting of a casein matrix interrupted by fat (de Kruif et al., 1995). The water in the cheese structure is entrapped within the protein phase. Low moisture part skim Mozzarella cheese is one of the most consumed cheeses in the world (Francolino et al., 2010). Mozzarella is commonly referred to as a fresh cheese; however, it often undergoes a period of ripening to attain a desired level of functionality (Kindstedt, 1993b). Mozzarella differs in structure to most other cheeses due to the thermo-mechanical stretching step that the cheese undergoes (Kindstedt et al., 2004; McMahon et al., 1999). This process leads to the formation of a fibrous texture where the continuous protein fibres are interrupted by channels of fat and serum (Kindstedt, 2007). Over time the composition of these channels changes as free water, formed during the heating/stretching process, within the channels is absorbed into the protein matrix (Kuo et al., 2001).

The state, or relative degree of freedom, that water exists in influences both the structural and functional properties of a food (e.g. melt in Mozzarella) (Godefroy et al., 2003). As the distribution of water changes within Mozzarella over this initial storage period, the functional properties of the cheese change (Kindstedt, 1995). Other structural changes that are occurring in Mozzarella during this initial period of storage include a redistribution of the calcium and other minerals within the cheese (McMahon and Oberg, 1998). This involves a slight increase in the proportion of soluble calcium in the Mozzarella during storage (McMahon and Oberg, 2011). This corresponds to a decrease in the number of calcium-mediated protein-to-protein interactions resulting in a looser gel (Joshi et al., 2004).

The transformation of the state of calcium during storage has been examined in a number of cheese varieties including Cheddar (O'Mahony et al., 2005) and Colby (Lee et al., 2010). This phenomenon has been described as the attainment of a pseudoequilibrium between the insoluble and soluble calcium within the cheese (Hassan et al., 2004). The solubilisation of colloidal calcium is a key factor affecting the functionality of Mozzarella cheese (Mizuno et al., 2009). However, the exact mechanism responsible for the pseudoequilibrium has not been elucidated. This shift in the distribution of calcium within cheese during storage has been correlated with changes in physical properties including hardness

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(O'Mahony et al., 2005). The quantity of soluble calcium present in cheese has been measured using extraction methods (Metzger et al., 2001). One common method involves the homogenisation of cheese in water, filtering or centrifuging and measuring the calcium content of the aqueous phase (Ayyash and Shah, 2011).

Nuclear magnetic resonance (NMR) has been used as a tool to monitor the change in the association of water with the protein within the cheese structure (Chaland et al., 2000; Gianferri et al., 2007; Godefroy et al., 2003; Hinrichs et al., 2004; Kuo et al., 2001). Another component within the cheese system that can be assessed using NMR is phosphorus (Gobet et al., 2010b). This can be achieved by utilising ^{31}P magic angle spinning solid state NMR (Rondeau-Mouro et al., 2009a). This method used with a combination of a ^{31}P single pulse, cross polarisation and dipolar dephasing enables the resonances of phosphates with different levels of mobility and proximity to protons to be distinguished (Bak et al., 2001). This allows the signals to be discriminated in regards to mobility, with immobile phosphorus in the insoluble phase and mobile phosphorus in the soluble phase (Gobet et al., 2010b).

2. Materials & methods

2.1. Materials

A commercial 10 kg block of Mozzarella cheese was obtained immediately after a 24 h rapid cool cycle following manufacture (Fonterra Cooperative Group, New Zealand). The 10 kg block was then cut into 20 equal sized blocks, vacuum packed and stored at 4 °C. The blocks were randomly selected on each day of the trial. The composition of the sample is described in Table 1 below.

2.2. Confocal microscopy

Thin sections of refrigerated cheese samples were cut using a scalpel blade. The protein stained with 1% Fast Green and the fat stained with 0.5% Nile Red. A small quantity of each of the dyes was mixed together in a ratio of 1:1. The staining of the sample was done by placing 10 μl of the mixed dyes on a cover slip and immersing the thin section of cheese in the dye. The sample was left to sit in the dye for a period of 20 min. A concave slide was then placed onto the cover slip so the sample of cheese and dye were in the concave section of the slide. Pressure was applied to the slide so excess dye on the cover slip created a seal that keeps the cover slip attached to the slide. The slide was then placed on the microscope stage for examination. The illumination was provided by an Argon laser at 488 nm and a Helium/Neon laser at 633 nm. Scanning was done sequentially to excite the two dyes with a zoom factor of 1. The samples were examined using a Leica SP5 Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems, Wetzlar, Germany) with a 10× and 20× dry objective as well as a 40× and a 62× oil immersion objective.

At least three images were collected on each day of the trial at magnifications of 20×, 40× and 62×. The images were collected and viewed using Leica LAS AF Lite software (Leica Microsystems, Wetzlar, Germany).

Table 1
Composition of Mozzarella cheese sample.

	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Lactose (%)	Calcium (mmol/kg)
Mozzarella Samples	49.0	25.4	20.2	3.17	1.52	148

2.3. Scanning electron microscopy

Cheese samples were examined using a FEI Quanta 200 Scanning Electron Microscope (FEI Electron Optics, Eindhoven, Netherlands). Cheese samples were defatted by washing in a series of acetone solutions increasing in concentration from 50%, 75%, 95% and 100% for half an hour per wash. The sample was then soaked in an additional 100% acetone for an hour. The samples were then fractured, prompted with a razor blade after critical point drying, using liquid CO_2 as the critical point fluid. The fractured samples were selected based on quality of the fracture (based on the presence of a flat fracture plane). The samples were mounted on aluminium specimen stubs and sputter coated in gold using a Baltec SCD 050 sputter coater (Bal-tec, Balzer, Germany). The samples were analysed using the high vacuum mode on the microscope. The samples were fractured in two directions: parallel and perpendicular to the protein fibres. Samples were prepared in triplicate and at magnifications of 120, 500, 1000 and 2000× on the quadrant of the SEM screen.

2.4. NMR spectroscopy

2.4.1. ^{1}H NMR

Samples of cheese (4 mm depth, 4 mm wide and 12 mm in height) were cut using a razor blade. The samples were placed in a 5 mm diameter ceramic NMR tube. The samples were then placed in a Bruker AMX 200 MHz horizontal wide-bore magnet (Bruker, Rheinstetten, Germany).

Transverse (T_2) relaxation constants were measured using the Carr Purcell Meiboom Gill (CPMG) spin echo pulse sequence (Carr and Purcell, 1954; Meiboom and Gill, 1958). The relaxation of the water present in the cheese was fitted to a bi-exponential model based on the equation (Chaland et al., 2000; Kuo et al., 2001):

$$I = Ae^{\left(\frac{-t}{T2a}\right)} + Be^{\left(\frac{-t}{T2b}\right)} \quad (1)$$

The T_2 constant is related to the molecular mobility of the water. The T_{2a} component relates to the fraction of water with the shortest relaxation time due to being associated with the protein (Altan et al., 2011). While the T_{2b} relates to the water in a free state which has a longer relaxation time (larger relaxation constant). I represents the intensity of the water peak. A and B represent the proton intensity proportional to the different populations of water with the corresponding relaxation constant, and t represents the relaxation time. The NMR measurements were conducted in triplicate at 22 °C.

2.4.2. ^{31}P NMR

Solid state phosphorus magic angle spinning (MAS) NMR experiments were conducted in accordance with the method disclosed by Gobet et al. (2010b). These experiments were conducted on a Bruker Biospec 200 MHz NMR (Bruker, Rheinstetten, Germany) using a 7 mm Bruker MAS probe (80.99 MHz ^{31}P).

Samples of cheese taken by inserting a 7 mm MAS rotor into the cheese, the core was broken off to fill the rotor.

A single pulse excitation (SPE) sequence was performed using 5 μs $\frac{\pi}{2}$ ^{31}P pulse for an acquisition time of 30 ms during while proton 45 kHz dipolar decoupling was applied. A recycling of 30 s was used for quantification conditions. The ^{31}P spectral information was deconvoluted in accordance with Gobet et al. (2010b).

2.5. Urea-polyacrylamide gel electrophoresis

PAGE was used to investigate the extent of breakdown of both

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