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Experimental evidence for previously unclassified calcium phosphate structures in the casein micelle

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ABSTRACT

^{1}H-^{31}P Cross-polarization magic angle spinning (CP-MAS) measurements of 40-d-old Mozzarella cheese and 20 m*M* EDTA-treated casein micelles revealed that each sample had immobile phosphorus with the same spectral pattern, which did not match that of native casein micelles. To identify the immobile phosphorus bodies, ^{1}H-^{31}P CP-MAS spectra and cross-polarization kinetics measurements were undertaken on native casein micelles, EDTA-chelated casein micelles, and reference samples of β-casein and hydroxyapatite. The results showed that the immobile phosphorus bodies in the mature Mozzarella cheese had the following characteristics: they are immobile phosphoserine residues (not colloidal calcium phosphate); they are not the product of phosphoserine to colloidal calcium phosphate bridging; the phosphate is complexed to calcium; their rigidity is localized to a phosphorus site; their rigidity and bond coupling is unaffected by protein hydration; and the immobile bodies share a narrow range of bond orientations. Combining these observations, the best explanation of the immobile phosphorus bodies is that bonding structures of phosphorus-containing groups and calcium exist within the casein micelle that are not yet clearly classified in the literature. The best candidate is a calcium-bridged phosphoserine-to-phosphoserine linkage, either intra- or inter-protein.
Key words: ^{1}H-^{31}P cross-polarization magic angle spinning (CP-MAS), casein micelle, calcium phosphate

INTRODUCTION

At first glance, casein micelles are nano-sized spherical assemblages (80–400 nm) of casein proteins (α_{S1}, α_{S2}, β, and κ) and minerals (Ca, P, Mg) in milk. Yet they have the characteristics of complex biological machines. They can exchange proteins and minerals, transport minerals (Ca and P) in solution at supersaturated concentrations, absorb approximately 6 times their weight in water, and dissemble and reassemble their structure in different chemical environments (Horne, 2011). Casein micelles contain phosphorus in 2 chemical forms: calcium phosphate deposits and phosphorylated serine (phosphoserine) in the caseins (Thomsen et al., 1995; Bak et al., 2001). The calcium phosphate deposits are also described as colloidal calcium phosphate (**CCP**) nanoclusters (Horne, 1998). One currently accepted view is that casein micelles are assembled by inter-protein bonding by van der Waals forces, hydrophobic bonds, and ionic calcium linkages around the CCP nanoclusters (Horne, 1998, 2011; Phadungath, 2005; Dalgleish, 2011). The calcium bridged CCP to phosphoserine linkage is illustrated in Figure 1.

The CCP is an immobile (rigid) amorphous inorganic salt (Horne, 1998). Thomsen et al. (1995) showed that phosphorus (**^{31}P**) solid-state nuclear magnetic resonance (**NMR**) could differentiate between the mobile and immobile phases of the phosphorus species in casein micelles. They used the NMR technique of proton-to-phosphorus (**^{1}H-^{31}P**) cross-polarization magic angle spinning (**CP-MAS**; Thomsen et al., 1995). They showed a major fraction of the phosphoserine residues in the casein micelle proteins were immobilized due to their cross-linking with rigid CCP clusters by caseinate calcium (calcium that links phosphoserine to the CCP). The CP-MAS technique involves polarization transfer from an abundant nucleus (^{1}H) to a dilute nucleus (^{31}P). The CP-MAS experiment acts as a filter for detecting only the immobile component (rigid species) in a heterogeneous sample. It does this because cross-polarization (**CP**) relies on a strong static component of the dipolar interaction between the proton and the other nucleus. This implies that if the molecule can re-orientate in space more than once during the CP period (typically 1 ms), then the net magnetization transfer will be zero. Hence, CP-MAS is efficient for detecting immobile, rigid molecular species but ineffective for mobile, fluid-like species (Kolodziejski and Klinowski, 2002).

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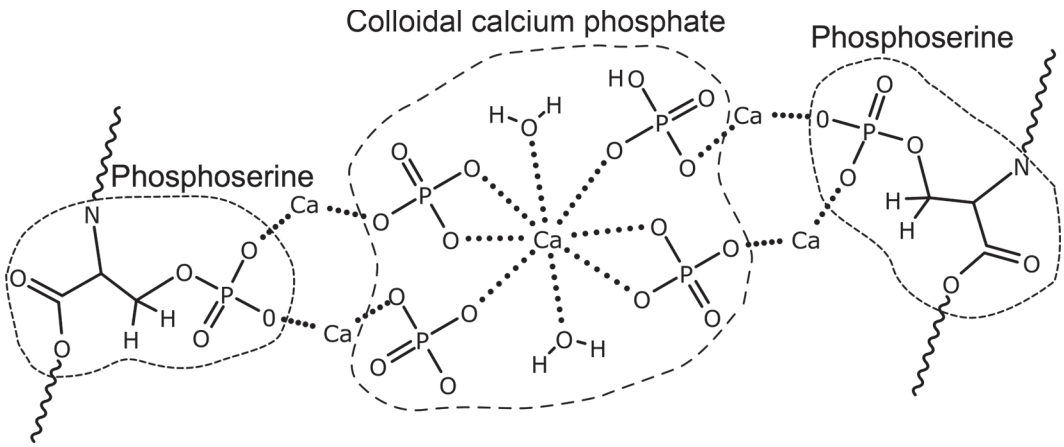

Figure 1. Representative structure of casein micelle inter-protein linkages by calcium-bridged phosphoserine to colloidal calcium phosphate. Structure derived from colloidal calcium phosphate structure proposed by Holt et al. (1989) and cross-linkage structure by Horne et al. (1998).

Gobet et al. (2010) applied the ^{1}H-^{31}P CP-MAS method to cheese. Their goal was to observe the state and distribution of immobile/mobile phosphorus species for different cheeses. Their results showed that mature cheese contained significant amounts of rigid CCP and phosphoserine phosphorus. This was an interesting discovery because the casein proteins in cheese are in a hydrated state but the phosphoserine residues were still held in an immobile state by their linkages to the CCP.

We applied ^{1}H-^{31}P CP-MAS to monitor the state of the phosphorus in maturing Mozzarella cheese during storage. Figure 2 shows the ^{1}H-^{31}P CP-MAS spectra of a Mozzarella sample over 40 d. Five peaks can be observed in each spectra; the center peak is the isotropic peak. Spanning either side of the isotropic peak are the first- and second-order spinning sideband peaks. The isotropic peak is the product of the isotropic chemical shift of the ^{31}P nuclei of the CCP and the phosphoserine. Due to peak broadening, the CCP and phosphoserine appear as one isotropic peak. Sideband peaks are offset from the isotropic peak in intervals of the spinning frequency of the MAS rotor (Wu et al., 1994; Gobet et al., 2010). The amplitude of the sideband peaks approximates the powder pattern of the chemical shift anisotropy (**CSA**) of each ^{31}P site. The peak intensity of the cross-polarized isotropic peak is proportional to the concentration of rigid phosphorus in the Mozzarella cheese. The decline in isotropic peak area shows the rigid phosphorus content of the Mozzarella decreased during storage. It is widely reported that the micellar CCP is mobilized into cheese serum during cheese aging (Hassan et al., 2004; Guinee and O'Kennedy, 2009). It is noteworthy that the spinning side-band peaks did not decline at the same rate as the isotropic peak during the Mozzarella aging. This means the CSA pattern of the phosphorus changed with mobilization of micellar phosphate. This was repeatable for 3 Mozzarella samples.

The CSA pattern can act as a chemical "fingerprint," because it is the product of the chemical environment of the nuclei. The chemical environment depends on the type and proximity of other nuclei and the bonding characteristics (strength and geometry). Protons have the strongest influence on the CSA pattern of phosphorus (strong coupling). Thus, protons produce high intensity spinning side bands (Wu et al., 1994). The chemical structure of CCP has yet to be defined but

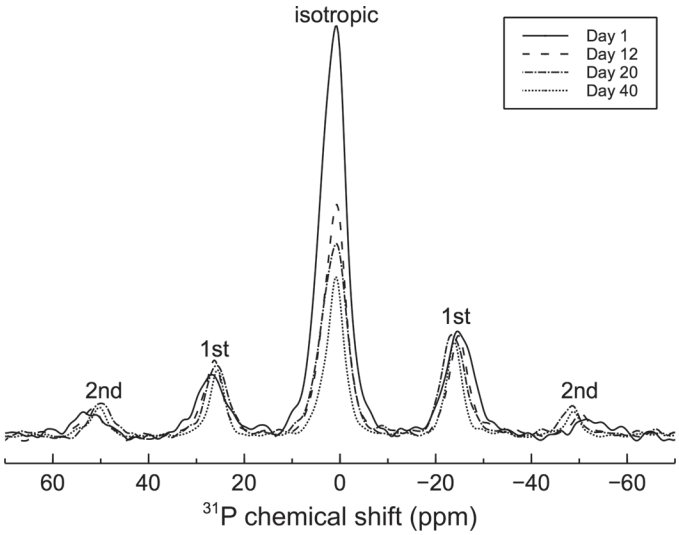

Figure 2. ^{1}H-^{31}P Cross-polarization magic angle spinning (CP-MAS) spectra of Mozzarella cheese stored at 4°C over 40 d. Spinning side bands are indicated by their order number (first, second) from the central isotropic peak of the phosphorus signal.