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Dephosphorylation of caseins in milk protein concentrate alters their interactions with sodium hexametaphosphate

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ABSTRACT

This study investigated the effects of dephosphorylation and sodium hexametaphosphate (SHMP) salt addition on the viscosity of milk protein concentrate (MPC) solutions. Dephosphorylation (DP) of casein was performed using bovine alkaline phosphatase. Nuclear magnetic resonance (NMR) spectra showed that dephosphorylation depleted the casein-bound phosphate region (CNP). SHMP addition (5 mM) had no impact on the ^{31}P NMR spectra of DP-MPC; addition of 5 mM SHMP to control MPC (C-MPC) resulted in a shift in peaks associated with the CNP region, possibly caused by SHMP sequestering calcium, leading to swelling of micelles. DP-MPC exhibited a lower viscosity compared to C-MPC, with SHMP addition at 12.5 and 25 mM causing gelation of C-MPC and DP-MPC solutions. This work confirmed the role that phosphate residues have in maintaining micelle structural stability and provides new insights into controlling viscosity of MPC solutions.

1. Introduction

Milk protein concentrate (MPC) powders are produced using separation processes such as ultrafiltration (UF) and diafiltration (DF) of pasteurised skim milk. They have protein contents ranging from 40 to 80% (w/w) (Havea, 2006, Sikand, Tong, Roy, Rodriguez-Saona, & Murray, 2011) and a similar casein to whey protein ratio as the original skim milk (i.e., ~80:20). After membrane filtration, the liquid MPC may be heat treated and evaporated before spray drying, and while the subsequent powders are used in a wide range of applications (including infant milk formula and sports nutritional beverages and foods), their reconstitution properties can be challenging (Crowley, Kelly, Schuck, Jeantet, & O'Mahony, 2016). Several factors contribute to their insolubility, such as the heat treatment of the skim milk, protein and mineral content, UF and DF conditions (e.g., volume concentration factor), spray drying temperatures, storage conditions of the subsequent powder (e.g., time and temperature) and the rehydration process (e.g., temperature of reconstitution medium and mixing conditions) (Gaiani et al., 2010; Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2010; Richard et al., 2012).

There are a number of novel methods available for increasing the solubility of high protein MPC powders. Previous work (Bhaskar, Singh, & Blazey, 2007) has shown that depleting calcium from MPC, using ion-exchange technology, can lead to significant increases in protein solubility. McCarthy et al. (2017) showed that the addition of calcium chelating salts to MPC solutions can increase protein solubility, but that there is a significant concomitant increase in viscosity. The structural stability of casein micelles is dependent on a delicate balance of attractive (e.g., hydrophobic interactions) and repulsive (e.g., electrostatic repulsion) forces between the constituent proteins. The colloidal calcium phosphate (CCP) associated with the proteins in casein micelles *via* phosphoserine residues can be depleted using approaches such as ion-exchange and mineral chelation, thereby reducing micelle integrity (Dalgleish & Corredig, 2012; de Kruif, Huppertz, Urban, & Petukhov, 2012; McMahon & Oommen, 2008; Horne, 2006; Wong, Camirand, Pavlath, Parris, & Friedman, 1996).

Chelating salts act by sequestering free calcium ions in milk-based systems, and can also potentially interact with calcium associated with the CCP located within the casein micelles, to an extent dependent on the electrostatic strength and ability of the chelating salt to bind calcium. The binding of calcium through the addition of chelating salts, alters the distribution of calcium between the colloidal and serum phases, which consequently influences electrostatic-mediated protein–protein interactions. This results in a greater net negative charge on the caseins, resulting in increased electrostatic repulsion, decreased protein–protein interaction and increased viscosity (De Kruif & Holt, 2003; Lin, Leong, Dewan, Bloomfield, & Morr, 1972; Odagiri & Nickerson, 1964). Depending on calcium chelating salt type and

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concentration used, casein micelles can dissociate into single or multiple protein clusters (De Kort, Minor, Snoeren, Van Hooijdonk, & Van Der Linden, 2009, 2011; Panouillé, Benyahia, Durand, & Nicolai, 2005; Pitkowski, Nicolai, & Durand, 2008).

The individual casein proteins (i.e., α_{s1}-, α_{s2}-, β- and κ-casein) have different degrees of phosphorylation, with α_{s1}-, α_{s2}-, β- and κ-casein having 8, 11/12, 5 and 1 phosphate residues, respectively (De Kruif & Holt, 2003 Horne, 2006). A number of previous studies (Yun, Ohmiya & Shimizu, 1982; Liu et al., 2016; McCarthy, Kelly, O'Mahony, & Fenelon, 2013) have studied the effects of dephosphorylation of casein on charge, mineral sensitivity, protein aggregation, curd forming properties and re-micellization of casein proteins.

The current study aimed to develop a mechanistic understanding of the role of phosphate groups, through dephosphorylation, on casein proteins in MPC solutions using the advanced analytical capability of phosphate nuclear magnetic resonance (^{31}P NMR) and to control viscosity of MPC suspensions in which calcium ion activity had been altered by the addition of a calcium-chelating salt. Minimising viscosity of concentrated casein systems, such as milk protein concentrate, is of academic and industrial interest in the development of protein-dense nutritional powders and beverages as high viscosity can negatively affect unit operations (e.g., UF performance, spray dryer atomisation) and finished product quality (e.g., beverage mouthfeel). Calcium chelating salts are commonly added to concentrated casein protein solutions to enhance powder solubilisation and heat stability; however, the concurrent swelling and viscosity increases limit their use and effectiveness in such applications.

2. Materials and methods

2.1. Materials

Milk protein concentrate (MPC) powder was obtained from a local dairy ingredient manufacturer. The protein, moisture, fat and ash content of the MPC were 81.4% (w/w), 4.30% (w/w), 1.40% (w/w) and 7.80% (w/w), respectively, as provided by the manufacturer. The lactose content was determined by difference as 5.10% (w/w). Bovine alkaline phosphatase (BAP) enzyme was obtained from Sigma Aldrich (Vale Rd, Ballyraine Lower, Arklow, Co. Wicklow, Ireland).

2.2. Reconstitution of milk protein concentrate

MPC powders (250 g, 10%, w/w) were dissolved in preheated (50 °C) distilled water aided by an overhead stirrer. Ultrasonication was carried out using an ultrasound device (Hielscher UIP1000hd, Hielscher Ultrasonics Gmbh, Warthestraβe 21 D-14513, Berlin, Germany) using the method previously described by McCarthy, Kelly, Maher and Fenelon (2014) at an amplitude of 50% for 10 min to promote rehydration of the MPC. Sodium azide (0.02% w/w) was added to the resulting solutions to prevent microbial growth. Solutions were stirred for 4 h at 22 °C prior to storage overnight at 4 °C with low speed magnetic stirring.

2.3. Dephosphorylation of casein and addition of sodium hexametaphosphate

Reconstituted MPC solutions (10%, w/w, protein) were incubated with BAP (1:20 w/w) at pH 6.5 and 37 °C for 3 h. A control MPC (C-MPC) solution was prepared under the same conditions but without the addition of the enzyme. After 3 h the reaction was terminated by heating at 70 °C for 5 min to inactivate the enzyme. SHMP was dissolved in 1 mL of water as a stock solution and the pH adjusted to 6.5 prior to addition to the C-MPC and DP-MPC solutions to give a final concentration of 5, 12.5 or 25 mM.

2.4. Protein profile analysis by electrophoresis

Urea-PAGE was used to separate casein proteins using the method of Ornstein and Davis (1964), with a separating gel composed of 7.5% acrylamide, 0.375 M Tris–HCl, pH 8.8 and 4 M urea. Protein samples were prepared at a concentration of 5 mg/mL in sample buffer, and 20 μL of sample was loaded onto the gel. After electrophoresis, the gels were stained overnight using 0.05% (w/v) Coomassie Brilliant Blue R-250 in 25% (v/v) isopropanol and 10% (v/v) acetic acid. After staining, the gels were de-stained using a 10% (v/v) isopropanol and 10% (v/v) acetic acid solution until a clear background was achieved.

2.5. Nuclear magnetic resonance

^{31}Phosphate nuclear magnetic resonance (^{31}P NMR) was conducted at 25 °C using a Bruker Advance III 400 NMR (Bruker UK Ltd., Coventry, UK) located at Trinity College (Dublin, Ireland) operating at 400.2 MHz for proton, 162.0 MHz for phosphorous-31 and 100.6 MHz for carbon-13 resonance. MPC powders (10–20 mg) were prepared in deuterium oxide (D_2O) solvent (1 mL) and NMR data was analysed using TopSpin 3.5 software (Bruker UK Ltd).

2.6. Rheological measurements of milk protein concentrate solutions

Rheological measurements of MPC solutions (10%, w/w, protein) were carried out using a controlled-stress rheometer (AR2000ex rheometer, TA Instruments, Crawley, UK), equipped with a concentric cylinder geometry. SHMP was prepared as described in Section 2.3 and added to 17 mL of MPC solutions (10%, w/w, protein). Samples were inverted ten times prior to testing to ensure a homogenous mixture. Viscosity measurements were carried out at 20 °C with pre-shearing at 100 s^{-1} for 10 s, followed by a peak hold step at a shear rate of 100 s^{-1} for 2 h. Low-amplitude oscillatory shear rheological measurements of the C-MPC and DP-MPC solutions containing 12.5 and 25 mM SHMP were also determined using a 60 mm parallel plate geometry. SHMP was added to the MPC solution to give 12.5 or 25 mM final concentration and stirred for 5 min to allow adequate mixing prior to analysis. The sample was then pre-sheared at 50 s^{-1} for 10 s at 20 °C to eliminate any shear history, followed by a time sweep at 0.25% strain and 1 Hz frequency over 6 h, during which the rheological parameters elastic (G') and viscous (G'') moduli were recorded.

2.7. Dynamic light scattering and particle size distribution analysis

Particle size measurements were performed on C-MPC and DP-MPC solutions with added SHMP concentrations of 0, 5, 12.5 and 25 mM using a Zetasizer nano (Malvern Instruments, Worcestershire, England). Measurements were conducted 1 h after SHMP addition to ensure equilibration. Solutions were diluted (1:50) in deionised water with a dispersant refractive index (RI) of 1.33 and viscosity of 0.89 mPa.s used. The refractive index used for the protein particles in the sample was 1.45 in conjunction with an absorption value of 0.001. Experiments were conducted in triplicate at 25 °C at a backscattering angle of 173°. Data was displayed as the z-average (nm), correlating to the intensity-weighted mean size of all particles present in the solution.

2.8. Statistical analysis

Rheological measurements were analysed using a Paired T-test with a 95% confidence interval. Particle size data was statistically analysed using one-way analysis of variance (ANOVA), with post hoc Tukey analysis. The level of significance was considered as $P < 0.05$. All statistical analysis was carried out using Minitab 17 (Minitab Inc, Coventry, United Kingdom).