Physicochemical properties of skim milk powder dispersions prepared with calcium-chelating sodium tripolyphosphate, trisodium citrate, and sodium hexametaphosphate

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ABSTRACT

Due to health benefits of proteins, the demand for protein beverages has grown rapidly. Translucent protein drinks with neutral pH may have advantages over acidic beverages that may cause dental erosion, and skim milk powder (SMP) is an affordable protein ingredient. Dissociating casein micelles by calcium chelators is a well-known method to reduce SMP dispersion turbidity, but much is to be studied for physicochemical properties as affected by chelator type and concentration. The objective of the present study was to characterize physicochemical properties of dispersions with 5% (wt/vol) SMP after addition of 0 to 30 mM sodium tripolyphosphate, trisodium citrate, or sodium hexametaphosphate. The turbidity was decreased with increasing chelator concentration, with the lowest turbidity observed in the SMP dispersions with sodium hexametaphosphate. The smallest hydrodynamic diameter was observed at an intermediate chelator concentration, resulting from the balance of casein micelle dissociation and aggregation of dissociated caseins induced at an elevated ionic strength. Heating at 90°C for 5 min increased turbidity but lowered hydrodynamic diameter of SMP dispersions, with some exceptions. The morphology of SMP dispersions differed for each chelator and was also affected by chelator concentration and heating. Trisodium citrate was the most effective to demineralize colloidal calcium phosphate in casein micelles, but the amount of dissolved calcium was not directly correlated with the decreased turbidity, indicating different chelating mechanisms by each chelator. Analysis of serum calcium and phosphorus concentrations also suggested that the type and concentration of soluble and insoluble calcium phosphates and their partitioning in the serum and casein micelles were dynamically changed by the studied parameters

to affect dispersion turbidity and structures of casein micelles. Findings from the present study may be used to formulate translucent beverages incorporating SMP and other casein micelle ingredients.

Key words: skim milk powder dispersion, casein micelle, calcium chelator, colloidal calcium phosphate

INTRODUCTION

The increased demand for proteins is expected with the projection that world population will rise to approximately 10 billion by 2050 (United Nations, 2015). In addition, the increased awareness that proteins contribute to healthier aging drives the increase in protein consumption, contributing to the steady growth of functional foods with proteins (Henchion et al., 2017). Incorporation of proteins, mainly whey protein isolate, into beverages has been attempted due to the functionalities of proteins, such as aiding muscle protein synthesis and increasing satiety (LaClair and Etzel, 2010; MacKenzie-Shalders et al., 2015). However, heat treatment used to pasteurize and sterilize beverages can cause aggregation and precipitation of whey proteins, thus lowering acceptance by consumers (Rittmanic, 2006). Even though beverages with whey protein isolate can be clear at acidic pH of around 3.0, consumption of acidic beverages can cause dental erosion even in young people (Seow and Thong, 2005; LaClair and Etzel, 2010), which calls for clear beverages with neutral pH.

Skim milk powder (SMP) is well dispersed in deionized water at neutral pH. Like milk, SMP provides casein and whey proteins at a ratio of approximately 4:1, with functionalities mostly determined by casein micelles and their structure (Dalglish and Corredig, 2012). Calcium and phosphorus are the most important minerals for the structure of casein micelles. Calcium and phosphorus in the serum phase of SMP dispersions are in equilibrium with colloidal calcium phosphate (CCP) in casein micelles (Walstra, 1999). Colloidal calcium phosphate in casein micelles functions as “cement” to link phosphorylated residues to form the net-

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work structure of casein micelles (Horne, 1998, 2006). Cationic calcium of CCP also neutralizes phosphoserine residues of caseins (Schmidt, 1982), and the weakened electrostatic repulsion allows hydrophobic patches of casein molecules to interact with each other, which is also important to casein micelle structure (Horne, 1998). With the average dimension of casein micelles being around 200 nm, SMP dispersions are turbid (Glantz et al., 2010), and the knowledge of the importance of CCP to casein micelle structure can be used to guide the dissociation of casein micelles to reduce dispersion turbidity. Such studies have been focused on disrupting CCP linkages by high hydrostatic pressure (Huppertz and De Kruif, 2007; Orlén et al., 2010; Bravo et al., 2013) or driving the calcium-phosphate balance from CCP in casein micelles to the continuous serum phase by addition of calcium-chelating agents (Panouillé et al., 2005; Kaliappan and Lucey, 2011; Lazzaro et al., 2017).

Chelators refer to materials that have high affinities to form complexes with cations and, therefore, can displace cations from original binding sites (Gaucheron, 2005). Calcium chelators can be added to milk or other dairy protein dispersions to improve rehydration properties of dairy powder or emulsifying capacity by altering physical interactions surrounding casein micelles (de Kort et al., 2011; Lazzaro et al., 2017; McCarthy et al., 2017). Various calcium chelators can dissociate casein micelles, including EDTA, citrate, and phosphate (Pitkowski et al., 2008; de Kort et al., 2011; Kaliappan and Lucey, 2011). Casein micelles are dissociated into small clusters, and specific caseins are released by mechanisms of chelators that govern protein-mineral equilibria, resulting in the reduced amount of diffusible calcium and depletion of CCP (de Kort et al., 2011). Therefore, the reduced turbidity of dispersions with casein micelles after addition of calcium-chelating agents has been reported frequently (Mizuno and Lucey, 2005; Pitkowski et al., 2008; Culler et al., 2017). The extent of turbidity reduction of casein micelle dispersions depends on the type and concentration of chelators with the decreasing chelating ability of polyphosphate > citrate > orthophosphate (Van Wazer and Callis, 1958). For example, milk after adding 1 mM sodium hexametaphosphate (SHMP) and 100 mM sodium tartrate at pH 7.8 had comparable turbidity after 30 min of equilibrium (Culler et al., 2017). The type of calcium chelators added also affects heat stability of casein micelle dispersions; better stability was reported for dispersions with added disodium uridine monophosphate than for those with added trisodium citrate (TSC) or SHMP after sterilization at 126°C, although the former is a weaker calcium chelator (de Kort et al., 2012). However, calcium and phosphorus compositions

of SMP dispersions as affected by different types of calcium chelators and heating and the correlation with casein micelle structures are still to be studied.

The overall objective of the present study was to characterize physicochemical properties of SMP dispersions at pH 6.8 after addition of calcium chelators and heating. Sodium tripolyphosphate (STPP), TSC, and SHMP were chosen due to the higher calcium binding affinity of polyphosphate and citrate than of orthophosphate (de Kort et al., 2009). Additionally, sodium sources were used to exclude possible effect of cationic counter ions in calcium chelators. Physicochemical properties of SMP dispersions were characterized for turbidity, hydrodynamic diameter (D_h), ionic composition using inductively coupled argon plasma-optical emission spectrometry (ICP-OES), zeta-potential, and morphology using transmission electron microscopy (TEM).

MATERIALS AND METHODS

Materials

Carnation SMP was a product of Nestlé USA (Solon, OH). The protein content of SMP was determined to be 35.4% based on the Kjeldahl method (AOAC International, 2005). The STPP (CAS no. 7758-29-4) was obtained from Acros Organics (Pittsburgh, PA), and TSC (CAS no. 68-04-2) and SHMP (CAS no. 10124-56-8) were obtained from Alfa Aesar (Ward Hill, MA). Hydrochloric acid was purchased from Fisher Scientific (Fair Lawn, NJ).

Sample Preparation

The SMP dispersions were prepared by hydrating SMP at 5% (wt/vol) in 10 mL of deionized water in 20-mL scintillation vials. Deionized water was dissolved with each calcium chelator at 0, 1, 2, 3, 5, 7, 10, 15, or 30 mM, with 0 mM being a control. Samples were incubated overnight at room temperature (RT; ~21°C) and then adjusted to pH 6.80 ± 0.02 using 1.0 M HCl or NaOH solution. After pH adjustment, samples were also heated for 5 min in a water bath pre-equilibrated to 90°C. Samples before and after heating were incubated at RT for about 12 h before measuring the following properties within 18 h.

Turbidity and D_h

The turbidity of SMP dispersions was determined at RT using a 2020 we/wi turbidimeter (LaMotte, Chestertown, MD) equipped with a tungsten lamp and a photodiode detector. The measurement was recorded

for nephelometric turbidity units (NTU). Dynamic light scattering was used to measure the particle size (model Zetasizer nano-ZS, Malvern Instruments, Malvern, UK). The viscosity used in dynamic light scattering was measured with a tuning-fork vibrational viscometer (SV-10, A&D Co., Tokyo, Japan). The z-average D_h from the cumulants analysis was reported. Measurements were done with a backscatter angle of 173° and an equilibrium temperature of 25°C .

Concentrations of Calcium and Phosphorus in the Serum Phase

To determine calcium and phosphorus concentrations in the serum phase of SMP dispersions, SMP dispersions were contained in the Amicon ultra-15 centrifugal filter unit equipped with an ultracel-10 membrane with a molecular weight cut-off of 10 kDa (Merck Millipore Ltd., Cork, Ireland) and centrifugated for 3 h at $4,500 \times g$ at 21°C (model RC-5B plus centrifuge, Sorvall, Newtown, CT). The permeate contained free ions and ion complexes smaller than the membrane pore, but not those bound with proteins. The permeate after dilution for 100 times with deionized water was analyzed using a Spectro Ciros ICP-OES instrument (Spectro Ciros, Mahwah, NJ). The ICP-OES standards (VHG Labs, Manchester, NH) were used in calibration, and the limit of detection was 0.01 mg/L for both calcium and phosphorus.

Zeta-Potential

The SMP dispersions were adjusted to $\text{pH } 6.80 \pm 0.02$ using 1.0 M HCl or NaOH. The zeta-potential of SMP dispersions was determined at 25°C with 120 s of equilibration time using the above Malvern instrument. The instrument measures electrophoretic mobility (U_E ; calculated as $\mu\text{m} \times \text{cm}/\text{V} \times \text{s}$) on the basis of laser doppler electrophoresis to derive zeta-potential from the Henry equation:

$$U_E = \left(\frac{2\varepsilon\zeta}{3\eta} \right) f(ka), \quad [1]$$

where ε is the dielectric constant of solvent (F/m), ζ is zeta-potential (mV), η is the viscosity of solution (Pa·s), and $f(ka)$ is Henry's function (de Kort et al., 2012).

TEM

The morphology of SMP dispersions was studied using TEM. A freshly glow discharged 400 mesh cop-

per grid with a thin carbon film was placed on 50 μL of an SMP dispersion droplet deposited on a piece of Parafilm (Bemis Co., Neenah, WI). After removing excess sample with a piece of Kimwipes (Kimberly-Clark, Irving, TX), the grid was washed briefly by placing on a drop of water. The washed grid was stained by placing on a drop of 1% uranyl acetate for 1 min. After removing excess stain using a piece of Kimwipes, the sample was dried at RT before imaging on a Zeiss Libra 200MC (Carl Zeiss Inc., Fort Lauderdale, FL) operating at 200 kV.

Statistical Analysis

Statistical analyses were conducted using SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC) with 1-way ANOVA. Fisher's least significance difference test was used to calculate mean $\pm$ standard deviation from the sample in duplicate on each test, and differences of calculated mean values were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Turbidity and D_h of SMP Dispersions as Affected by Chelators

As expected, increasing the concentration of calcium chelators resulted in visually more transparent SMP dispersions (Figure 1). The decrease in the turbidity of SMP dispersions with increasing calcium chelator concentration was observed for all 3 chelators (Figure 2). With 1 mM chelator, the turbidity of SMP dispersions was 1,125, 1,429, and 571 NTU for STPP, TSC, and SHMP treatments, respectively, reflecting their differences in calcium-chelating ability. The binding affinity of chelators to calcium relies on temperature, pH, and valency of chelators (Lucey et al., 2011). With the temperature and pH being similar in the present study, the SMP dispersion turbidity after addition of 1 mM chelator is correlated with the number of negative charges of STPP ($n = 5$), TSC ($n = 3$), and SHMP ($n = 6$; McCarthy et al., 2017). However, the difference in the turbidity of SMP dispersions was minimized at the chelator concentration of 30 mM, showing 238, 251, and 232 NTU for STPP, TSC, and SHMP treatments ($P < 0.05$), respectively, suggesting that 30 mM chelators may be close to a plateau concentration at which most available casein micelles are dissociated regardless of the chelator charge. A previous study (McCarthy et al., 2017) reported that the turbidity of dispersions with 0.5% (wt/vol) milk protein concentrate was not significantly different for treatments with 10 mM TSC (68.18 NTU) and 5 mM SHMP (68 NTU), which had the same

level of equivalent chelating capacity (mEq/L). In the present study, a significant difference in turbidity was observed for the SMP dispersions with 10 mM TSC (277 NTU) and 5 mM SHMP (249.5 NTU). The difference between these 2 studies can be explained by the higher micellar casein concentration in the present study (~1.4% wt/vol) requiring a higher concentration of chelators to reduce the turbidity of SMP dispersions.

When D_h was examined (Figure 3), the decrease in D_h with an increase in chelator concentration was observed only at a lower chelator concentration range (0–3 mM for STPP, 0–7 mM for TSC, and 0–2 mM for SHMP). For each chelator, the smallest D_h was observed at an intermediate chelator concentration, which is different from the monotonic decrease in SMP dispersion turbidity with increasing calcium chelator concentration (Figure 2). The turbidity of a dispersion is a function of particle dimension as well as concentration and compactness (i.e., the ability to reflect light) of particles (Jonassen et al., 2012). At an increased ionic strength, the electrostatic repulsion is weakened due to charge screening effect (Saricay et al., 2019) to cause aggregation of dissociated caseins and therefore the increased D_h (Figure 3), whereas casein aggregation lowers particle concentration to cause a continued decrease in turbidity (Figure 2).

Turbidity and D_h of SMP Dispersions with Chelators as Affected by Heating

Heating influenced both D_h and turbidity of SMP dispersions with calcium chelators (Figures 1–3). Some dispersions experienced a significant change in visual appearance after heating, such as treatments with 2, 15, and 30 mM STPP (Figure 1). An increase in turbidity was observed after heating SMP dispersions at all studied STPP concentrations (Figures 2a). Particularly, the addition of STPP at 15 or 30 mM increased turbidity more than 10-fold after heating, indicating the formation of larger structures. The precipitation observed for these 2 treatments after heating suggested the possible formation of insoluble calcium–STPP complexes that could also involve proteins. Similarly, heating increased the turbidity of most dispersions with TSC except at 3 and 30 mM (Figure 2b). As for the SHMP treatments, the turbidity after heating increased at 1 and 2 mM SHMP but decreased at 10 to 30 mM SHMP ($P < 0.05$), whereas the heating effect was insignificant ($P > 0.05$) at 3 to 7 mM SHMP (Figure 2c).

The effect of heating on D_h (Figure 3) showed a different trend than turbidity (Figure 2). Most treatments showed a decrease in D_h (Figure 3) after heating, except at 15 and 30 mM STPP and 5 to 15 mM TSC. This is

further discussed below for effects of heating on changes in calcium and phosphorus concentrations.

After heating, pH of the dispersion without chelators decreased ($P < 0.05$), but the trend for dispersions with chelators depended on the type of chelators (Figure 4). No significant changes in pH were observed for TSC treatments ($P > 0.05$). A significant decrease in pH after heating was observed for dispersions with 1 to 5 mM STPP or 1 to 15 mM SHMP ($P < 0.05$), with the lowest pH observed at an intermediate chelator concentration, whereas a significant increase ($P < 0.05$) in pH was observed at 30 mM STPP (Figure 4). The overall pH change trend after heating SMP dispersions with different concentrations of chelators generally agreed with changes of D_h for STPP and SHMP treatments but not those of TSC (Figure 3). There was also no correlation between pH change (Figure 4) and turbidity change (Figure 2) after heating.

The pH can affect turbidity and D_h of SMP dispersions in 2 different ways. In general, the calcium binding affinity of chelators is higher at a higher pH (Lucey and Fox, 1993; de Kort et al., 2009; McIntyre et al., 2016), and a slight increase in turbidity can be expected at a decreased pH (after heating) due to decomplexation of calcium and chelators. In preliminary experiments, sedimentation occurred rapidly after heating the SMP dispersions with 15 and 30 mM STPP at pH 6.80, whereas no precipitation was observed after heating SMP dispersion at pH 8.50 even with 50 to 200 mM STPP. It then appears that the cause of pH changes after heating is the ionic composition in the serum phase, discussed below, which is further complicated by dynamic binding with proteins (de Kort et al., 2009).

Calcium and Phosphorus Concentrations in Serum Phase of Dispersions as Affected by Chelators and Heating

Calcium and phosphorus concentrations in the serum phase of SMP dispersions were studied using ICP-OES, presented in Tables 1 and 2, respectively. In milk, calcium can be present as either ions or soluble nonionic species (e.g., chelates and complexes) in the serum or as colloidal particles in casein micelles (DeMan et al., 1999). At low chelator concentrations in SMP dispersions, changes in both calcium and phosphorus concentrations in the serum were insignificant ($P > 0.05$) for all 3 calcium chelators, whereas significant ($P < 0.05$) increases in both calcium and phosphorus concentrations were observed at higher concentrations of chelators except for phosphorus concentration with TSC. However, due to the presence of phosphate in STPP and SHMP, the contribution of CCP to the increase of

Figure 1. The appearance of 5% (wt/vol) pH 6.80 skim milk powder dispersions with 0 to 30 mM sodium tripolyphosphate (a, b), trisodium citrate (c, d), or sodium hexametaphosphate (e, f) before (a, c, e) and after (b, d, f) heating at 90°C for 5 min.

Figure 2. The turbidity of 5% (wt/vol) pH 6.80 skim milk powder dispersions with 0 to 30 mM sodium tripolyphosphate (a), trisodium citrate (b), or sodium hexametaphosphate (c) before and after heating at 90°C for 5 min. Different letters (a–p) above bars indicate significant difference ($P < 0.05$). Error bars are SD ($n = 2$). NTU = nephelometric turbidity units.

Figure 3. The z-average hydrodynamic diameter of 5% (wt/vol) pH 6.80 skim milk powder dispersions with 0 to 30 mM sodium tripolyphosphate (a), trisodium citrate (b), or sodium hexametaphosphate (c) before and after heating at 90°C for 5 min. Different letters (a–n) above bars indicate significant difference ($P < 0.05$). Error bars are SD ($n = 2$).

Figure 4. The pH changes of 5% (wt/vol) pH 6.80 skim milk powder dispersions with 0 to 30 mM sodium tripolyphosphate (a), trisodium citrate (b), or sodium hexametaphosphate (c) before and after heating at 90°C for 5 min. Asterisks above bars indicate significant difference ($P < 0.05$) before and after heating. Error bars are SD ($n = 2$).

phosphorus concentration in the serum phase of SMP dispersion was not clear. Therefore, the phosphorus concentration contributed by STPP and SHMP was calculated (Table 2). For STPP treatments, the serum phosphorus concentration was higher than the amount of STPP added, whereas the opposite was observed for SHMP treatments, indicating that phosphates from SHMP bound with proteins or formed insoluble inorganic complexes larger than the membrane pores used to separate the serum for mineral analysis.

Calcium phosphates can exist as various forms comprising different amounts of calcium and phosphates (Kezia et al., 2017). The mass ratio of calcium and phosphorus (Ca/P) was 0.62 in the SMP dispersion serum without chelators. The Ca/P ratio gradually decreased to 0.1683 and 0.0880 when STPP and SHMP concentration increased to 30 mM, respectively. On the other hand, the Ca/P ratio gradually increased to 1.27 with an increase in TSC added into SMP dispersions. The formation of various calcium phosphate compounds is affected by pH and temperature as well as concentrations of calcium and phosphates and ionic strength (Dorozhkin, 2014). Therefore, it is probable that the increased concentration of phosphates and the increased ionic strength after adding STPP or SHMP exerted the counteracting effects on dynamic equilibria among various calcium phosphate compounds. Calcium phosphates can be soluble in various forms in aqueous dispersions with different Ca/P ratios—for example, 0.5 for monocalcium phosphate monohydrate, 1.0 for dicalcium phosphate dihydrate, 1.2 to 2.2 for amorphous calcium phosphates, and 1.5 to 1.7 for calcium-deficient hydroxyapatite (Kezia et al., 2017). The increased Ca/P ratio with increasing TSC addition level suggests the increased tendency of calcium and phosphorus from casein micelles to form amorphous calcium phosphates or calcium-deficient hydroxyapatite in the serum of SMP dispersions (Kezia et al., 2017). The opposite trend may be the case for STPP and SHMP treatments.

Calcium chelators bind to calcium ions with different affinity forming soluble or insoluble complexes (de Kort et al., 2009). The reason why SMP dispersions had different turbidity at the same concentration of different calcium chelators (Figure 2) is probably because each chelator has a different calcium-binding capacity and causes different dynamic ionic equilibria in the serum and different casein structures via several factors. First, soluble calcium and casein-bound calcium are complexed by chelators, with the extent depending on the chelator type and concentration. Then, the bonding between CCP and casein in micelles is disrupted, causing disintegration of casein micelles and the increased calcium and phosphorus concentrations in the serum phase with increased chelator concentration (Tables 1

Table 1. Calcium concentration (mg/L) in the serum phase of 5% (wt/vol) pH 6.80 skim milk powder dispersions after addition of 0 to 30 mM sodium tripolyphosphate (STPP), trisodium citrate (TSC), or sodium hexametaphosphate (SHMP) before or after heating at 90°C for 5 min¹

Chelator concentration (mM)	STPP		TSC		SHMP	
	Before heating	After heating	Before heating	After heating	Before heating	After heating
0	142.0 ^{nopqr}	140.4 ^{nopqr}	142.0 ^{nopqr}	140.4 ^{nopqr}	142.0 ^{nopqr}	140.4 ^{nopqr}
1	131.6 ^{opqr}	119.2 ^{pqr}	166.3 ^{lmnop}	164.0 ^{lmnop}	118.7 ^{pqr}	121.2 ^{pqr}
2	116.9 ^{pqr}	103.2 ^{qr}	184.8 ^{klmno}	191.7 ^{klmn}	131.7 ^{opqr}	121.6 ^{pqr}
3	112.3 ^{pqr}	91.4 ^r	201.5 ^{ijklm}	208.7 ^{hijklm}	134.1 ^{nopqr}	129.9 ^{opqr}
5	126.7 ^{opqr}	83.9 ^r	250.9 ^{ghi}	257.0 ^{fghi}	171.1 ^{klmnop}	166.0 ^{lmnop}
7	159.3 ^{mnopq}	95.5 ^r	300.5 ^{defg}	301.5 ^{defg}	222.4 ^{hijkl}	226.2 ^{hijk}
10	219.0 ^{hijkl}	136.8 ^{nopqr}	337.0 ^d	346.7 ^d	262.5 ^{efgh}	298.0 ^{defg}
15	486.6 ^b	247.9 ^{ghij}	407.2 ^c	425.5 ^c	316.7 ^{de}	310.1 ^{def}
30	596.5 ^a	167.1 ^{lmnop}	519.2 ^b	509.6 ^b	312.1 ^{def}	310.3 ^{def}

^{a-r}Different superscript letters within the table indicate significant difference ($P < 0.05$).

¹Numbers are mean values from duplicates.

and 2), which agrees with a previous study (Mizuno and Lucey, 2005). The amounts of casein-bound calcium and phosphorus may have decreased with the addition of TSC (Tables 1 and 2). The same may have been the case for STPP and SHMP treatments, except forming different types of calcium phosphate compounds that are different from those in TSC treatments, as suggested by different Ca/P ratios. The lowest calcium concentration in SHMP treatments and the lower serum phosphorus concentration than the amount due to SHMP addition suggest that both minerals may have bound with proteins or formed insoluble complexes. The former is more likely because the turbidity was lower at a higher SHMP concentration (Figure 2). The speculation agrees with the reported binding of some calcium with proteins after addition of a small amount of SHMP in a previous study (Mizuno and Lucey, 2005).

For SMP treatments added with TSC, SHMP, and low concentrations of STPP, heating had no significant effect on the amount of solubilized calcium and

phosphorus in the serum phase of SMP dispersions (Tables 1 and 2). When STPP concentration was 7 mM and higher, the concentration of solubilized calcium decreased significantly after heating, whereas the significant decrease of solubilized phosphorus after heating was observed only at 15 and 30 mM STPP. Based on decreased calcium and phosphorus concentrations (Tables 1 and 2) and both increased turbidity (Figure 2a) and D_h (Figure 3a) of SMP dispersions with 15 and 30 mM STPP, it is likely that heating resulted in the reformation of the dissociated caseins as larger particles involving STPP but not TSC or SHMP (further examined using TEM below).

Zeta-Potential of SMP Dispersions with Chelators

The magnitude of negative zeta-potential of SMP dispersions at pH 6.80 overall decreased ($P < 0.05$) with increasing concentrations of STPP, TSC, and SHMP (Table 3), but the changes were insignificant at

Table 2. Phosphorus concentration (mg/L) in the serum phase of 5% (wt/vol) pH 6.80 skim milk powder dispersions after addition of 0 to 30 mM sodium tripolyphosphate (STPP), trisodium citrate (TSC), or sodium hexametaphosphate (SHMP) before or after heating at 90°C for 5 min¹

Chelator concentration (mM)	STPP		TSC		SHMP	
	Before heating	After heating	Before heating	After heating	Before heating	After heating
0	228.3 ⁿ (0)	231.4 ⁿ	228.3 ⁿ	231.4 ⁿ	228.3 ⁿ (0)	231.4 ⁿ
1	257.8 ^{mn} (92.9)	253.1 ⁿ	236.8 ⁿ	240.7 ⁿ	259.5 ^{mn} (185.8)	279.2 ^{lmn}
2	301.0 ^{lmn} (185.8)	292.9 ^{lmn}	244.1 ⁿ	263.9 ^{lmn}	364.2 ^{lmn} (371.6)	378.9 ^{klmn}
3	365.1 ^{lmn} (278.7)	341.9 ^{lmn}	251.3 ⁿ	257.2 ^{mn}	470.2 ^{ijklm} (557.5)	518.6 ^{ijklm}
5	522.7 ^{kl} (464.6)	473.3 ^{ijklmn}	274.5 ^{lmn}	281.8 ^{lmn}	707.7 ^{hij} (929.1)	798.4 ^{ghi}
7	707.5 ^{hij} (650.4)	640.1 ^{ijk}	292.2 ^{lmn}	294.6 ^{lmn}	1,039.7 ^{fg} (1,300.7)	1,164.8 ^{ef}
10	1,036.0 ^{fg} (929.1)	934.7 ^{gh}	313.8 ^{lmn}	322.9 ^{lmn}	1,385.3 ^{de} (1,858.2)	1,581.0 ^d
15	2,283.5 ^c (1,393.7)	1,557.6 ^d	347.3 ^{lmn}	353.9 ^{lmn}	2,131.8 ^c (2,787.3)	2,195.6 ^c
30	3,544.0 ^a (2,787.3)	3,045.6 ^b	408.8 ^{klmn}	400.3 ^{klmn}	3,546.4 ^a (5,574.6)	3,748.5 ^a

^{a-n}Different superscript letters within the table indicate significant difference ($P < 0.05$).

¹Numbers are mean values from duplicates. Numbers in parentheses are phosphorus concentrations (mg/L) calculated based on the amount of chelator added in the dispersion.

Table 3. Zeta-potential of 5% (wt/vol) skim milk powder dispersions after addition of 0 to 30 mM sodium tripolyphosphate (STPP), trisodium citrate (TSC), or sodium hexametaphosphate (SHMP) and adjusting pH to 6.80

Chelator concentration (mM)	Theoretical ionic strength with complete dissociation of chelator (mM)			Zeta-potential ¹ (mV)		
	STPP	TSC	SHMP	STPP	TSC	SHMP
0	0	0	0	-13.73 ± 1.61^{de}	-13.73 ± 1.61^{de}	-13.73 ± 1.61^{de}
1	15	6	21	-14.07 ± 1.58^{cde}	-12.90 ± 1.65^e	-15.00 ± 1.16^{abcd}
2	30	12	42	-15.00 ± 0.77^{abcd}	-14.17 ± 1.01^{bcde}	-15.25 ± 1.35^{abc}
3	45	18	63	-15.88 ± 1.45^a	-14.08 ± 1.13^{cde}	-15.43 ± 1.16^{ab}
5	75	30	105	-15.27 ± 0.99^{abc}	-14.68 ± 1.25^{abcd}	-15.13 ± 1.49^{abc}
7	105	42	147	-15.60 ± 0.92^a	-15.70 ± 1.88^a	-11.40 ± 0.35^f
10	150	60	210	-11.22 ± 0.59^f	-14.78 ± 0.95^{abcd}	-10.54 ± 0.72^{fgh}
15	225	90	315	-9.31 ± 1.57^h	-10.83 ± 0.78^{fg}	-10.48 ± 0.59^{fgh}
30	450	180	630	-9.80 ± 1.15^{gh}	-10.20 ± 0.74^{fgh}	-9.57 ± 0.76^{gh}

^{a-h}Different superscript letters within the table indicate significant difference ($P < 0.05$).

¹Numbers are mean $\pm$ SD ($n = 2$).

low chelator concentrations. At an increased chelator concentration, calcium and phosphorus concentrations in the serum of SMP dispersions increased (Tables 1 and 2). The increased ionic strength lowers electrophoretic mobility and therefore the measured zeta-potential magnitude (Ricq et al., 1998). When different chelators are compared, the contribution of calcium chelators to the ionic strength follows the decreasing order of SHMP > STPP > TSC (Table 3). However, the zeta-potential magnitude was similar ($P > 0.05$) at the same chelator concentration. The zeta-potential data suggest a portion of chelator ions bound with caseins, which agrees with the measured serum Ca and P concentrations in Tables 1 and 2. Additionally, the interpretation of zeta-potential results is further complicated by the dynamic equilibria between various forms of calcium phosphates (Kezia et al., 2017), which determines the Ca/P ratio in the serum to affect the ionic strength and therefore the measured zeta-potential. At this point, understanding the specific forms of calcium phosphates is still needed. In addition, not all calcium and phosphorus released from casein micelles is soluble after addition of chelators; this complicates the actual ionic strength in SMP dispersions and therefore interpretation of the measured zeta-potential.

Structural Changes of Calcium-Chelated Casein Micelles Studied Using TEM

Casein micelles in SMP dispersions without addition of calcium chelators were mostly spherical (Figure 5a) and had a diameter of around 150 to 200 nm. Some aggregated particles larger than 400 nm (Figure 5a-3) were also observed, which can be contributed to by the incomplete hydration of SMP and resulted in the mean D_h of ~ 280 nm (Figure 3). After heating, such

aggregated structures were not observed (Figures 5a-4, a-5, and a-6). As casein micelles in milk are stable after thermal sterilization (Singh, 2004; Tsioulpas et al., 2010), TEM images suggest the enhanced hydration of SMP powder after heating, which agrees with the reduced D_h after heating (Figure 3).

The TEM images of SMP dispersions with STPP, TSC, or SHMP at the concentrations of 1, 7, and 30 mM before and after heating are shown in Figure 5b, 5c, and 5d, respectively. At 1 mM chelator, SMP dispersions with STPP and TSC had a morphology (Figure 5b-1 and c-1) comparable with that of casein micelles without chelators (Figure 5a). In contrast, 1 mM SHMP resulted in structures much smaller than intact casein micelles, some appearing as aggregates of dissociated caseins (Figure 5d-1). The TEM images support the smaller D_h (Figure 3) and turbidity (Figure 2) of SMP dispersion with 1 mM SHMP than those with the same concentration of STPP or TSC. As chelator concentration increased from 1 to 7 mM, smaller structures were observed (Figure 5b-2, c-2, and d-2), indicating further dissociation of casein micelles and agreeing with the reduction in D_h (Figure 3) and dispersion turbidity (Figure 2). When the chelator concentration was further increased to 30 mM, darker images with higher density were observed for STPP (Figure 5b-3) and TSC (Figure 5c-3) treatments, indicating the aggregated caseins, whereas the SHMP treatment showed highly aggregated structures (Figure 5d-3). Sodium hexametaphosphate has 6 negative charges distributed evenly around the molecule and therefore the ability to crosslink caseins (de Kort et al., 2011), resulting in the aggregated structures at 30 mM SHMP (Figure 5d-3). The aggregated caseins may be responsible for the larger D_h of dispersions with 30 mM chelators than 7 mM chelators, particularly for the SHMP treatment (Figure

Figure 5. Transmission electron microscopy images of 5% (wt/vol) skim milk powder dispersion at pH 6.80 without chelator (a) or with 1 mM (1 and 4), 7 mM (2 and 5), or 30 mM (3 and 6) sodium tripolyphosphate (b), trisodium citrate (c), or sodium hexametaphosphate (d) before (1–3) and after (4–6) heating at 90°C for 5 min. Dispersions were diluted to 1% (wt/vol) skim milk powder with the serum before imaging.

Figure 5 (Continued). Transmission electron microscopy images of 5% (wt/vol) skim milk powder dispersion at pH 6.80 without chelator (a) or with 1 mM (1 and 4), 7 mM (2 and 5), or 30 mM (3 and 6) sodium tripolyphosphate (b), trisodium citrate (c), or sodium hexametaphosphate (d) before (1–3) and after (4–6) heating at 90°C for 5 min. Dispersions were diluted to 1% (wt/vol) skim milk powder with the serum before imaging.

3). The reduced ability of dissociated caseins, with a less dense structure, compared with casein micelles to scatter light corresponded to the further decrease in turbidity at 30 mM chelator (Figure 2), even for highly aggregated structures of the SHMP treatment.

In general, heating caused a greater extent of casein micelle dissociation for dispersions with chelators (Figure 5), agreeing with the decreased D_h after heating (Figure 3). The exceptions in increased D_h and turbidity after heating the SMP dispersion with 30 mM STPP and 7 mM TSC were supported by formation of slightly larger aggregated particles (Figure 5b-6 and c-5). Morphological changes after heating SMP dispersions with different amounts and types of chelators again suggest the complex dynamics of calcium phosphate compounds in the serum and their binding with caseins, as discussed previously. In the future, detailed analyses of different forms of calcium phosphate compounds in the serum and their binding with caseins will be needed to clearly establish correlations between macroscopic turbidity and mesoscopic structures probed in light scattering and TEM.

CONCLUSIONS

The turbidity of 5% (wt/vol) SMP dispersions was effectively lowered using STPP, TSC, and SHMP. A higher chelator concentration resulted in a less turbid dispersion due to a greater extent of chelating calcium from CCP to result in dissociation of casein micelles. Trisodium citrate was the most effective in releasing calcium from casein micelles, whereas SHMP decreased the turbidity of the dispersions the most, suggesting differences in the mechanism of calcium chelation and the possible complex formation between calcium and chelators. The D_h of SMP dispersions started to decrease with the initial increase of the chelator concentration, mostly contributed by the chelating effect. However, at higher concentrations of chelators, chelators caused the aggregation of dissociated casein micelles, as observed in dynamic light scattering and TEM. In particular, heating greatly increased the turbidity and D_h of SMP dispersions with 15 and 30 mM STPP but decreased D_h of the dispersion with SHMP. Differences in macroscopic turbidity of SMP dispersions and mesoscopic structures point to the need to study specific forms of calcium phosphates in the serum phase and their binding with proteins as affected by chelator type and concentration. Nevertheless, findings in this study may be used to formulate shelf-stable and heat-stable beverages with improved clarity containing significant contents of casein micelles.

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