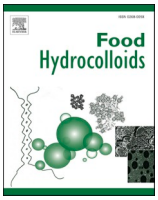
Food Hydrocolloids 153 (2024) 109970

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd



Influence of citrate- and phosphate-based calcium sequestering salts on the disruption of casein micelles

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ARTICLE INFO

Keywords:
Micellar casein isolate
Soluble Ca
Hexametaphosphate
Pyrophosphate
Tripolyphosphate

ABSTRACT

The disruption of casein micelles through the addition of calcium sequestering salts (CSS) disodium phosphate (DSP), disodium pyrophosphate (DSPP) and tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium hexametaphosphate (SHMP) and trisodium citrate (TSC) at 10, 15, 20 and 30 mEq/L levels was investigated in 5% micellar casein isolate (MCI) solution. All the CSS, except DSPP and TSPP, showed a decrease in particle size and viscosity with increasing concentration. The addition of 10–30 mEq/L of CSS to MCI decreased the protein-bound sedimentable Ca and P at pH 6.5 10 kDa-permeable Ca decreased with increasing concentration of DSP, DSPP, TSPP, STPP and SHMP. These results indicate that orthophosphates, pyrophosphates and polyphosphates combined with Ca to form insoluble Ca phosphate complexes together with casein. Higher amount of 10 kDa permeable Ca for MCI samples with TSC confirms the formation of soluble Ca-citrate complexes.

1. Introduction

In milk, most caseins exist in spherical aggregates known as casein micelles, which consist of the four individual caseins, α_{S2}-, κ-, β- and α_{S1}-casein, in a relative ratio of 10:15:35:40, respectively. The diameter of casein micelles varies between 100 and 300 nm, whereby κ-casein is predominantly located on the surface of the micelle, the α_S-caseins in the interior and β-casein is found throughout the micelles (Dalgleish & Corredig, 2012). Protein-protein interactions and micellar calcium phosphate (MCP) nanoclusters bridge these caseins; α_{S1}-, α_{S2}- and β-Casein link with MCP nanoclusters via their phosphoserine centres (Garcia et al., 2023). In general, Ca levels and pH affect the protein-protein interactions, which in turn influence the structure and texture of milk-protein gels like cheese (Dalgleish & Corredig, 2012; Wang & Moraru, 2021).

Ca exists in two forms in milk, which are in equilibrium: soluble Ca and micellar Ca. Within soluble Ca, a part is ionic Ca, whereas soluble Ca is also present as soluble complexes with citrate, phosphate and other anions. Micellar Ca is largely present in amorphous MCP nanoclusters (radius ~2.5 nm) inside the casein micelles or as Ca ions bound to phosphoserine residues. Micellar Ca participates in neutralizing the proteins phosphoseryl residues and bridging of caseins (Dalgleish & Corredig, 2012; Xu et al., 2016). Like Ca, phosphate is also present in different forms in milk: inorganic phosphate (P_i), associated with Ca in serum as well as casein micelles, and organic phosphate (P_o), which is covalently bound to caseins, lipids, and carbohydrates (Belloque et al., 2000). P_i can be free (HPO_4^{2-} and $H_2PO_4^-$) or combined with Ca and Mg to form Ca phosphate salts. P_i and P_o (phosphoseryl residues) can combine with Ca to form MCP (Gaucher et al., 2007).

Ca and P can be partially removed from casein micelles to dissociate them to different extent and develop dairy products with tailored functionalities (Xu et al., 2016). Casein micelles are very stable, but their structure can be destabilized by changes in pH, thermal treatment, proteolytic enzymes, and addition of calcium sequestering salts (CSS). Milk acidification is accompanied with dissociation of minerals, including micellar Ca, P_i, Mg and citrate. The dissolution of MCP is higher at lower pH and almost all MCP is solubilized at pH 5.1 (Zhong et al., 2007).

CSS act by sequestering free Ca ions present, and depending on their structure, they can also interact with Ca from MCP. CSS shift the protein-mineral equilibria, leading to the depletion of MCP, decrease in free Ca ion concentration and the dissociation of individual caseins from the

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https://doi.org/10.1016/j.foodhyd.2024.109970
Received 14 January 2024; Received in revised form 23 February 2024; Accepted 6 March 2024
Available online 11 March 2024

micelle (de Kort et al., 2011). However, the extent to which CSS interact with Ca ions and affect the casein micelle structure varies. In a recent study, it was suggested that disodium phosphate (DSP) acts through forming insoluble Ca-phosphate complexes and trisodium citrate (TSC) forms soluble Ca-citrate complexes and solubilises phosphate (Deshwal et al., 2023). On the other hand, sodium hexametaphosphate (SHMP) has been suggested to disrupt MCP nanoclusters, bind serum phase Ca and form SHMP-Ca-casein complexes (Garcia et al., 2023; Mizuno & Lucey, 2007). However, it is difficult to understand the exact nature of interaction between CSS anion and casein owing to the involvement of multiple phenomena, like complexation of free Ca, chelation of micellar Ca, peptization of MCP nanoclusters and pH shifts induced by CSS addition (Kaliappan & Lucey, 2011). Therefore, milk protein systems with individual CSS and constant pH should be investigated to understand their mechanism of action.

Several studies have evaluated the effect of CSS on solubilization of casein and minerals after ultracentrifugation of the samples and measuring the amount of casein fractions and minerals in supernatant (Choi & Zhong, 2020; Deshwal et al., 2023; Mizuno & Lucey, 2005, 2007). However, non-sedimentable fraction can include both soluble and protein-associated salts, which does not seem to provide the elaborated information on the disruption of casein micelles (Mizuno & Lucey, 2005, 2007). Such information may be obtained by selectively concentrating the soluble minerals by ultrafiltration through a semi-permeable membrane with a pore size less than 10 kDa (Holt, 2004). Using this approach, the addition of 12 or 24 mEq/L SHMP to micellar casein isolate caused large increase in non-sedimentable Ca, but only small increase in 10 kDa permeable Ca relating to the formation of SHMP complex with casein (Garcia et al., 2023). Hence, full understanding of the potential mode of action of CSS on casein micelles, particularly on salt speciation, cannot be gained from non-sedimentable fractions alone and required also consideration of soluble fractions, which was undertaken in this study.

The aim of the present study was to understand how different types of CSS disrupt the structure of casein micelles. Also, the ability of CSS to sequester free Ca^{2+} and Ca from the casein micelles at different concentrations and pH was studied. The orthophosphate salt DSP, the pyrophosphate salts disodium pyrophosphate (DSPP) and tetrasodium pyrophosphate (TSPP), the polyphosphate salts sodium tripolyphosphate (STPP) and sodium hexametaphosphate (SHMP), and trisodium citrate (TSC) were selected as CSS. All CSS were sodium salts, since the type of counter-ion can affect the protein-mineral interactions (de Kort et al., 2011). A 5% micellar casein isolate (MCI) solution was used and adjusted to pH 5.5 and 6.5 after the addition of CSS. The present study provides a better understanding of the mechanism of different CSS in dairy formulations.

2. Materials and methods

2.1. Materials

MCI (moisture 4.2%, fat 1.3%, protein 86.0%, lactose 2.9% and ash 7.3%) and sodium caseinate (NaCN; moisture 5.3%, fat 0.8%, protein 90.7%, lactose 0.3% and ash 4.0%) powder were obtained from FrieslandCampina (Amersfoort, The Netherlands). The CSS DSP, DSPP, TSPP, STPP, SHMP and TSC were procured from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample preparation and fractionation

Suspensions of MCI and NaCN were prepared by mixing MCI or NaCN powder with deionised water (50 °C) to a final concentration of 5 g protein per 100 g, at 300 rpm at 40 °C for 120 min. Solutions were stored at refrigerated temperature overnight for complete hydration and warmed again with continuous stirring at 300 rpm at 40 °C for 60 min. Thereafter, solutions were allowed to cool to room temperature, followed by addition of different amounts of CSS to reach the final concentration: 0, 10, 15, 20 and 30 mEq/L (corresponding mmol/kg concentrations in Table 1). The pH of the samples was adjusted to 5.5 or 6.5 by dropwise addition of 2 N HCl or 0.1 N NaOH under continuous stirring. Samples were stored overnight at 5 °C for equilibration and minor pH adjustments were performed at room temperature subsequently, when required.

To separate the non-sedimentable and sedimentable fraction, MCI samples prepared as described above were ultracentrifuged at 100,000×*g* for 1 h at 20 °C. The sedimentable (pellet) and non-sedimentable (supernatant) fraction were separated by decanting and weighed. A portion of supernatant was transferred to an Amicon® Ultra-15 centrifugal filter tube with a 10 kDa molecular mass cut-off membrane (Merck KGaA, Darmstadt, Germany) and centrifuged at 4000×*g* for 20 min at 20 °C. The obtained permeate was, called the 10 kDa permeable fraction, and used for mineral analysis.

2.3. Particle size analysis

Particle size, expressed as the Z-average hydrodynamic diameter (in nm), was determined by dynamic light scattering in samples diluted 100-fold with demineralised water, using a Zetasizer Nano (Malvern Instruments, Malvern, UK) at a scattering angle of 173° and a temperature of 25 °C.

2.4. Calcium, phosphorus and citrate analysis

The free Ca ion concentration was determined using a Ca ion-selective electrode (SENSION+ 9660 combination Calcium ISE, Hach, Little Ireland, Ireland) as described by (Crowley et al., 2014). Ca and P content in the whole sample, the ultracentrifugal supernatant and the 10 kDa permeate were determined using inductively coupled plasma optical emission spectrometry (Agilent Technologies, CA, USA) as described previously (Deshwal et al., 2023a). Prior to analysis, samples were digested in microwave assisted digester at 180 °C (1600 W) for 15 min. The digested samples were allowed to cool and volume was made up to 100 mL using deionised water. Ca and P in 10 kDa permeate represented the soluble Ca and P. The concentration of Ca and P in non-sedimentable and 10 kDa permeable fraction are expressed as % of total Ca and P, respectively. Protein-bound non-sedimentable Ca was calculated by subtracting non-sedimentable Ca from 10 kDa permeable Ca. Similarly, protein-bound sedimentable Ca was calculated by subtracting non-sedimentable Ca from total Ca in whole sample. Citrate concentration was determined as per the NEN-EN-17294 method using ion-chromatography with conductivity detection. Samples were clarified and filtered prior to separation of citrate using an Aminex column and 5 mM sulphuric acid as the mobile phase.

2.5. Reversed-phase high performance liquid chromatography (RP-HPLC)

The individual caseins in whole samples and ultracentrifugal

Table 1
Concentration (in mmol/kg) of the calcium sequestering salts (CSS) disodium phosphate (DSP), disodium pyrophosphate (DSPP), tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium hexametaphosphate (SHMP) and trisodium citrate (TSC) added to achieve concentrations of 10, 15, 20 or 30 mEq/L.

CSS	Formula	Charge	10 mEq/L	15 mEq/L	20 mEq/L	30 mEq/L
DSP	Na_2HPO_4	−3	3.33	5.00	6.67	10.00
DSPP	$Na_2H_2P_2O_7$	−4	2.50	3.75	5.00	7.50
TSPP	$Na_4P_2O_7$	−4	2.50	3.75	5.00	7.50
STPP	$Na_5P_3O_{10}$	−5	2.00	3.00	4.00	6.00
SHMP	$Na_6(PO_3)_6$	−6	1.67	2.50	3.33	5.00
TSC	$Na_3C_6H_5O_7$	−3	2.50	5.00	6.67	10.00

supernatants were determined by RP-HPLC as previously described (Deshwal et al., 2023). Whole samples and supernatants were mixed with equal volumes of a buffer containing 0.1 M Bis-Tris buffer (pH 6.8), 6 M guanidine hydrochloride, 5.37 mM sodium citrate, and 19.5 mM dithiothreitol (DTT) adjusted to pH 7.0. The mix was incubated at room temperature for an hour and then diluted to 1:3 using 4.5 M guanidine hydrochloride buffer having pH 2. The samples were then filtered through a 0.22 μm PES filter (Apex Scientific, Maynooth, Ireland) for loading onto the HPLC column. The percentage of non-sedimentable casein for each casein fraction was calculation by expressing the peak area for the respective casein in the supernatant as a percentage of that in the whole sample.

2.6. Viscosity

The viscosity of MCI and NaCN solutions was measured at 20 °C with an Anton Paar rheometer using a cup and bob geometry (Garcia et al., 2023). Samples were conditioned at 20 °C for 2 min, followed by shearing at 0.1/s for 1 min, 0.1 to 1000/s in 5 min, 1000 to 0.1/s in 5 min, and finally at 0.1/s for 1 min. Data points were collected after every 5 s and viscosity is presented at 100/s in mPa.s in upward curve (0.1–1000/s).

2.7. Statistical analysis

All the experiments were performed in triplicate and the average and standard deviations are reported. Statistical difference of the mean values of the samples was determined using ANOVA at 5% level of significance using SPSS software (version 29, IBM, Armonk, NY, USA) following Duncan's test.

3. Results

3.1. Particle size

Particle size of the MCI dispersion significantly ($P < 0.05$) decreased when 15–30 mEq/L of STPP, SHMP and TSC was added at both the studied pH values, but for DSPP, significantly ($P < 0.05$) larger aggregates were formed at pH 5.5 (Table 2). At the highest concentration of added CSS (30 mEq/L), samples containing the polyphosphates STPP and SHMP showed the smallest particle size at both pH 6.5 (81–85 nm) and 5.5 (80–115 nm). At pH 6.5, the particle size of the MCI dispersion with DSPP increased from 200 nm at 10 mEq/L concentration to 337.40 nm at 30 mEq/L added DSPP (Table 2). For the pyrophosphates DSPP and TSPP, addition of 10 mEq/L at both pH 5.5 and 6.5 decreased particle size significantly ($P < 0.05$) but higher levels of these added salts progressively increased the particle size (Table 2). Pitkowski et al. (2008) showed that adding polyphosphate above a critical concentration required for complete dissociation of casein formed small micellar particles containing 10–15 caseins and hydrodynamic radius of around 10 nm. Similarly, addition of 1 mM of SHMP to a 5% skim milk powder dispersion reduced the particle size to around 60 nm, which was much smaller than normal casein micelles (150–200 nm) (Choi & Zhong, 2020). Except for samples with added SHMP, the particle size of all the MCI samples added with CSS was higher at pH 5.5 than pH 6.5 (Table 2).

3.2. Protein partitioning

The non-sedimentable casein fractions in the MCI solution added with different CSS at pH 6.5 and pH 5.5 are shown in Fig. 1. Non-sedimentable κ-casein, α_{S2}-casein, α_{S1}-casein and β-casein increased with increasing concentration of CSS added; α_{S1}-casein and β-casein behaved similar in terms of dissociation from casein micelles for all CSS (Fig. 1), yielding a linear relationship ($R^2 = 0.98$) between levels of non-sedimentable α_{S1}-casein and β-casein over the entire sample set (Fig. 2A). The increases in levels of non-sedimentable caseins were dependent on the type and concentration of CSS and followed the following order: polyphosphates (STPP, SHMP) > pyrophosphates (DSPP, TSPP) > citrate (TSC) > orthophosphate (DSP). In previous studies on milk protein concentrate solutions, the addition of DSP also resulted in little change in non-sedimentable casein (Kaliappan & Lucey, 2011; Mizuno & Lucey, 2005, 2007). At equal concentrations of CSS (10–30 mEq/L), TSC showed higher amount of non-sedimentable caseins than DSP but lower than DSPP, TSPP, STPP and SHMP (Fig. 1). Out of the polyphosphates, SHMP showed the highest amount of non-sedimentable caseins at pH 5.5, while STPP and SHMP showed similar levels at pH 6.5 (Fig. 1).

The levels of non-sedimentable casein were higher at pH 6.5 than at pH 5.5. However, at pH 5.5, samples with added TSC showed higher levels of non-sedimentable caseins than samples with added DSP and pyrophosphates (DSPP and TSPP) (Fig. 1B–D, F, H). Additionally, samples with added pyrophosphates showed lower levels of non-sedimentable caseins than those with DSP at pH 5.5 (Fig. 1). Interestingly, for the samples with added pyrophosphates at pH 6.5, samples with added TSPP showed higher amount of non-sedimentable caseins, and also higher non-sedimentable Ca and P, as will be discussed in the next section, than the counterpart pyrophosphate DSPP (Fig. 1), indicating stronger disruption of casein micelles in the former.

3.3. Partitioning of calcium, phosphorus and citrate

Free Ca^{2+} ion concentration decreased with increasing concentration of added CSS up to 30 mEq/L (Fig. 3). All the MCI solutions containing phosphate based CSS had higher free Ca^{2+} ion concentration at pH 5.5 than pH 6.5, which corroborates with the previous findings of (Gaucher et al., 2007).

The addition of 10–30 mEq/L of pyrophosphates, polyphosphates and citrate to MCI significantly ($P < 0.05$) decreased the protein-bound sedimentable Ca (Fig. 4, Table S1) and P (Fig. 5, Table S4) at pH 6.5. At pH 5.5, protein-bound sedimentable Ca increased significantly ($P < 0.05$) with increasing concentration of DSPP, TSPP, and STPP and decreased significantly ($P < 0.05$) with increasing concentration of

Table 2
Particle size (in nm) of 5% micellar casein isolate (MCI) solutions with 0–30 mEq/L of added calcium sequestering salts (CSS) disodium phosphate (DSP), disodium pyrophosphate (DSPP), tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium hexametaphosphate (SHMP), or trisodium citrate (TSC) at pH 6.5 and pH 5.5. Values are mean ± standard deviation (n = 3).

CSS	pH 6.5					pH 5.5				
	0 mEq/L	10 mEq/L	15 mEq/L	20 mEq/L	30 mEq/L	0 mEq/L	10 mEq/L	15 mEq/L	20 mEq/L	30 mEq/L
DSP	211 ± 9^{abA}	319 ± 10^{dF}	241 ± 3^{cD}	224 ± 3^{bE}	199 ± 5^{aC}	381 ± 9^{aA}	631 ± 10^{cC}	507 ± 19^{bE}	464 ± 15^{bD}	401 ± 38^{aC}
DSPP	211 ± 9^{abA}	200 ± 3^{aB}	225 ± 9^{bcC}	234 ± 3^{cF}	337 ± 12^{dE}	381 ± 9^{abA}	332 ± 21^{aB}	430 ± 33^{bD}	1654 ± 12^{cE}	2450 ± 46^{dD}
TSPP	211 ± 9^{cA}	109 ± 5^{aA}	126 ± 5^{bA}	133 ± 2^{bC}	234 ± 6^{dD}	381 ± 9^{cA}	231 ± 14^{aA}	237 ± 4^{aB}	293 ± 17^{bC}	365 ± 15^{cC}
STPP	211 ± 9^{dA}	218 ± 4^{dC}	126 ± 3^{cA}	94 ± 2^{bA}	81 ± 2^{aA}	381 ± 9^{eA}	238 ± 6^{dA}	193 ± 2^{cA}	172 ± 3^{bB}	115 ± 1^{aA}
SHMP	211 ± 9^{dA}	231 ± 3^{eD}	198 ± 1^{cB}	111 ± 4^{bB}	84 ± 3^{aA}	381 ± 9^{eA}	218 ± 4^{dA}	163 ± 5^{cA}	108 ± 3^{bA}	78 ± 2^{aA}
TSC	211 ± 9^{cA}	271 ± 5^{dE}	201 ± 8^{bcB}	195 ± 2^{bD}	159 ± 7^{aB}	381 ± 9^{dA}	317 ± 5^{cB}	293 ± 6^{bC}	186 ± 6^{aB}	176 ± 7^{aB}

^{abcde}Mean values in a row for a specific pH not sharing a common lowercase superscript letter are significantly different ($P < 0.05$).
^{ABCDEF}Mean values in a column not sharing a common uppercase superscript letter are significantly different ($P < 0.05$).

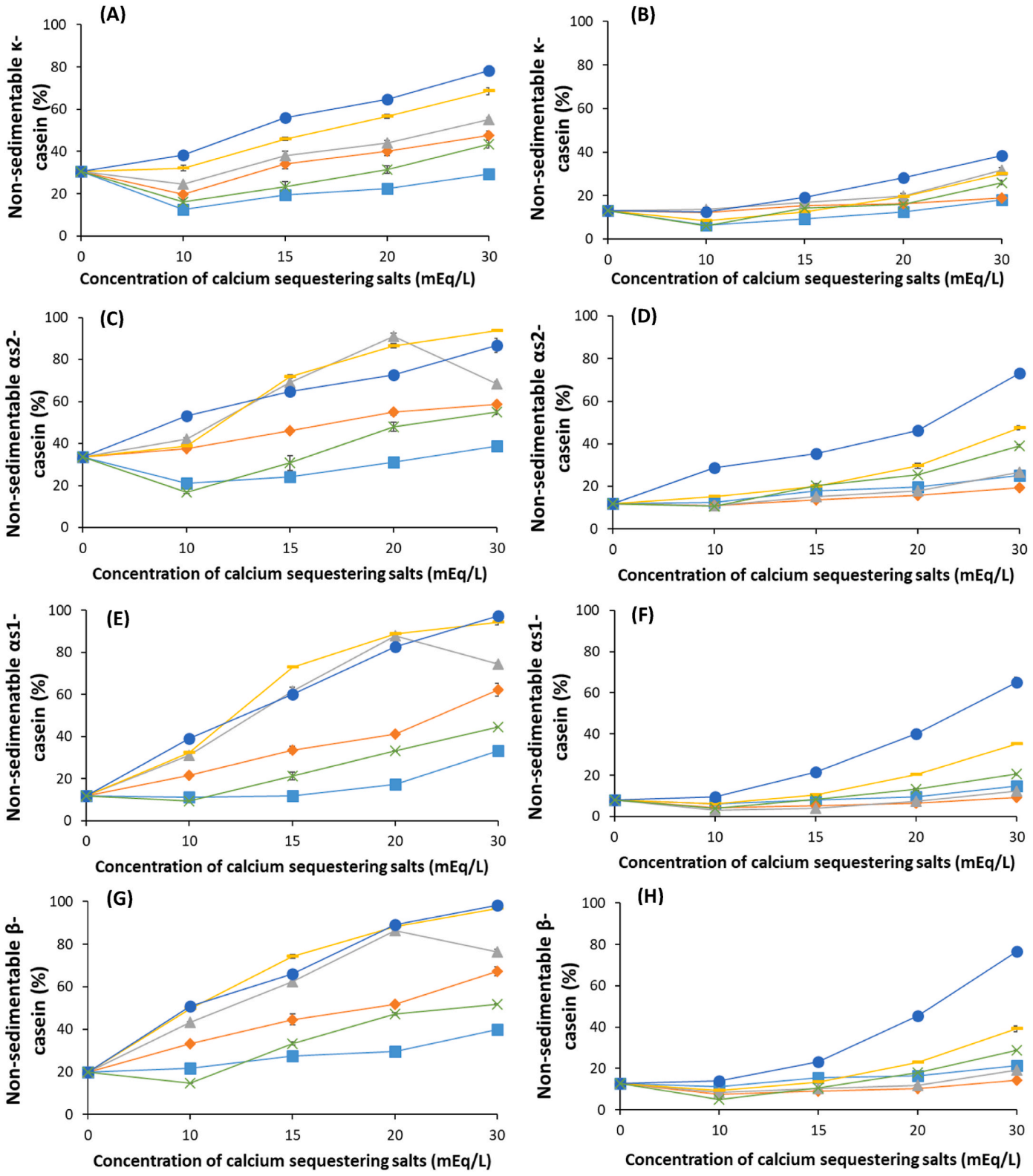

Fig. 1. Effect of the addition of the calcium sequestering salts disodium phosphate (DSP; ■), disodium pyrophosphate (DSPP; ◆), tetrasodium pyrophosphate (TSPP; ▲), sodium tripolyphosphate (STPP; –), sodium hexametaphosphate (SHMP; ●) or trisodium citrate (TSC; ✖) on the level of non-sedimentable (100,000×*g* for 60 min at 20 °C) κ-casein (A, B), α_{S2}-casein (C, D), α_{S1}-casein (E, F) and β-casein (G, H) in 5% micellar casein isolate suspensions at pH 6.5 (A, C, E, G) and 5.5 (B, D, F, H). Values are means (n = 2) with the standard deviation indicated by vertical error bars.

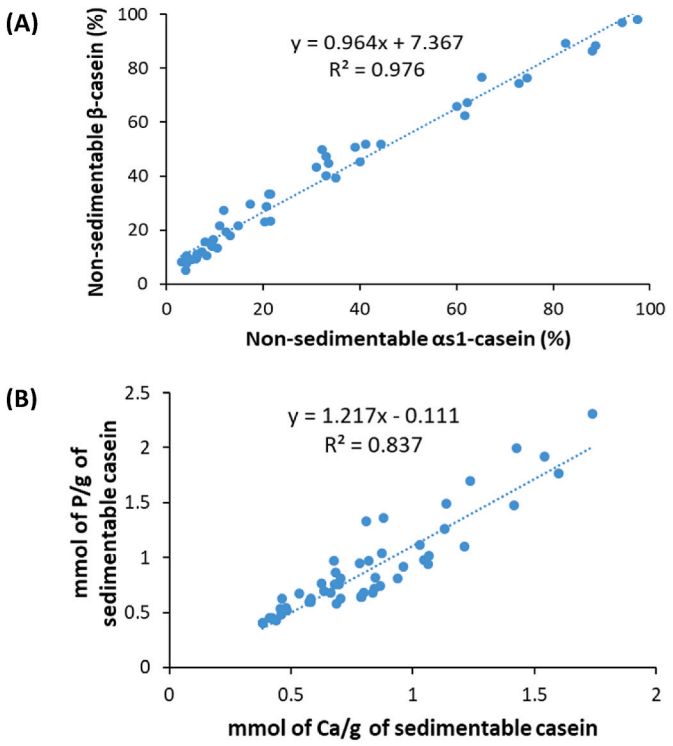

Fig. 2. Correlations between levels of non-sedimentable α_{S1}-casein and non-sedimentable β-casein (A) and mmol of Ca and P/g of sedimentable casein (B) for CSS at pH 6.5 and 5.5.

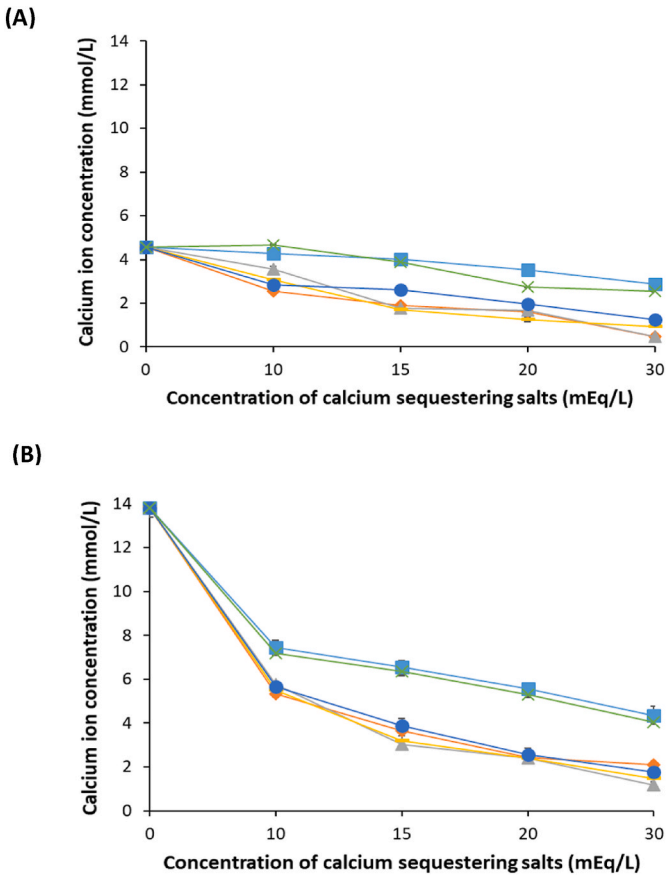

Fig. 3. Effect of addition of 0–30 mEq/L of the calcium sequestering salts disodium phosphate (DSP; ■), disodium pyrophosphate (DSPP; ◆), tetrasodium pyrophosphate (TSPP; ▲), sodium tripolyphosphate (STPP; —), sodium hexametaphosphate (SHMP; ●) or trisodium citrate (TSC; ✖) on the concentration of ionic calcium at pH 6.5 (A) and 5.5 (B) in 5% MCI. Values are means (n = 3) with the standard deviation indicated by vertical error bars.

SHMP and TSC (Fig. 4; Table S1). Protein-bound sedimentable P showed similar trends with increasing CSS concentration. At 30 mEq/L, samples with added SHMP and TSC showed significantly ($P < 0.05$) lowest levels of protein-bound sedimentable Ca (Fig. 4, Table S1) and P (Fig. 5; Table S4) at pH 5.5.

10 kDa-permeable Ca decreased significantly ($P < 0.05$) with increasing concentration of DSPP, TSPP, STPP and SHMP, whereas it increased significantly ($P < 0.05$) for TSC at both pH 5.5 and 6.5 (Fig. 4; Table S3). These findings are in line with previous reports showing that addition of 20 mmol/L TSC to milk strongly increased the 10 kDa permeable Ca from 32 to 65% of total Ca (Vujicic et al., 1968) and that the addition of 12 mEq/L of SHMP to MCI (9% protein) increased the 10 kDa permeable Ca from 11 to 17% of total Ca (Garcia et al., 2023). 10 kDa-permeable P significantly ($P < 0.05$) increased for all the phosphate based CSS, except DSP at pH 6.5 (Fig. 5, Table S3). However, DSP showed ~11% decrease, pyrophosphates, and polyphosphates showed ~70–80% decrease in 10 kDa permeable Ca at pH 5.5 (Fig. 5, Table S3). A significantly ($P < 0.05$) higher concentration of non-sedimentable Ca was observed with increasing concentration of SHMP at pH 5.5 and 6.5, but 10 kDa-permeable Ca showed less significant ($P < 0.05$) decrease at pH 6.5 (Fig. 4, Table S2). This suggests that SHMP can directly form complex with casein, also in the absence of Ca, corroborating previous findings (Culler et al., 2017).

In MCI suspensions without added CSS, 10 kDa-permeable Ca was nearly 5-fold higher at pH 5.5 (16.7 mmol/L) than pH 6.5 (3.3 mmol/L) (Fig. 4, Table S3). Similar findings were observed for concentration of free Ca^{2+} ions (Fig. 3), which is consistent with the findings of (Ho et al., 2018). The concentrations of 10 kDa-permeable Ca (Fig. 4; Table S3) and P (Fig. 5; Table S6) were also higher at pH 5.5 than at pH 6.5 for all samples with added CSS, indicating the pH-induced solubilization of MCP at pH 5.5. In spite of large pH-induced differences in the level of 10 kDa-permeable Ca in MCI, added STPP and SHMP reduced the 10 kDa permeable Ca at pH 5.5 and pH 6.5 almost to the same level (Fig. 4; Table S3), which suggests the formation of insoluble Ca-polyphosphate complexes with casein. Nakajima et al. (1975) reported a similar decrease in diffusible Ca, which can be considered comparable to 10 kDa-permeable Ca determined in this study, on addition of STPP to casein micelles isolated from skim milk. Almost all the citrate (≥95%) was present in the non-sedimentable fraction, of which ≥75% was 10 kDa permeable. pH and level of TSC addition did not affect the percentage of citrate in non-sedimentable and 10 kDa permeable fraction (data not shown).

The mineralisation of the sedimentable casein fraction, expressed in mmol of sedimentable Ca and P per g of sedimentable casein, increased with increasing concentration of added CSS, except for TSC, for which the mineralisation of sedimentable casein fraction remained constant (Fig. 6). According to these results, the higher the concentration of phosphate-based CSS, the higher amount of Ca is transferred to non-sedimentable fraction, suggesting the formation of insoluble Ca-phosphate complexes with casein. For control samples, the mineralisation level was 0.79 mmol Ca per g casein and 0.64 mmol P per g casein, which are in line with previous studies (Huppertz et al., 2021; Malacarne et al., 2014). Overall, higher mineralisation of sedimentable casein was observed at pH 6.5 than pH 5.5. At 30 mEq/L of added CSS, the mineralisation level for Ca was highest for SHMP (1.74 mmol Ca per g casein) at pH 6.5 and for STPP (1.14 mmol Ca per g casein) at pH 5.5 (Fig. 6). Furthermore, a strong correlation ($R^2 = 0.837$) was observed between Ca and P mineralisation of sedimentable casein (Fig. 2B). In the current study, when the degree of casein mineralisation, expressed as concentration of MCP increased, the amount of non-sedimentable individual casein fractions also increased.

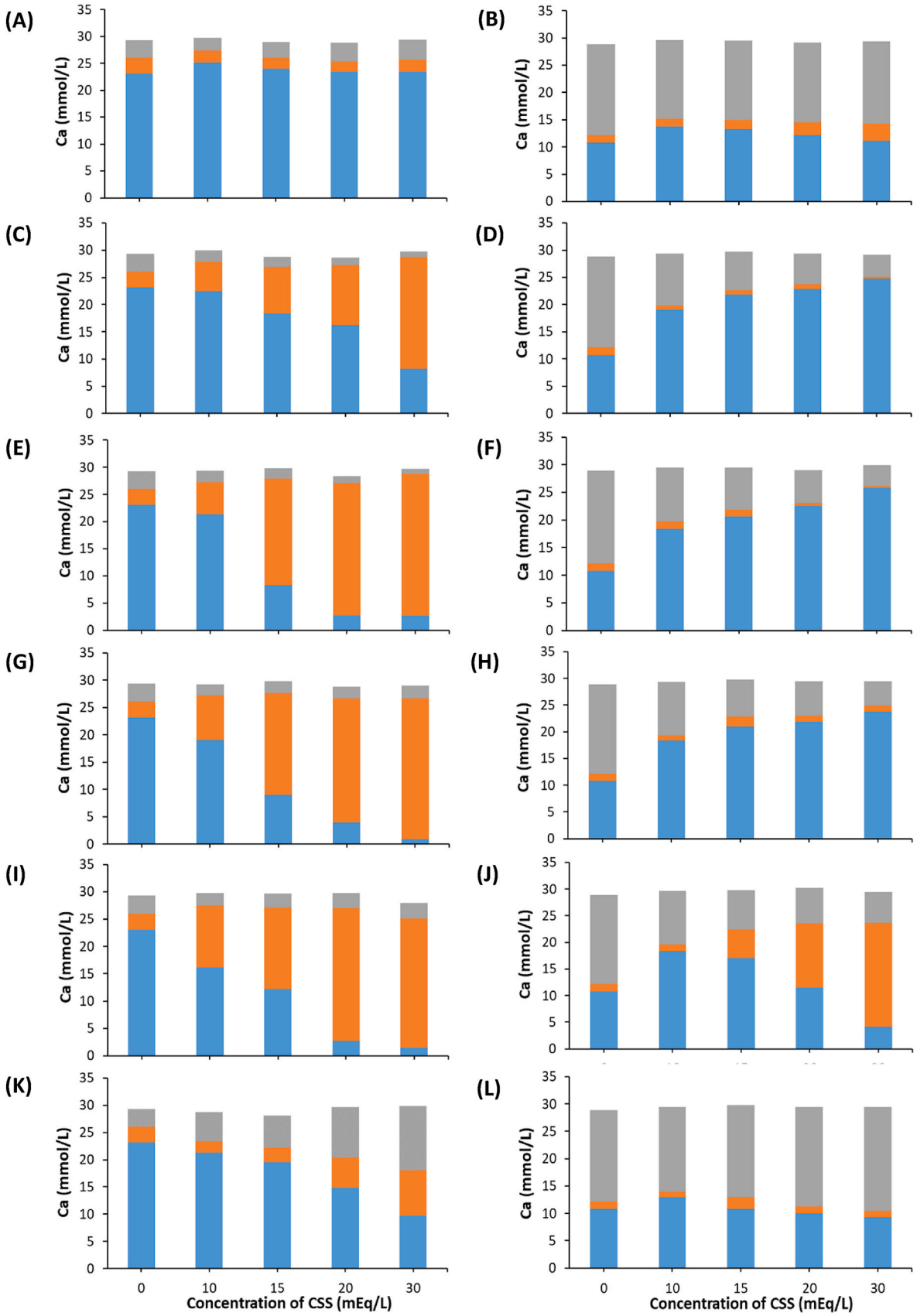

Fig. 4. Effect of addition of 0–30 mEq/L of the calcium sequestering salts (CSS) disodium phosphate (DSP; A, B), disodium pyrophosphate (DSPP; C, D), tetrasodium pyrophosphate (TSPP; E, F), sodium tripolyphosphate (STPP; G, H), sodium hexametaphosphate (SHMP; I, J), trisodium citrate (TSC; K, L) on the level of protein-bound sedimentable (■), protein-bound non-sedimentable (■) and 10 kDa-permeable (■) calcium at pH 6.5 (A, C, E, G, I, K) and 5.5 (B, D, F, H, J, L) in 5% MCI. Values are means (n = 3). Statistical significance of data is indicated in Supplementary Tables S1–S3.

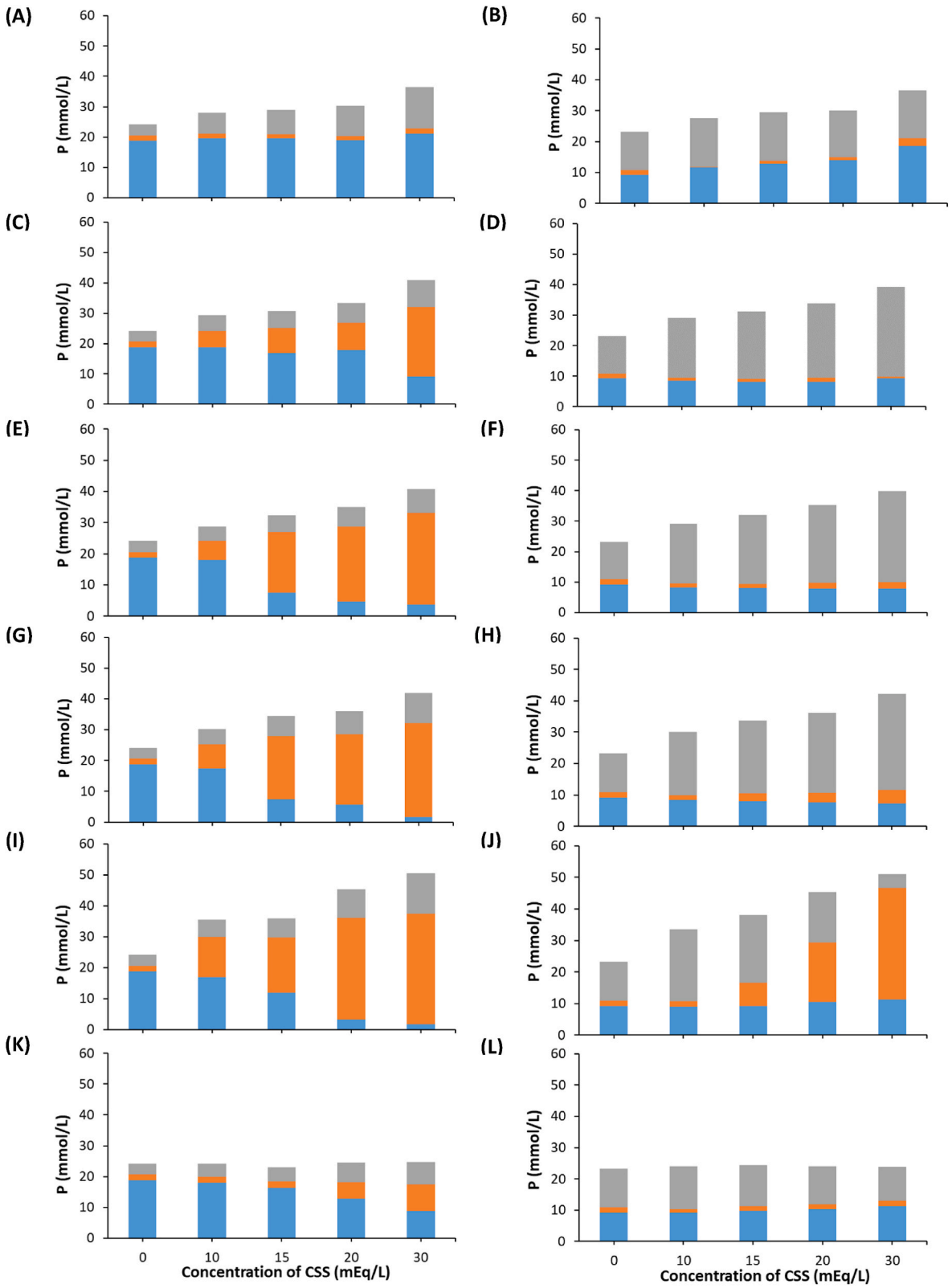

Fig. 5. Effect of addition of 0–30 mEq/L of the calcium sequestering salts (CSS) disodium phosphate (DSP; A, B), disodium pyrophosphate (DSPP; C, D), tetrasodium pyrophosphate (TSPP; E, F), sodium tripolyphosphate (STPP; G, H), sodium hexametaphosphate (SHMP; I, J), trisodium citrate (TSC; K, L) on the level of protein-bound sedimentable (■), protein-bound non-sedimentable (■) and 10 kDa-permeable (■) phosphorus at pH 6.5 (A, C, E, G, I, K) and 5.5 (B, D, F, H, J, L) in 5% MCI. Values are means (n = 3). Statistical significance of data is indicated in Supplementary Tables S4–S6.

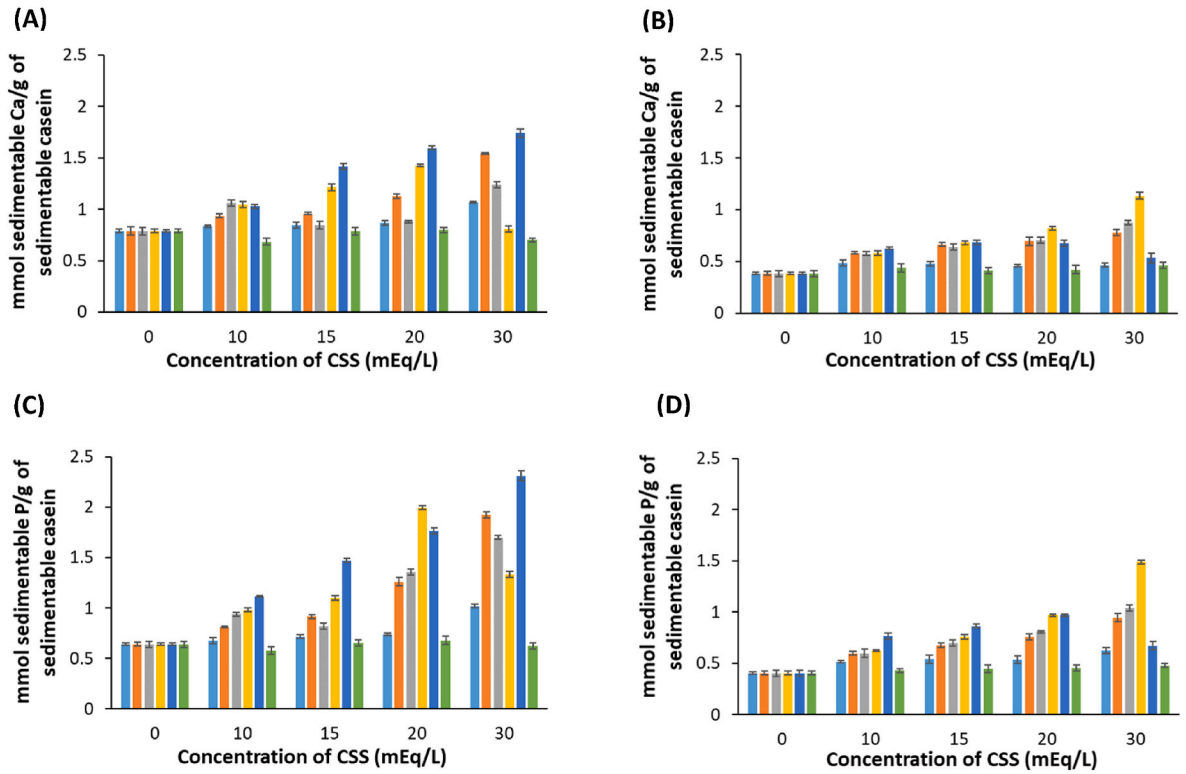

Fig. 6. Effect of addition of 0–30 mEq/L disodium phosphate (DSP;), disodium pyrophosphate (DSPP;), tetrasodium pyrophosphate (TSPP;), sodium tripolyphosphate (STPP;), sodium hexametaphosphate (SHMP;), trisodium citrate (TSC;) on the level of sedimentable calcium (A, B) and phosphorus (C, D) per gram of sedimentable casein at pH 6.5 (A, C) and 5.5 (B, D) in 5% MCI. Values are means (n = 2) with the standard deviation indicated by vertical error bars.

3.4. Viscosity

Measuring the changes in viscosity upon addition of CSS to MCI (Ca-rich) and NaCN (Ca-free) suspensions can help elucidate whether CSS binds directly to caseins or Ca ions are involved in this binding. The addition of orthophosphates, polyphosphates and citrates significantly ($P < 0.05$) decreased MCI viscosity with increasing concentration at pH 6.5 (Table 3). On the contrary, the addition of pyrophosphates significantly ($P < 0.05$) increased MCI viscosity with increasing concentration at both pH 6.5 and 5.5 (Table 3). For NaCN solutions at pH 6.5, all studied CSS caused a significant ($P < 0.05$) decrease in viscosity with increasing concentration, except SHMP which showed significant ($P < 0.05$) increase in viscosity (Table 4). This decrease may be related to the increase in ionic strength on addition of CSS. Huppertz et al. (2017) reported decreased viscosity of sodium caseinate suspensions (2.8% w/w) with increasing ionic strength and the effects were more prominent at high pH and less notable at lower pH. When SHMP was added to NaCN solutions, viscosity increased significantly ($P < 0.05$) from 4.72 mPa ·s to 5.5 mPa ·s at 30 mEq/L at pH 6.5, suggesting that SHMP also interacts with casein in the absence of Ca (Table 4).

At 30 mEq/L added DSPP and TSPP, MCI samples showed an abrupt ~10-fold and ~2-fold increase in viscosity, respectively (Table 3). The viscosity of MCI with 30 mEq/L added DSPP increased significantly ($P < 0.05$) to 53.3 mPa s at pH 6.5 and 20.8 mPa s at pH 5.5. Such high viscosity indicate the possibilities of casein aggregate structures of large size and in sufficient number to occlude the water. As the concentration

Table 3
Viscosity (in mPa.s) of 5% micellar casein isolate (MCI) solutions with 0–30 mEq/L of the added calcium sequestering salts (CSS) disodium phosphate (DSP), disodium pyrophosphate (DSPP), tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium hexametaphosphate (SHMP), or trisodium citrate (TSC) at pH 6.5 and pH 5.5. Values are mean ± standard deviation (n = 3).

CSS	pH 6.5					pH 5.5				
	0 mEq/L	10 mEq/L	15 mEq/L	20 mEq/L	30 mEq/L	0 mEq/L	10 mEq/L	15 mEq/L	20 mEq/L	30 mEq/L
DSP	2.72 ± 0.05^{aA}	4.54 ± 0.06^{eE}	3.86 ± 0.01^{dF}	3.26 ± 0.01^{cD}	2.95 ± 0.03^{bB}	1.98 ± 0.07^{aA}	4.08 ± 0.01^{eE}	3.54 ± 0.02^{dE}	3.07 ± 0.01^{cD}	2.55 ± 0.04^{bB}
DSPP	2.72 ± 0.05^{aA}	2.97 ± 0.13^{abB}	3.35 ± 0.02^{bD}	4.91 ± 0.06^{cE}	53.33 ± 0.58^{dE}	1.98 ± 0.07^{aA}	2.72 ± 0.03^{bC}	2.97 ± 3.93^{bC}	3.93 ± 0.01^{cE}	20.84 ± 0.72^{dD}
TSPP	2.72 ± 0.05^{aA}	3.15 ± 0.02^{bC}	3.66 ± 0.01^{cE}	5.27 ± 0.04^{dF}	6.38 ± 0.05^{eD}	1.98 ± 0.07^{aA}	2.77 ± 0.08^{bC}	3.54 ± 0.03^{cE}	5.05 ± 0.03^{dF}	6.15 ± 0.01^{eC}
STPP	2.72 ± 0.05^{bA}	3.54 ± 0.02^{eD}	3.20 ± 0.02^{dC}	2.81 ± 0.03^{cC}	2.37 ± 0.02^{aA}	1.98 ± 0.07^{aA}	3.34 ± 0.02^{dD}	3.14 ± 0.03^{cD}	2.35 ± 0.03^{bC}	2.02 ± 0.01^{aAB}
SHMP	2.72 ± 0.05^{cA}	3.31 ± 0.08^{eC}	2.91 ± 0.01^{dB}	2.28 ± 0.01^{bB}	2.12 ± 0.06^{aA}	1.98 ± 0.07^{aA}	2.31 ± 0.02^{dA}	2.19 ± 0.03^{cB}	2.08 ± 0.01^{bB}	2.01 ± 0.01^{abAB}
TSC	2.72 ± 0.05^{cA}	2.74 ± 0.09^{cA}	2.15 ± 0.03^{bA}	2.04 ± 0.02^{abA}	1.95 ± 0.02^{aA}	1.98 ± 0.07^{bA}	2.56 ± 0.04^{cB}	1.92 ± 0.07^{bA}	1.90 ± 0.04^{bA}	1.73 ± 0.04^{aA}

^{abcde}Mean values in a row for a specific pH not sharing a common lowercase superscript letter are significantly different ($P < 0.05$).
^{ABCDEF}Mean values in a column not sharing a common uppercase superscript letter are significantly different ($P < 0.05$).

Table 4
Viscosity (in mPa.s) of 5% sodium caseinate solutions with 0–30 mEq/L of the added calcium sequestering salts (CSS) disodium phosphate (DSP), disodium pyrophosphate (DSPP), tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium hexametaphosphate (SHMP), or trisodium citrate (TSC) at pH 6.5 and pH 5.5. Values are mean ± standard deviation (n = 3).

CSS	pH 6.5					pH 5.5				
	0 mEq/L	10 mEq/L	15 mEq/L	20 mEq/L	30 mEq/L	0 mEq/L	10 mEq/L	15 mEq/L	20 mEq/L	30 mEq/L
DSP	4.01 ± 0.04^{eA}	3.89 ± 0.01^{dC}	3.78 ± 0.01^{cC}	3.72 ± 0.01^{bD}	3.63 ± 0.01^{aC}	3.10 ± 0.03^{cA}	2.90 ± 0.03^{bB}	2.88 ± 0.03^{bAB}	2.85 ± 0.03^{bB}	2.77 ± 0.02^{aA}
DSPP	4.01 ± 0.04^{eA}	3.71 ± 0.03^{dB}	3.64 ± 0.03^{cB}	3.58 ± 0.01^{bC}	3.45 ± 0.04^{aB}	3.10 ± 0.03^{dA}	2.95 ± 0.03^{cB}	2.88 ± 0.03^{bAB}	2.80 ± 0.02^{aA}	2.77 ± 0.03^{aA}
TSPP	4.01 ± 0.04^{dA}	3.61 ± 0.03^{cA}	3.52 ± 0.02^{bA}	3.51 ± 0.01^{bB}	3.44 ± 0.03^{aAB}	3.10 ± 0.03^{cA}	2.80 ± 0.03^{aA}	2.82 ± 0.01^{abA}	2.83 ± 0.03^{abAB}	2.87 ± 0.03^{bB}
STPP	4.01 ± 0.04^{eA}	3.71 ± 0.03^{dB}	3.63 ± 0.04^{cB}	3.49 ± 0.01^{bB}	3.36 ± 0.04^{aA}	3.10 ± 0.03^{cA}	2.91 ± 0.03^{bB}	2.92 ± 0.02^{bBC}	2.87 ± 0.01^{bB}	2.76 ± 0.02^{aA}
SHMP	4.01 ± 0.04^{aA}	4.72 ± 0.06^{bD}	4.87 ± 0.08^{cD}	5.16 ± 0.04^{dE}	5.50 ± 0.09^{eD}	3.10 ± 0.03^{aA}	3.40 ± 0.03^{bD}	3.56 ± 0.03^{cD}	3.62 ± 0.01^{dD}	3.64 ± 0.03^{dC}
TSC	4.01 ± 0.04^{dA}	3.66 ± 0.04^{cAB}	3.51 ± 0.04^{bA}	3.41 ± 0.01^{aA}	3.36 ± 0.05^{aA}	3.10 ± 0.03^{cA}	3.07 ± 0.02^{cC}	2.95 ± 0.07^{bC}	2.85 ± 0.03^{aB}	2.82 ± 0.01^{aB}

^{abcde}Mean values in a row for a specific pH not sharing a common lowercase superscript letter are significantly different ($P < 0.05$).
^{ABCDEF}Mean values in a column not sharing a common uppercase superscript letter are significantly different ($P < 0.05$).

of added DSP, STPP, SHMP and TSC was increased, more Ca was chelated, leading to higher casein disruption and subsequent decrease in viscosity (Table 3). A similar mechanism was explained by McCarthy et al. (2017) for 5% micellar casein isolate solution added with SHMP and TSC. Mizuno and Lucey (2007) reported no gelation in 10% milk protein concentrate solutions at TSPP concentrations below 2.9 mM and above 10.5 mM. The viscosity of MCI samples added with CSS was lower at pH 5.5 than pH 6.5 (Table 3). Ho et al. (2018) and Karlsson et al. (2005) showed reduction in viscosity on changing the pH of milk protein concentrate from 6.5 to 5.8, as observed in present study. For rennet casein gels with phosphate-based CSS, based on microscopy and rheological measurements, Zhong et al. (2007) reported fewer protein aggregates with less cross-links at pH 5.8 than pH 6.7, corresponding to a weaker gel and more porous aggregate structure at pH 5.8.

4. Discussion

Forces involved in the internal stability of casein micelles include attractive forces, involving Ca phosphate cross-links, hydrogen bonds, hydrophobic and charge interactions, and repulsive forces, comprising electrostatic interactions. Of these forces, Ca phosphate cross-links are the main contributor and immobilizes the flexible hydrophilic parts of caseins, thereby imparting more rigid structure to casein micelles (Horne, 1998). The addition of CSS to MCI suspensions results in dispersion of caseins induced by loss of Ca phosphate cross-links and formation of different type of Ca-CSS complexes or casein-Ca-CSS complexes (Mizuno & Lucey, 2007). This micellar disruption is also apparent from the reductions in particle size (Table 2) and the increases in non-sedimentable casein (Fig. 1) in MCI suspensions with added CSS. CSS have been reported to follow the following order of Ca sequestration: polyphosphates > pyrophosphates > citrates > orthophosphates (Deshwal et al., 2023b; Mizuno & Lucey, 2005). Orthophosphates, like DSP, can form insoluble Ca-phosphate complexes (e.g. $Ca_3(PO)_4)_2$ or $CaHPO_4$). Pyrophosphates (DSPP and TSPP) and polyphosphates (STPP and SHMP), on the other hand, have been suggested to interact with cations and caseins simultaneously, forming casein-Ca-pyrophosphate and casein-Ca-polyphosphate complexes, respectively (De Kort et al., 2009; Mizuno & Lucey, 2005, 2007). TSC has been suggested to form soluble Ca-citrate complexes and solubilises phosphate (Deshwal et al., 2023; Mizuno & Lucey, 2005). De Kort et al. (2009) reported that Ca^{2+} reacts with DSP in a ratio of 3:2 to form $Ca_3(PO4)_2$ complexes, and with SHMP in a ratio of 3:1 to form $Ca_3(PO_4)_6$. The formation of soluble Ca-citrate complexes is indeed in line with increased levels of 10 kDa--permeable Ca in samples with added TSC (Fig. 4) and the fact that virtually all added citrate in these samples was also found in the 10 kDa-permeable fraction (data not shown).

There are three different ways to disrupt a casein micelles by CSS: binding the micellar Ca, disrupting the protein-protein interactions and peptization of nanoclusters (Garcia et al., 2023; Huppertz et al., 2017). Solubilization of MCP can be assessed from soluble minerals. This, however, needs to be assessed from the 10 kDa-permeable fraction and not the non-sedimentable fraction, because the non-sedimentable mineral fraction includes both soluble and protein-associated minerals; therefore, it does not provide detailed information of the casein micelles disruption. In order to achieve such information, measuring minerals in permeate of samples obtained using ultrafiltration membranes capable of permeating dissolved salts without protein and associated salts, could be performed (Garcia et al., 2023). The results of non-sedimentable and 10-kDa permeable Ca (Fig. 4; Tables S2 and S3) and P (Fig. 5; Tables S5 and S6) indicate that orthophosphates, pyrophosphates and polyphosphates combined with Ca to form insoluble Ca phosphate complex together with casein (Mizuno & Lucey, 2007). On the other hand, significantly ($P < 0.05$) higher amount of 10 kDa permeable Ca for MCI samples with TSC (Fig. 4; Table S3) confirms the formation of soluble Ca-citrate complexes, as previously suggested (Deshwal et al., 2023). Levels of 10 kDa-permeable Ca were less affected by increasing concentration of added phosphate-based CSS (Fig. 4; Table S3). This clearly indicates that added inorganic phosphate ions did not solubilize MCP from the micelle to the soluble phase like citrate. At low concentrations (50 mM), orthophosphates did not displace the MCP because affinity of Ca is higher for phosphoseryl residues and inorganic phosphate present in MCP than inorganic phosphate ions of CSS (Le Ray et al., 1998). On saturation of soluble phase with Ca phosphate, precipitation and/or interaction of the casein micelle with Ca phosphate can occur (Gaucher et al., 2007).

The level of protein-bound sedimentable Ca (Fig. 4; Table S1) and P (Fig. 5; Table S3) corresponded inversely with the decreasing trend of non-sedimentable caseins for all the CSS at pH 6.5. The addition of TSC to MCI significantly ($P < 0.05$) increased the 10 kDa permeable Ca and P suggesting that TSC binds micellar Ca, thereby solubilizing both Ca (Fig. 4; Table S3) and inorganic P (Fig. 5; Table S6). Addition of the phosphate-based CSS (DSP, DSPP, TSPP, STPP and SHMP), however, increased non-sedimentable Ca (Fig. 4; Table S2) and non-sedimentable casein (Fig. 1), without a notable increase in 10 kDa permeable Ca (Fig. 4; Table S3) or P (Fig. 5; Table S6). This indicates that the phosphate-based CSS do not induce the observed disruption of casein micelles by solubilization of MCP, but rather act by either disrupting protein-protein interactions or by peptization of nanoclusters. The only slight change in viscosity of NaCN on addition of DSP, DSPP, TSPP and STPP (Table 4) clearly suggests the requirement of Ca ions for complexation between caseins and these phosphate-based CSS. Only the increase in NaCN viscosity with added SHMP (Table 4) suggests a direct

interaction of this CSS with caseins. Arginine, histidine, lysine and the α-NH_2 terminal groups are positively charged amino acids in NaCN, therefore might be binding site for SHMP (de Kort, 2012). This suggests that for SHMP, part of the micellar disruption observed (Fig. 1, Table 2) may be via direct interaction of SHMP with caseins. For the other phosphate-based CSS, i.e., DSP, DSPP, TSPP and STPP, the lack of increases in 10 kDa-permeable Ca (Fig. 4; Table S3) and NaCN viscosity (Table 4) indicates that neither solubilization of MCP nor direct interactions with caseins are likely drivers for micellar disruption and that CSS-induced peptization of MCP nanoclusters is more likely. Further studies in CSS-induced peptization of MCP nanoclusters are required.

At pH 5.5, in spite of decreasing non-sedimentable Ca (Fig. 4; Table S2) and P (Fig. 5; Table S5), caseins (Fig. 1) increased. This suggest that the nature of Ca-(poly/pyrophosphate)-casein interaction depends on the concentration of CSS and pH. SHMP, a polyelectrolyte molecule, can interact with positively charged sites on casein proteins. The presence of multiple negative charges due to SHMP molecules results in excessive charge repulsion (Ellinger, 2018). At high levels (34 mEq) of SHMP addition to MCI, formation of soluble CaHMP complexes resulted in decrease of casein-bound Ca and P (Kaliappan & Lucey, 2011), which is also observed in present study (Figs. 4 and 5). Zittle (1966) stated that pyrophosphates and polyphosphates binds with the positively charged residues on casein. This may result in casein aggregation or precipitation leading to increase in viscosity even in dilute protein solution. In present study, MCI samples with added DSPP and TSPP showed increases in viscosity with increasing concentration (0–30 mEq/L; Table 3), attributed to aggregation of dispersed caseins. Pyrophosphates are able to cross-link casein better than polyphosphates in the presence of Ca (Mizuno & Lucey, 2007). This is because multiple negative charges in polyphosphates causes higher caseins repulsion causing difficulties in casein re-association via hydrophobic interactions (Mizuno & Lucey, 2005, 2007). This also explains the decrease in viscosity and particle size with increased level of addition of STPP and SHMP to MCI solutions (Tables 2 and 3).

Non-sedimentable caseins are more sensitive to Ca-induced aggregation compared to micellar casein, as casein present on the micellar surface (κ-casein) are less sensitive to aggregation than caseins inside the micelles (α_S- and β-casein) (Eshpari et al., 2017). Therefore, it is hypothesized that higher amount of non-sedimentable caseins results in formation of more complexes between casein and CSS involving Ca at pH 6.5 than pH 5.5. This is further confirmed by higher amount of non-sedimentable Ca and lower amount of 10 kDa-permeable Ca at pH 6.5 than pH 5.5 (Fig. 4; Tables S2 and S3). Pitkowski et al. (2008) reported that the natural tendency of polyphosphates to chelate Ca relative to casein is lower at pH 6.0 than at pH 6.7. The higher dissociation of MCP at pH 5.5 increases the ionic Ca, resulting in increased binding of ionic Ca to the dispersed caseins, thus reducing their charge and increasing their solubility (Post et al., 2012). This higher solubility of complexes between casein-CSS involving Ca is responsible for lower viscosity of DSPP suspensions at pH 5.5 than pH 6.5.

5. Conclusions

This study demonstrated that interactions between CSS and casein micelles induced changes in distribution of casein and Ca, particle size and viscosity. Firstly, soluble Ca and casein-bound Ca can be complexed by CSS, which is dependent on CSS type (orthophosphate, pyrophosphate, polyphosphate and citrate) and concentration. Thereafter, MCP bonding with caseins is disrupted leading to disintegration of casein micelles and enhanced Ca, P and CSS concentration in soluble phase. The formation of Ca-CSS complexes with or without casein is strongly affected by pH. Based on the amount of 10 kDa permeable Ca, TSC was the most effective in solubilizing micellar Ca. The limited changes in 10 kDa permeable Ca levels after adding phosphate-based CSS signifies non-displacement of Ca ions from the micelle and indicated different routes of micelle dissociaton. The viscosity and particle size findings of DSPP and TSPP suggests that after dissociation of casein fractions by CSS, protein, Ca and phosphate can re-associate to form new complexes causing viscosity increase.

Funding

Gaurav Kr Deshwal is a recipient of a Walsh Scholarship from Teagasc, the Irish Agricultural and Food Development Authority (Ref 2020213).

CRediT authorship contribution statement

Gaurav Kr Deshwal: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Mark Fenelon:** Writing – review & editing, Supervision. **Laura G. Gómez-Mascaraque:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Thom Huppertz:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We are highly thankful to Liesbeth Van der Meulen for her help in citrate analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.109970.

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