Fortification of Concentrated Milk Protein Beverages with Soy Proteins: Impact of Divalent Cations and Heating Treatment on the Physical Stability

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Abstract

This study investigated the effects of adding calcium and magnesium chloride on heat and storage stability of milk protein concentrate-soy protein isolate (8:2 respectively) mixtures containing 10% w/w total protein subjected to the in-container sterilization (115°C x 15 min).

Increasing concentration of divalent cation salts resulted in an increase in protein particle size, dry sedimentation and sediment height and a decrease in pH, heat stability and hydration in milk protein-concentrate-soy protein isolate mixtures solutions on sterilization at 115°C. Fortification of divalent cation salts in milk protein-concentrate-soy protein isolate mixture solutions resulted in an accelerated protein sedimentation and two unique sediment regions during accelerated storage stability testing. Moreover, the heat stability decreased upon sterilization at 115°C, with addition of MgCl2 causing a greater increase in sedimentation velocity and compressibility than CaCl2. Increasing pH value of protein milk concentrate-soy protein isolate mixtures solutions from 6.7 to 7.2 resulted in an increase in viscosity following the heat treatment.

The study demonstrated that the type and concentration of divalent cation salts used strongly impacts heat and storage stability of milk protein concentrate-soy protein isolate mixture nutritional beverages.

Keywords: Milk protein concentrate; Soy protein isolate; Divalent cation salts; Heat stability; Storage stability.

Introduction

Over the past 20 years, the vegetable protein ingredients have been mixed with milk protein for preparation of nutritional beverages. These nutritional beverages are fundamentally to prevent dehydration, to supply energy, to provide protein and electrolytes to replace losses [1]. For this reason, various minerals such as calcium, magnesium, sodium, potassium and chloride are fortified in commercial infant and enteral formulations to achieve a balanced nutritional profile. Calcium and magnesium are the major intracellular divalent cations and play a major role in many biochemical processes including, protein and nucleic acid metabolism, neuromuscular transmission, bone growth and metabolism, ion channel stabilisation, energy metabolism, regulation of blood pressure, contraction of myocardial muscle cells and as a cofactor in a large number of enzymatic reactions [2-4]. USDA [5] reported that 57% of the US population may have an inadequate intake of magnesium. In eastern Asia, less than 5% of China population met the adequate calcium intake in 2002 [6]. Therefore, there is an interest in enriching the dairy-based nutritional beverages with calcium and magnesium which may be appropriate for some people [7,8].

Lindberg et al., [9] suggested that calcium or magnesium salts with the greatest water solubility resulted in the highest bioavailability of calcium and magnesium. However, fortification with soluble calcium or magnesium salts causes multiple physico-chemical changes in dairy-based nutritional products, including increased concentration of ionic concentration, reduced zeta potential and super saturation of those divalent cations, which can speed the sediment formation during the storage and fouling of heat exchangers during the processing, particularly when heated at retort sterilization (115 to 120°C for 10-20 min) or ultra-high-temperature (UHT) processing (135-150°C for 3–5 seconds) [10].

Due to the cost effective source of protein, nutritional benefits and functional properties, soy protein isolate has been popularly used as the food fortified ingredients. However, an inherent soybean organoleptic taste and poor solubility greatly limited the commercial application in the formulation of dairy-based foodstuff. For this reason, only a small portion of soy protein isolates are formulated in the dairy-derived liquid or powdered products. Milk proteins, vegetable proteins and their mixtures have been studied for the physicochemical and rheological properties in protein solutions and emulsions [11-14]. Liang et al. [11] reported that it was possible to produce heat stable oil-in-water emulsions formed with 10% (w/w) mixed micellar casein and soy protein at 6:4 weight ratio. With increased of soy protein content in the protein mixture, the heat stability decreased along with increased viscosity was observed. In addition, the heat stability was found to be pH dependent [11]. Therefore, by understanding the effect of different milk protein-concentrate-soy protein isolate ratios on the emulsion and heat stabilities, one would be able to develop a novel dairy technology to tailor the functional properties of products containing dairy and vegetable proteins. A great deal of attentions in the previous researches have focused on a dairy technology of soy protein fortification for milk-based concentrate drinks, however there have been few studies on the heat stability of mixed protein systems with added divalent cations. Therefore, the comparison effect of calcium and magnesium cations on
the heat stability of the mixed protein systems is of particular interest. The distributions of magnesium and calcium in various milk products are summarized by Oh and Deeth [15]. Two major differences are apparent: the total magnesium contents are all much lower than the total calcium levels and the distribution between the serum and colloidal phases of milk are different. A large proportion (~2/3) of magnesium is in the serum fraction [15-17], while a similarly large proportion of calcium is in the colloidal fraction associated with the casein micelles.

It has been reported that adding multivalent cations in milk and concentrated milk can adversely affect the heat stability of casein micelles [18,19], whey proteins [20], glycmin and conglycinin [21]. Ca\(^{2+}\) and Mg\(^{2+}\) are effective in neutralizing the negative charges, and reducing the electrostatic double-layer force of the milk proteins. As a result, the surface charge of protein molecules decreases, and the hydrophobic interactions become more extensive between the proteins resulting in coagulum formation during heating [20,22]. Liu et al. [21] reported that the addition of divalent cations at 10 mM significantly increased the amount of the primary aggregate of soy globulins and the number of primary particles induced by calcium chloride was smaller than that by magnesium chloride. Addition of calcium and magnesium chloride up to 19 mM resulted in a decrease in the pH and supernatant casein of casein micelles dispersion (2.59% protein content), with the effect of the calcium being greater than that of magnesium [23]. Addition of calcium chloride also caused a reduction in the casein micelle hydration whereas magnesium chloride made no significant change [23].

The objective of this study was to extend the findings of Liang et al. [11]. To the authors’ knowledge, there are no published studies systematically studying the influence of concentration of divalent cation salts on the physico-chemical properties of soy protein fortified high protein nutritional beverages. A better understanding of the mixed protein and the protein-mineral interactions will facilitate the development of a strategy to improve the end product functional properties.

Therefore, this study aims to determine the changes in physical properties when divalent cations are added into the mixed protein systems. The sediment height and sedimentation rate are also evaluated for determining the storage stability. The effect of fortification of calcium and magnesium cation at concentrations ranging from 2 to 8 mM, which is typically found in high protein based nutritional formulation, was studied.

Materials and Methods

Materials

Milk protein concentrate (MPC), containing 82.0% w/w protein, 1.80% w/w fat, 7.20% w/w ash, 2.60% w/w lactose, 2.22% w/w calcium, was supplied by Murray Goulburn Co-operative (Brunswick, Victoria, Australia). Intact isolated soy protein (SPI) (87.8% w/w protein, 4.37% moisture and 0.10% ash) was supplied by Dupont (Memphis, Tennessee, USA). Divalent cation salts (calcium chloride and magnesium chloride) and all other chemicals used were of analytical grade and were supplied by Sigma Aldrich (Sigma-Aldrich, Singapore), unless otherwise specified.

Preparation of mixed protein emulsions

Mixed protein solutions of 10% (w/w) were prepared by hydrating the mixtures of milk protein concentrate (MPC) and soy protein isolate (SPI) at 8.2, 7.3, 6.4 and 5.5 ratios in distilled water at 50°C for at least 60 minutes. These solutions were then subjected to high shear agitation using an Ultra-Turrax T25 (IKA-Weker GmbH & Co. KG, Staufen, Germany) for 3 min at 21,000 rpm for aid of protein dissolution. Soya oil (10% w/w) was mixed with the protein solutions and was then pre-homogenized at 14,000 rpm for 1 min using an Ultra-Turrax T25 to form a coarse emulsion. The coarse emulsion was further homogenized by a Microfluidizor (M110Y, Microfluidics, Newton, MA) at a pressure of ~4300 psi (30 MPa) for one pass to form the final emulsion. The pH of each emulsion was adjusted to 6.8 with 1 M NaOH or 1 M HCl. Sodium azide was added to the emulsion samples as an antimicrobial agent (0.02% w/w). All of the emulsions were stored at 4°C until further use.

Particle size distribution and mean droplet sizes of the emulsions

The particle size distribution of the model emulsions was measured by static light scattering using a Beckman Coulter LS 13302 (Brea, CA USA). The refractive indices of 1.33 for water and 1.456 for an oil droplet were used for emulsion samples. To differentiate between the particle sizes measured with and without dissociating buffer [a mixture of EDTA (0.04 mol/L) and Tween 20 (0.5% w/v), pH 10.0], the particle size measured in the dissociating solution was referred to as the “primary” particle size and that measured directly in water was referred to as the “effective” particle size in this study. The average droplet size was expressed as the volume-weighted mean diameter D[4,3] (µm).

Emulsions microstructures

Images of the emulsions were captured using a Zeiss LSM 510 META (Carl Zeiss Microscopy GmbH, Jena, Germany) confocal laser scanning microscope. A 0.5 mL aliquot of each emulsion sample was transferred into a 2 mL centrifuge tubes before a 5 µL each of Nile Red, Fast Green or Nile Blue (approximately 0.1% w/w) were added. After thorough mixing, a drop of each sample was placed on a microscope slide (Menzel-Glser, Germany). Nile Red was excited with the 543 nm lines from a Helium-Neon 1.2 mW laser, and the filters were set to collect the emitted light between 560 and 615 nm. Fast Green was excited with the 488 nm line from an Argon 30 mW laser, and the emitted light was collected from 505-530 nm. Nile Blue was excited with the 633 nm line from a Helium-Neon 5 mW laser and the emitted light was collected from 657-754 nm. The images were scanned at a constant 7 µm below the level of the coverslip.

Fortifications of divalent cation salts with mixed protein solutions

Mixed protein solutions (8.2=MP:SPI, 10% w/w protein content) were prepared by hydrating soy protein isolate (SPI) and milk protein concentrate (MPC) using a polytetrafluoroethylene magnetic stirring bar (50×8 mm) in Milli-Q water at 55°C before add divalent cation salts for at least 60 min. Calcium and magnesium chloride at concentration of 2, 4, 6 and 8 mM were then added and the protein mixture was subjected to high shear agitation using an Ultra-Turrax T25 (IKA-Weke GmbH & Co. KG, Staufen, Germany) for 3 min at 21,000 rpm for better protein hydration [24]. The pH of mixed protein solutions was adjusted to 6.8 with 1 M NaOH or 1 M HCl. The mixed protein solution was stored at 4°C until further use. Each mixed protein solution was prepared at least in duplicate. MP-C, MP-2Ca, MP-4Ca, MP-6Ca, MP-8Ca, MP-2Mg, MP-4Mg, MP-6Mg and MP-8Mg solutions’ were defined as 10% mixed protein solutions (8.2=MP:SPI) contained 2 mM CaCl\(_2\), 4 mM CaCl\(_2\), 6 mM CaCl\(_2\), 8 mM CaCl\(_2\), 2 mM MgCl\(_2\), 4 mM MgCl\(_2\), 6 mM MgCl\(_2\), and 8 mM MgCl\(_2\), respectively.
In-container sterilization

Mixed protein solutions (300 mL) were placed in glass bottles (500 mL, Schott Duran, VWR Singapore) and stabilized using a Hirayama Laboratory autoclave (Hiclave HV-85, Hirayama, Japan) under static conditions at 115 ± 1°C for 15 min. The heat-up time was 40 min and samples were cooled until the temperature reached 90°C (after approximately 30 min), after which, the samples were removed from the autoclave and further cooled by immersing in cold running water to reach 22°C. All in-container stabilized solutions were stored at 4°C prior to analysis.

Determination of apparent viscosity

The apparent viscosity of the mixed protein solutions before and after sterilization was determined as a function of pH in the range 6.5 to 7.2 using a Brookfield RVDV-II+ viscometer (Brookfield Engineering, Middleboro, MA), equipped with the Spindle LV-1 61. Solutions were transferred into glass beaks (length, 130 mm; external diameter, 50 mm; wall thickness, 2 mm) and allowed to equilibrate at room temperature for 2 min before analysis. The viscosity was measured at shear rate 100 s⁻¹ over 5 min.

Dry sediment

The quantity of sediment in the mixed protein solutions was measured using a centrifugation method as described by Chen et al. [25].

Accelerated storage stability

Accelerated storage stability testing of the mixed protein solutions was conducted using a LUMiSizer analytical centrifuge (Lum GMBH, Berlin, Germany), equipped with the software package SepView 4.1 (Lum GMBH). The operational principle of the LUMiSizer has been described by Tobin, Fitzsimons, Kelly, & Fenelon. Samples (0.4 mL) were filled into polycarbonate cells (PC 110-131XX; Lum GMBH) using a syringe with wide-bore needle and centrifuged at 2218g for 2 min before analysis. The viscosity was measured at shear rate 100 s⁻¹ over 5 min.

Protein size distribution

The protein particle size distribution of the samples was measured by dynamic light scattering using a Beckman particle analyser (Beckman Coulter, DelsaTM Nano C, California, US), equipped with the software package SepView 4.1 (Lum GMBH). The mean protein particle size, viscosity, protein hydration, and dry sediment percentage were measured as described by Crowley et al. [26].

Protein hydration

The ultracentrifugation method was used for measurement of protein hydration. After ultracentrifugation at 50,000 g for 2 h at 22°C (Beckman Coulter, Avanti J centrifuge, JA25.50, fixed angle rotor, California, USA), the supernatant was discarded and pellets were drained for 30 min at 22°C prior to oven drying at 103°C for a minimum of 24 h. Protein hydration was determined as described by Crowley et al. [27].

Statistical analysis

All emulsions and solutions were prepared in two independent trials and analysed in triplicate. Statistical analysis of all data was conducted using Statistical Package for the Social Sciences (SPSS 18; IBM Corporation, New York, United States) software version 18. Mean values, number of determinations, standard deviation was calculated using SPSS 18.

Results and Discussion

Emulsion stability

The effect of heat treatment (120°C 20 min) on the effective particle size of emulsions stabilized by milk protein concentrate (MPC)-soy protein isolate (SPI) mixtures is shown in Figure 1. The extent of change in particle sizes has an implication on the heat stability of the emulsions. The larger particle sizes indicate protein/droplet aggregation and its association with poorer heat stability, and increased viscosity after heating above 90°C [11]. The effective particle size does not change when emulsions are heated at pH 6.7 to 7.3 suggesting the MPC-SPI stabilized emulsions are fairly heat stable irrespective of the mixed protein ratio. From a visual observation, the emulsion samples remained liquid after the heat treatment. Additionally, the representative confocal micrographs captured in MPC-SPI (8:2) emulsion sample showed homogenous distributed emulsion droplets and protein particles and these results are consistent for all emulsions stabilized by MPC–SPI in ratio from 8:2 to 5:5. For this reason, a ratio of MPC-SPI (8:2) is selected for evaluating the impact of divalent cations salts on the pH, protein size, viscosity, protein hydration, and dry sediment percentage and sedimentation velocity of mixed protein solutions.

pH

The initial pH in the MP-C solution was 6.74 and decreased greatly upon addition of CaCl₂ and MgCl₂ to levels of 6.61 and 6.63 in the MP-6Ca and MP-4Mg solutions, respectively (Table 1). The similar results were reported by Chen et al. [28] in a study of cow’s milk. It should be noted that all MP-solutions were adjusted to pH 6.8 prior to treatments of in-container sterilization.

For the MP-6Mg, MP-8Mg and MP-8Ca solutions, visible coagulation occurred during in-container sterilization thus these samples were not analysed further. The pH of other MP solutions containing divalent cations salts decreased by average 0.14 units (Table 1) due to the heat induced precipitation of tertiary calcium phosphate [29], lactose degradation and acid formation by the Maillard reaction [30]. In line with the present study, many researches [31] showed that the pH of soy protein system was decreased after heat treatment due to the formation of phytic acid [32] and association between the ionic calcium and soy proteins [33].

Protein particle size distribution

The mean protein particle diameter of the MP-C solution was 194 nm before heat treatment and increased slightly upon addition of divalent cations salts to 206, 206, 231, 194 and 222 nm in the MP-2Ca, MP-4Ca, MP-6Ca, MP-2Mg and MP-4Mg solutions, respectively. Similar results have been reported by the previous study [34] of cow’s milk. The mean protein sizes measured in MP solution were within the normal range found for milk protein size which was in agreement with the study by Beliciu and Moraru [24] who reported that the particle size was closer to the values measured for casein micelles than soy proteins due to the higher intensity of the signal than the smaller soy protein particles.

The mean protein particle size increased slightly further on...
Liu et al. [21] reported that the addition of divalent cation salts significantly increased the amount of the primary aggregate of globulins and altered their aggregation process in soy protein that, particularly, the number of primary particles induced by Mg\(^{2+}\) was bigger and faster than that by Ca\(^{2+}\). So, a bigger primary aggregate formation and a faster aggregation rates arising from the added MgCl\(_2\) are believed to contribute to the bigger protein size in the MP-Mg samples than in MP-Ca dispersions.

**Viscosity**

The pH-viscosity profiles of the MP containing divalent cation solutions after sterilization were given in Figure 2. There was no great difference in viscosity between the pH units for MP-C solution, with values ranging from 7.20 to 12.0 mPa.s after sterilization. For the solutions containing divalent cation salts after sterilization, they all had similar shapes that the apparent viscosity increased pH values. No values are available for the MP solutions containing divalent cation sterilization (Table 1) for all MP solutions and the magnitude of increase in mean particle size increased with increasing concentration of the addition of divalent cation salts due to the reduction of electrostatic and steric repulsion [35] which was in line with the study of Pathomrungsiyounggul et al. [31] while there was no great difference in poly disperse index (PDI) between all unheated and heated solutions (Table 1). This increase in protein particle size could probably mainly be attributed to heat-denatured globular proteins, especially β-lactoglobulin, glycinn and conglycinin complexing with κ-casein on the surface of casein micelles, along with possible deposition of calcium phosphate onto the micelles [36] and self-aggregation of globular proteins [37] and small casein micelles, or further association of globular-casein protein aggregates [38]. It is known that in-container sterilization at 120°C for 15 min caused complete denaturation of globular proteins [39].

**Table 1:** pH, protein particle size, poly disperse index (PDI) and hydration of 10% mixed protein solutions (milk protein concentrate and soy protein isolate, 8:2) with and without added divalent cation salts before and after sterilization. Values are presented as average data from two independent trials.

<table>
<thead>
<tr>
<th>pH adjustment</th>
<th>Mean protein particle size (d.nm)</th>
<th>Poly disperse index (PDI)</th>
<th>Hydration (g H(_2)O/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before pH adjustment</td>
<td>After sterilization</td>
<td>Before sterilization</td>
<td>After sterilization</td>
</tr>
<tr>
<td>MP-C</td>
<td>6.74 ± 0.00</td>
<td>6.68 ± 0.00</td>
<td>194 ± 3.54</td>
</tr>
<tr>
<td>MP-2Ca</td>
<td>6.71 ± 0.01</td>
<td>6.67 ± 0.00</td>
<td>206 ± 18.4</td>
</tr>
<tr>
<td>MP-4Ca</td>
<td>6.65 ± 0.00</td>
<td>6.65 ± 0.00</td>
<td>206 ± 24.0</td>
</tr>
<tr>
<td>MP-6Ca</td>
<td>6.61 ± 0.00</td>
<td>6.66 ± 0.00</td>
<td>231 ± 5.59</td>
</tr>
<tr>
<td>MP-2Mg</td>
<td>6.61 ± 0.00</td>
<td>6.67 ± 0.00</td>
<td>194 ± 19.1</td>
</tr>
<tr>
<td>MP-4Mg</td>
<td>6.63 ± 0.00</td>
<td>6.63 ± 0.01</td>
<td>222 ± 7.35</td>
</tr>
</tbody>
</table>

Noted that, for the MP-6Mg, MP-8Mg and MP-8Ca solutions, visible coagulation occurred during in-container sterilization so these samples were not analysed further.
at pH 6.5 and 6.6 since it coagulated as a result of the in-container stabilized treatment. The maximum viscosity at pH 7.2 of MP-2Ca solution was 23.5 mPa.s and increased slightly to 28.5, 33.4, 27.3 and 33.2 mPa.s in the MP-4Ca, MP-6Ca, MP-2Mg and MP-4Mg solutions, respectively. The increase in protein particle size (Table 1) and ionic strength arising from the addition of divalent cation salts are known to increase the luminosity of casein micelle [40,41] and thus viscosity.

Our previous study [11] showed that the initial heating pH had a significant effect on the heat-induced increases in the viscosity of emulsions (micellar casein isolate-soy protein isolate mixture emulsions); the viscosities of the micellar casein isolate-soy protein isolate mixture (6:4)-stabilized emulsions heated at pH 7.2 were approximately 30 times higher than those heated at pH 6.9. The slight increase in viscosity from pH 6.7 to 7.2 may arise from the hydration increase of casein micelle. An increase in pH will increase the negative charge of casein micelle which will lead to a swell of the electrical double layer and hydrodynamic radius, and hence volume fraction and viscosity [35,40]. This pH-viscosity pattern (Figure 2) in the present study was not reported in the literature previously for sterilization. Values are presented as average data from two independent trials.

**Protein hydration**

Hydration, which plays an integral role in the stability of proteins, was 3.00 g H₂O/g before heat treatment. Addition of divalent cation salts up to 6 mM resulted in a progressive decrease in hydration values of the MP solutions (Table 1). The decrease in hydration was in line with a previous study of micellar casein isolate solution [42] and soy protein dispersion [43] and it may be caused by an increase in Ca²⁺ concentration arising from the addition of divalent salts [44].

There was no dramatic difference in the value of hydration between the unheated and heated MP solutions. Compared with the addition of CaCl₂, adding MgCl₂ to the MP solutions caused a greater decrease in hydration values (Table 1). This greater hydration reduction in MP-Mg solutions than in MP-Ca solutions may arise from the lower pH (Table 1) since casein micelle hydration is strongly pH dependent [44]. It has been reported that a reduction in pH value arising from the increasing dextrose equivalent of the glucose polymers resulted in a greater reduction in hydration of milk protein isolate (8% protein content) [45].

**Dry sediment percentage**

Sediment formation in a protein nutritional beverage is one manifestation of poor heat stability and it is mainly caused by protein aggregation. The sediment formation of the MP-C solution on sterilization was 1.40%, and was promoted substantially by the addition of divalent cation salts, with MgCl₂ causing a slightly higher increase in sediment formation than CaCl₂ (Figure 3). It has been reported that sediment formation arising from in-container sterilization of formulated milk protein isolate-maltodextrin (8.0% protein content) nutritional beverages was 1.63% [45], but no data was given for mixed protein solutions contained milk and soy proteins. Based on our previous study of cow’s milk [46], there are generally less than 5.00% minerals on a dry weight basis and a range of 1.43:1 and 1.67:1 fat/protein ratio in the dry sediment.

When evaluating the heat stability of the MP solutions, a number of factors related to the soluble phase are key contributors to the decrease of heat stability due to the addition of divalent cation salts in MP solutions. The most notably reasons are Ca²⁺ and Mg²⁺ concentration, protein particle size, hydration and buffering capacity [47].

An increase in Ca²⁺ and Mg²⁺ concentration and the decrease in hydration effect caused by addition of divalent cation salts would be identified as a primary reason for decreased heat stability due to an enhancement in Ca/Mg-induced protein-protein aggregation. The negative effect of increased Ca²⁺ and Mg²⁺ concentration on the heat stability of dairy and soy products has been extensively reported [16,21,25,31,48].

Another factor, the increasing protein particle size arising from the aggregate formation of casein micelle and/or soy protein by addition of divalent cation salts (Table 1), also had a negative effect on the heat stability of the MP solutions. It has been elucidated by Beliciu and Moraru [14] that heat treatments at 95°C for 15 minutes did not induce any chemical interactions between soy proteins and casein micelles in the mixed casein micelle isolate-soy protein isolate solutions (5:5). Therefore, the aggregation of small casein micelles, soy protein or further association of whey-casein protein aggregates is probably attributed to increase protein particle size. It has been reported [49] that heat stability of dairy based nutritional products is inversely proportional to the casein micelle [25,50] and soy protein size [31].
Addition of the MgCl$_2$ to the MP solutions caused a slighter increase in protein particle size than of CaCl$_2$ on heat sterilization (Table 1) and this probably resulted in a slightly higher percentage of dry sediment in the MP-Mg solutions. It has been reported that the particle size of Mg-containing emulsions stabilized by whey protein hydrolysate (4 wt. %) were greater than those of emulsions containing Ca at the same levels of addition [51].

In addition, it seems that there are two different binding sites, phosphoserine for calcium, and glutamic and aspartic acids for magnesium, in the association behaviour of calcium and magnesium with casein and soy protein [52,53]. The affinity of Ca$^{2+}$ and Mg$^{2+}$ to proteins depend on the availability of the binding sites, which can be made more or less available by modulating the hydrophobic–hydrophilic balance of the protein interactions [54]. Therefore, one explanation is that a cooperative mechanism between the Ca$^{2+}$ and Mg$^{2+}$ is existed [54] that the presence of calcium in MP solution probably makes more sites available for the binding of magnesium that in turn significantly decrease the electrostatic and steric repulsion, and thus heat stability (Figure 4). In addition, the presence of magnesium ions caused a partial displacement of the calcium in the casein micelles and this displacement in high protein concentration of mixed solution may more weaken the integrity of casein micelle structure than the addition of calcium ions alone and thus heat stability. The larger size and lower degree of protein hydration in MP-Mg than in MP-Ca solutions in Table 1 also support this statement.

**Accelerated storage stability testing**

The sedimentation profiles of all MP solutions containing the divalent cation salts post sterilization are presented in Figure 5. Centrifugation of all MP solutions created different sediment heights (Figure 4), as indicated by the formation of an optically dense (low transmission) region at the base of the centrifuge tube (R2 in Figure 5), with concomitant dispersion in the region (R1 in Figure 5) between the meniscus and the top of the sediment layer. Simultaneously, another sediment region was gradually formed (R3 in Figure 5) in some solutions during 3 h centrifugation, with such sediment height (R3) being more narrowed in the MP solutions fortified with high concentration of divalent cation salts than in the MP solutions with low concentration of divalent cation salts or MP-C (Figure 5). These unique two sediment regions have been never reported before.

![Figure 3: Dry sediment percentage of 10% mixed protein solutions (milk protein concentrate and soy protein isolate, 8:2) with no added divalent cation salts, and 10% mixed protein solutions (milk protein concentrate and soy protein isolate, 8:2) contained 2 mM CaCl$_2$, 4 mM CaCl$_2$, 6 mM CaCl$_2$, 2 mM MgCl$_2$ and 4 mM MgCl$_2$ after sterilization. Values are presented as average data from two independent trials.](image1)

![Figure 4: Sediment height (R2) of 10% mixed protein solutions (milk protein concentrate and soy protein isolate, 8:2) with no added divalent cation salts, and 10% mixed protein solutions (milk protein concentrate and soy protein isolate, 8:2) contained 2 mM CaCl$_2$, 4 mM CaCl$_2$, 6 mM CaCl$_2$, 2 mM MgCl$_2$ and 4 mM MgCl$_2$ after sterilization. Values are presented as average data from two independent trials.](image2)
When considering this unique sediment region (R3) in the mixed protein solutions, the accelerated storage stability of MPC or SPI (10% protein content) alone was analysed separately (results not shown). Interestingly, there is no ‘second’ sediment. One possible explanation is that in the MP solution there are two classes of sedimenting particles which are strongly repulsive and thus an intermediate clear layer between them is observed (Figure 5). The fact that it does not occur in either MPC or SPI alone supports this statement. In addition, the narrower sediment region R3 at higher divalent cation salts concentration could also suggest that this reduces repulsion between the particle classes.

Alternatively, according to the study of Sobisch and Lerche [55], the layer between the sediment R3 and R2 may also be ‘transparent’ grid like structure which is formed by very tiny particles. In the consequence, the incident transmitting light can channel through the regular grid that leads to a ‘transparent’ sediment layer. This ‘transparent’ sediment layer has a denser but less turbid structure than the sediment region R3. The more turbid sediment in the regions R3 and R2 would possibly be related to a more polydisperse mixture, while the “tiny particles” in the transparent layer between the R3 and R2 are very likely of nearly same dimension and ordered in a very regular grid. The results of poly disperse index (PDI) in Table 1 also support this statement as increasing concentration of divalent cations in the soy protein fortified milk protein concentrate solutions resulted in a narrower PDI and wider transparent layers between the R3 and R2. This transparent layer would primarily possibly relate to the formation of primary aggregates of soy protein [21] and/or the soy-whey protein aggregates [56] due to the weak attractive of non-covalence bonds. Liu et al. [21] reported that after the pH adjustment from 8.0 to 5.5, the size of soy protein aggregates is constant at average 50 µm which are formed by the highly homogenised particles.

Increasing concentration of divalent cation salts also caused great increases in sedimentation velocity (Figure 6) and initial sediment region (R2 in Figure 5) of MP solutions; with Mg fortified MP causing a slightly higher sedimentation velocity than Ca fortified MP solutions at the same levels. It was reported that, regardless of addition concentration, approximately 80% of calcium became associated with casein micelles when adding CaCl2 into the skim [27]. Thus, increased colloidal calcium phosphate levels [48] and protein particle size (Table 1), together with the decreased casein micelle hydration (Table 1) in MP solutions fortified with soluble divalent cation salts may have

![Figure 5: Representative sedimentation profiles for 10% mixed protein solutions (milk protein concentrate and soy protein isolate, 8:2) with no added divalent cation salts, and 10% mixed protein solutions (milk protein concentrate and soy protein isolate, 8:2) contained 2 mM CaCl2, 4 mM CaCl2, 6 mM CaCl2, 2 mM MgCl2, and 4 mM MgCl2 after sterilization, showing transmission of NIR light through sample cells as a function of time and position in the sample cell at 22ºC for 3 h (4000RPM=2218 g). Profiles were recorded every 60 s during each run, with every 15th profile (moving from first to last) presented for clarity. Regions of stable dispersion (R1) and sediment (R2 and R3) during centrifugation are presented. Schematic representations in sedimentation sample tube are shown for last (3 h) profiles of centrifugation. Noted that, for the MP-6Mg, MP-8Mg and MP-8Ca solutions, visible coagulation occurred during in-container sterilization so these samples were not analysed further. Phase separation profiles are from one trial but are representative of data from two individual trials.](image-url)
formed denser casein micelles which caused a higher sedimentation velocity (Figure 6).

Compared with the storage stability profiles of MP-C solution, there was a sharp, vertical, intersection characteristic of the phase boundary of aqueous/sediment in the profile of MP-4Mg and MP-6Ca solutions and, as centrifugation time extended, the gap distance (Figure 5) between each profile after 3 h in the profile of MP-4Mg and MP-6Ca was constant (but narrower gap distance was observed in the MP-C solution). These characteristics suggested that fortification of MP with soluble divalent cation salts caused storage and heat instability, with MgCl₂ fortified solutions having a slightly faster sedimentation rate than the CaCl₂ fortification (Figure 6).

**Conclusion**

In conclusion, the present study demonstrated that the type and concentration of divalent cation salts impacted strongly on the physicochemical stability of mixed milk protein concentrate-soy protein isolate beverages during sterilization. Regardless of the mixed protein ratio, there was no significant difference in microstructure and particle size in emulsions between pH 6.7 and pH 7.3 when subjected to heating treatment. Incorporation of divalent cation salts in mixed milk protein concentrate-soy protein isolate beverages decreased the heat stability greatly, increase protein sedimentation and created two sediment regions during accelerated storage stability testing. With addition of MgCl₂ mixed protein beverages had shown poorer heat stability and higher sedimentation velocity than CaCl₂ fortified ones.

In order to resolve the undesired properties arising from the addition of divalent cation salts, stabilizing salts, such as phosphates and citrates, are often added to dairy based nutritional products to enhance the heat and storage stability. The type, concentration and mixed ratio of chelating salts in stabilizing the heat stability of mixed milk protein concentrate and soy protein isolates are poorly understood, thus further study will be warranted to determine the functionality of stabilizing salts.

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**References**


