Colloidal Dispersion of Casein Micelles with Pea Protein via Homogenization

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Introduction:

Caseins are the predominant protein present in bovine milk. Caseins account for 80% of the total proteins in milk and self-assemble into casein micelles (CM). There are three major subgroups of caseins: Alpha (α), Beta (β), and Kappa (κ). Although there are several proposed models of the CM structure, it is agreed that κ-caseins associate on the surface of the micelle, and contribute to stability of the micelle as a colloidal dispersion. (Broyard & Gaucheron, 2015). On the other hand, α – and β-caseins are hydrophobic and center themselves in the core of the micelle. The CM structure is held together between protein-protein and calcium phosphate interactions such as hydrophobic, hydrogen, and electrostatic binding. (Broyard & Gaucheron, 2015). The structure of CM can be disrupted through environmental changes such as the addition of chelating agent, NaCl, and cations, along with changes in temperature and pH (Broyard & Gaucheron, 2015). These structural changes ultimately affect the final technological and functional properties of CM. The unique structure and hydrophobic core of CM allows it to act as a primary carrier for bioactive compounds through encapsulation. CM serve as a natural delivery system, as it provides essential nutrients such as calcium, phosphate, and protein through its unique structure (Semo et al., 2007). CM have been successfully used to encapsulate hydrophobic nutraceuticals such as fat-soluble Vitamin D2 for potential enrichment of low-fat foods. (Semo et al., 2007).

The presented research aims to utilize CM to deliver another type of hydrophobic compound: plant-based protein. Plant-based proteins have risen in demand as consumers are desiring more environmentally conscious, and healthy high protein products (Boland et al., 2013). Pea protein (PP) - chosen as the plant-based protein for this study – has grown in its popularity due to its fairly balanced amino acid composition and branched chain amino acids that assist in muscle synthesis and expansion (Babault et al., 2015). PP is a non-allergenic, gluten-free, and cost-effective plant protein. However, it can be a challenge to use in food systems because of its low water solubility and undesirable bitterness. PP have a high percentage of hydrophobic proteins (65-80%), making it a good model hydrophobic plant-protein to use in this study (Lu et al., 2020; Nehete et al., 2013) Plant-based proteins such as soy, wheat, corn, and sunflower also have high contents of non-polar, hydrophobic amino acids, resulting in their low solubility in water (Nehete et al., 2013) The presented research creates a potential for the use of additional hydrophobic plant-based proteins in a CM delivery system.

This study suggests the use of CM as a particular system optimal for protein blending with high sensory acceptability to curtail the challenges of PP. A protocol to incorporate PP with CM was established by utilizing the intrinsic structural properties of CM along with cold-temperature homogenization. The CM/PP blends were created using common dairy pilot plant equipment to allow for easy reproducibility in any dairy facility. The objective of this study was to create a stable colloidal dispersion of CM with PP, prioritizing two goals: 1) improve the solubility of PP in aqueous solution, and 2) preserve or improve the functional properties of CM. The hypothesis of this study states that cold temperature homogenization can be applied to an unstable dispersion of CM and PP, and the resulting blend will be a stable colloidal dispersion in which the insoluble PP associate with CM in suspension. This research highlights a promising application for other plant-based proteins to be utilized within the dairy industry to help drive future product innovation while also meeting current processing conditions and consumer demands.

Materials and Methods:

Sample Preparation:

The milk sample was prepared by dissociating CM with 0.02 M sodium citrate (Heertje et al., 1985). 5.16g/L sodium citrate (It's Just! Co.) was dissolved in commercial fat-free milk (Dean's Dairy Pure) and equilibrated at 4°C for 24 hours. A 2.6% PP slurry was created by combining PP isolate (Mettle Nutrition LLC.) with DI water and continuously agitated at 25°C for 24 hours. The milk sample and the PP slurry were combined at various casein-to-PP ratios: 90% casein-10% PP, 80% casein-20% PP, and 50% casein-50% PP. The samples were combined to a final volume of 500 mL.

Homogenization and pasteurization of CM/PP blends:

The protein blends underwent three cycles of homogenization using a bench-top GEA 2-stage homogenizer at 3500 psi (1^{st} stage)- 500 psi (2^{nd} stage) at 4-10°C. After homogenization, they were batch pasteurized at 63°C and held for 30 minutes to eliminate pathogenic growth. The sample blends were stored at -20°C until further analysis.

Particle size analysis:

The hydrodynamic diameter (nm) of the soluble particles in the liquid CM/PP blends and control samples were measured using NanoBrook ZetaPALS Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) Three mL filtered DI water were added to a clean cuvette and 100 µL of liquid sample was added. Results were measured in quintet.

SDS-PAGE:

To determine the protein profile of the CM/PP blends and control samples, 10% SDS-PAGE (polyacrylamide gel electrophoresis) were prepared (non-reducing gel). 45 µL of sample were mixed with 45 µL 2x Laemmli sample buffer (Bio-Rad). 30 µL of the mixed sample, containing 45 µg protein, were loaded into each well. The gel ran for 70 minutes at 120 volts. After the running time, the gel was stained in Coomassie blue solution, based on manufacturer's instructions. An image of the gel was taken using Chemidoc-Touch Imaging System (Bio-Rad).

Reversed Phase HPLC:

The CM/PP blends and control samples were analyzed via Reversed Phase-HPLC (RP-HPLC-Shimaduzu) with Aeris 3.6 µm Widepore XB-C8 (LC Column 250 x 4.6 mm) and a fluorescence detector. Solvent A was 0.1% TFA in HPLC grade water and Solvent B was 0.1% TFA in HPLC grade acetonitrile. Both solvents were degassed for 10 minutes prior to being vacuum filtered through a 0.22 µm nylon filter. The flow rate of the solvents was 1mL/minute. From 0-20 minutes, Solvent B was 25-50%. From 20 minutes to 30 minutes, Solvent B was 50- 80 %. From 30 minutes to 32 minutes, Solvent B was 80- 25 %. The excitation wavelength and emission wavelength were 280 nm and 348 nm, respectively, as these are the optimal wavelengths to detect tryptophan. The column oven was 40°C, and the sample injection volume was 5 µL. The samples were prepared by using the method described by Ma et al. 2017 with some modifications (Ma et al., 2017) In brief, 800 µL of protein sample was combined with 800 µL of Solution A. Solution A contained 5.37 mmol/L sodium citrate, 19.5 mmol/L DLdithiothreitol, 6 mol/L GDnHCL and 0.1 mol/L BisTris buffer. Solution A was pH=7 and filtered through a 0.22 µm nylon filter. The combined sample was vortexed for 10 seconds and then incubated at room temperature for 1 hour. 500 µL of the sample/solution A mixture was combined with 1000 µL of Solution B. Solution B contains 4.5 mol/L GdnHCl with 899 mL water, 100 mL Acetonitrile, and 1 mL TFA. Solution B was filtered through a nylon 0.2 µm filter. The 1.5 mL sample/solution B mixture was filtered through a 0.22 µm filter into an amber HPLC vile and stored at 4°C until ready for analysis.

Amino acid sequencing:

PPs of interest were amino acid sequenced via liquid-chromatography mass-spectrometry (LC-MS/MS). Peptide fragments generated by tandem MS were compared with the MASCOT database to obtain the amino acid sequence. Proteins with at least two matching peptide fragments to the database were considered reliable identification matches.

Coagulation of CM/PP blends:

1% $CaCl_2$ and diluted CHY-MAX M 127223 (CHR-HANSEN) rennin was added to the protein blends to form protein curds. The protein curds were measured for texture analysis and SDS-PAGE. The texture of the curd was analyzed using the TA-Texture analyzer. The parameters used were followed for the two-bite analysis used by (Li & Wang, 2012) with slight modifications. In brief, a cylinder probe was used for a double bite compression test conducted at three different locations of the curd's surface. 2.0 mm/s was used as the before speed, 0.5 mm/s analysis speed, and 0.5 mm/s after speed. The measuring run was 5.0mm and the initial force was 5.0g (a slight modification from the original process, to adhere to the equipment available). The TA-54 4 mm diameter, 35 mm tall probe was used. Hardness, adhesiveness, resilience, cohesion, springiness, gumminess, and chewiness were measured in triplicates. The curds were made uniform by compressing as much whey out as possible via a cheese cloth. Once the majority of the whey was expelled, the curd was placed in a 30mm diameter cylinder and compressed for 30 minutes under the weight of 100g. Any excess whey was expelled under this compression. The curds were stored in 4°C until analysis.

Statistical Analysis:

All experiments were conducted in quintet (particle size analysis) or triplicate (texture analysis). One-way ANOVA analyses followed by Tukey multiple comparison were conducted using JMP software.

Results and Discussion:

Hydrodynamic diameter (nm) results of soluble particles in skim milk show the successful disruption of caseins through the addition of sodium citrate and the decrease of temperature (Figure 1). Significantly larger sized caseins were observed in the 4°C milk sample compared to the 60°C milk sample, with hydrodynamic diameters of 197.11 ± 2.07 nm and 163.15 ± 5.28 nm, respectively ($p<0.05$). Additionally, upon the addition of sodium citrate to the milk sample, the soluble particle size increased significantly ($p<0.05$). The hydrodynamic diameter of the milk only and milk + citrate samples were 197.11 ± 2.07 nm and 220.06 ± 2.34 nm respectively. These results support the use of cold temperature homogenization and sodium citrate addition to disrupt the caseins to leach out hydrophobic caseins into the soluble phase.

Three CM/PP blends with varying percentages of casein to PP pre- and post-homogenization are shown in Figure 2. Samples labeled “A” are pre-homogenization. There is clear separation between the protein segments in the unstable colloidal dispersions. Samples labeled “B” are post-homogenization blends, which are homogenous, stable blends with no separation. These blends remain stable at 4°C for up to 3 weeks with no visible sedimentation.

The hydrodynamic diameter (nm) of the soluble particles were measured of each CM/PP blends pre- and post-homogenization (Figure 3). Skim milk and pea protein slurry were measured as controls. The process of homogenization increased significantly ($p<0.05$) the soluble particle size of the 80-20 and 50-50 CM/PP blends. Before homogenization, the 80-20 and 50-50 CM/PP blends soluble particle size were 179.62 ± 3.34 nm and 200.04 ± 5.21 nm respectively. Post-homogenization, the particle size for the 80-20 and 50-50 CM/PP blends were 220.21 ± 4.81 nm and 322.10 ± 4.56 nm respectively. The particle size of CM in the skim milk control did not significantly change between pre-and post-homogenization: 220.06 ± 2.34 nm and 216 ± 8.35 nm respectively. This indicates that the change in particle size in the CM/PP blends is due to changes in the PP, rather than the CM. The PP control pre-homogenization particle size was significantly greater than the PP control post-homogenization: 956.80 ± 10.30 nm and 323.18 ± 5.85 nm respectively. These results indicate PP is incorporating in the soluble phase. However, these results do not show how the PP are interacting in the soluble phase or if they are incorporating with any specific milk components.

SDS-PAGE analysis was used to qualitatively understand how the PP was incorporating in the soluble phase. Skim milk and PP slurry controls were analyzed in via SDS-PAGE (gel not shown). Around 50kDa molecular weight, there was an intense band from the PP slurry. This 50kDa PP is used as an indicator to determine where the PP is incorporating itself: whey proteins or CM. This molecular weight protein is used as a PP indicator because the skim milk sample showed no protein bands in the general molecular weight region. The CM/PP blends and controls were coagulated with rennin to separate the whey proteins from CM. The CM/PP blends, and milk coagulated under these conditions, however the pea protein slurry did not. The total uncoagulated blend, the liquid whey component and the solid casein curd component were analyzed through SDS-PAGE (Figure 4). The total 90-10 blend (Lane 1) contained the 50kDa PP, indicating that the homogenization process incorporated this PP into the soluble phase. The whey component of the 90-10 blend (Lane 2) did not have a band representing the 50kDa protein, however, it was visible in the casein curd component (Lane 3). This indicates that the 50kDa PP incorporated with the CM, rather than the whey proteins. The similar results were observed in the 80-20 CM/PP blend (Lanes 4-6) and the 50-50 CM/PP blend (Lanes 7-9). Amino acid sequencing was conducted via LC-MS/MS of the 50kDa pea protein of interest. The results indicate that there is 77% coverage and 44 peptides identified to match PP vicilin. Vicilin is a predominant globulin, storage pea protein that is insoluble in water. This confirms the incorporation of an insoluble PP with CM via cold temperature homogenization.

RP-HPLC with fluorescence detection was used to further understand the interaction between PP and CM in the blend. Figure 5 shows the chromatogram of the same samples that were analyzed in the SDS-PAGE. There are two fluorescence peaks produced by PP, at the retention times 6.7- and 12.8-min. Similar peaks were detected in all three CM/PP blends, indicating that this PP is in the total blend. However, the respective whey components of the CM/PP blends did not have fluorescence peaks at these retention times, indicating that these PP incorporated with the CM, rather than the whey proteins.

Figure 6 represents the Hardness (g) of the coagulated curds from the CM/PP blends compared to commercial queso fresco and hard tofu. As the percentage of PP increases, the hardness significantly increases as well. Interestingly, the 90-10 CM/PP blend has significantly similar hardness to the commercial hard tofu: 43.83 ± 11.16 and 44.22 ± 0.58 respectively. The 80-20 CM/PP blend and commercial queso fresco have significantly similar hardness levels at 89.33 ± 5.36 and 103.74 ± 7.13 respectively. These results highlight good potential for these blends to be used in curded products, as they already have similar textures to current commercial products that already have consumer acceptance.

Conclusion:

The presented research indicates that cold-temperature homogenization can be successfully used to create a colloidal dispersion in which insoluble PP (vicilin) incorporates with CM in solution. The developed CM/PP blends show promising results for the use as mixed protein, functional ingredients in the form of liquid, gel, or powder. Future experiments will include sensory analysis of the CM/PP blends and quantitative molecular analysis of the interaction occurring between CM and PP. These results show a promising application for other plant-based proteins with low solubility to be utilized within the dairy industry. As fluid milk sales have been declining in recent years, the presented research may serve as a creative innovation in which the dairy industry can still provide highly nutritious dairy products while also capitalizing on plant-based protein trends.

Figures:

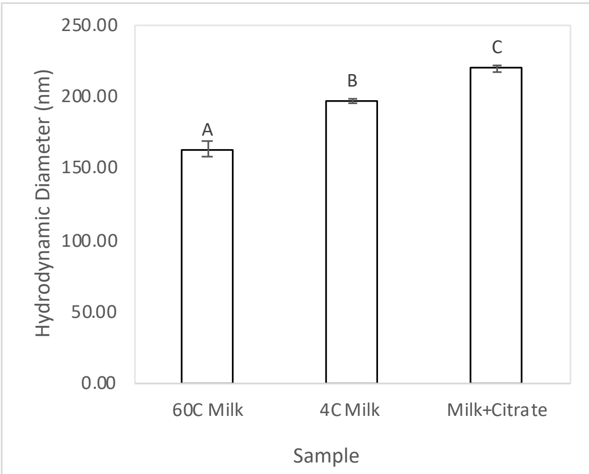

Figure 1: Hydrodynamic Diameter (nm) measured by DLS of milk samples under three different treatments: Heated to 60°C, Cooled to 4°C, and the addition of sodium citrate

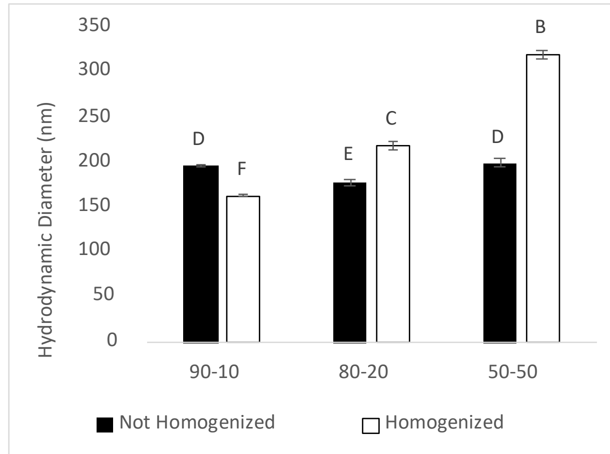

Figure 3: Hydrodynamic Diameter (nm) measured by DLS of CM/PP blends (90-10, 80-20, 50-50) pre- and post-homogenization

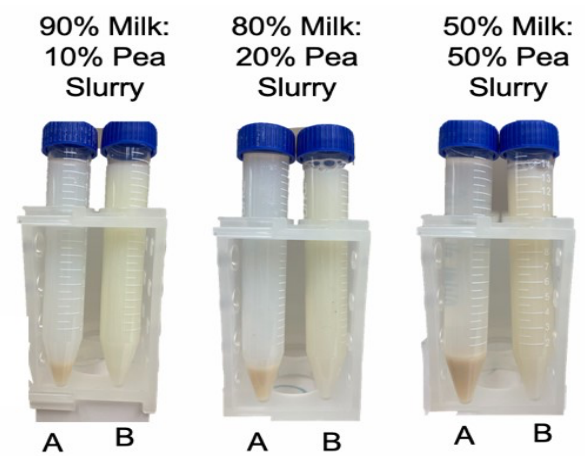

Figure 2: Visual representation of CM/PP blends (90-10, 80-20, 50-50) pre-homogenization (A) and post-homogenization (B)

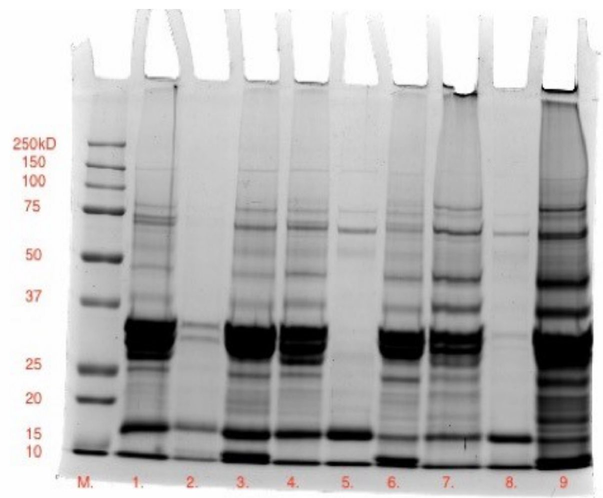

Figure 4: 10% SDS-PAGE stained in Coomassie blue solution representing protein profile of CM/PP blends. Lane 1: 90-10 total blend; Lane 2: 90-10 whey; Lane 3: 90-10 casein; Lane 4: 80-20 total blend; Lane 5: 80-20 whey; Lane 6: 80-20 casein; Lane 7: 50-50 total blend; Lane 8: 50-50 whey; Lane 9: 50-50 casein

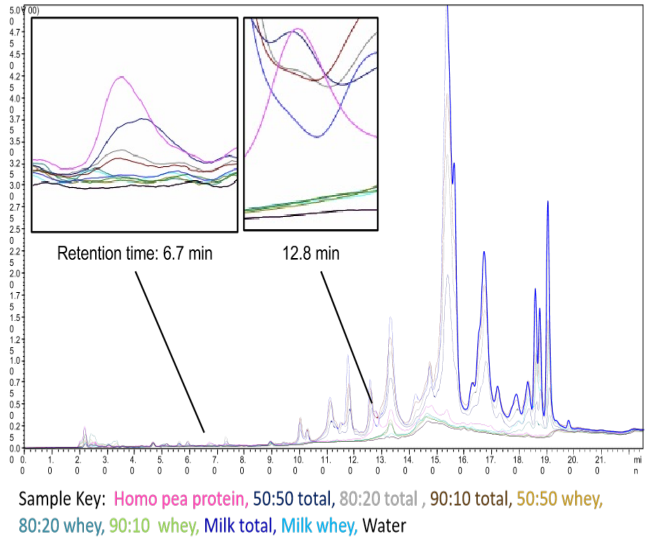

Figure 5: RP-HPLC with fluorescence detection chromatogram of PP, Skim milk, CM/PP total blends (90-10, 80-20,50-50) and respective whey components

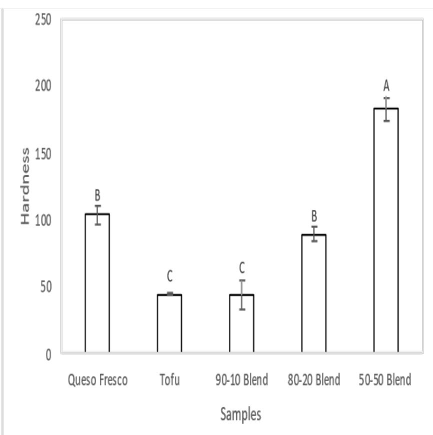

Figure 6: Hardness (g) measured via Texture Profile analysis. Samples: Commercial queso fresco, commercial hard tofu, and CM/PP blends (90-10, 80-20, 50-50)

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