Application of zinc and calcium acetate to precipitate milk fat globule membrane components from a dairy by-product

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ABSTRACT

There has been a great deal of interest in developing isolated dairy lipid fractions that are rich in phospholipids (PL), due to their health benefits and functional properties. Dairy by-products that contain elevated levels of PL and milk fat globule membrane (MFGM) proteins can be an excellent source for these isolates. The β stream, a by-product of anhydrous milk fat production, is an excellent candidate because it contains a higher concentration of PL than many other dairy by-products. In this study, we investigated an economically feasible processing method to obtain these valuable components from the β stream. The use of zinc acetate and calcium acetate, along with mild heat treatment and pH adjustment, was effective in precipitating PL and proteins into a pellet fraction. With an additional extraction from the pellet using ethanol (90% at 70°C), a PL-enriched lipid fraction was obtained. The effective precipitation conditions were zinc acetate of 25 mM concentration at pH greater than 6.5 at 30°C, and calcium acetate of greater than 75 mM concentration at pH greater than 6.5 at 60°C. With ethanol extraction, PL recovery of $97.7 \pm 1.7\%$ from the zinc acetate precipitate and $94.9 \pm 3.7\%$ from calcium acetate precipitate were achieved.

Key words: dairy lipid, ³¹P nuclear magnetic resonance, sphingomyelin, phospholipids, ethanol extraction

INTRODUCTION

The milk fat globule membrane (MFGM) and its components have attracted significant interest because of potential health benefits and functional properties. This membrane encompasses the triacylglycerol fat globule and is the richest source of phospholipids (PL), glycolipids, gangliosides, and glycoproteins in milk (Jimenez-Flores and Brisson, 2008). Although dairy PL

represent only 0.5 to 1.0% of total milk lipids (Fong et al., 2007), they are of particular interest because of their higher levels of sphingomyelin (SM; 4.1 to 29.2% of total PL) and phosphatidylserine (PS; 2.0 to 16.1% of total PL) compared with other PL sources such as soybeans or egg yolk (Burling and Graverholt, 2008; Contarini and Povolito, 2013). Reported to play important roles in cell regulation, SM is referred to as a tumor suppressor (Rombaut et al., 2006; Dewettinck et al., 2008; Contarini and Povolito, 2013). Cognitive performance improvement, which is of a particular significance to Alzheimer's disease treatment, has been attributed to PS (Pepeu et al., 1996; Rombaut and Dewettinck, 2006; Burling and Graverholt, 2008; Dewettinck et al., 2008; Contarini and Povolito, 2013).

The use of salts, such as calcium chloride and zinc acetate, to precipitate MFGM components has been reported for dairy whey by-products (Rombaut and Dewettinck, 2007; Damodaran, 2010). These salts can precipitate MFGM components such as PL and proteins before ultrafiltration, to increase processing efficiency and prevent fouling of membranes during whey protein concentrate (WPC) production (Rombaut and Dewettinck, 2007). If the MFGM components are not removed from whey, the resulting WPC can develop a stale or oxidized off-flavor and brown discoloration during storage due to a series of complex and inter-related chemical reactions that include lipid oxidation and Maillard browning (Morr and Ha, 1991). These off-flavors can limit the use of WPC in the food industry (Carunchia Whetstine et al., 2005) and reduce the functionality of WPC by leading to high turbidity, impaired foaming, and reduced emulsification properties (Damodaran, 2005). These drawbacks have led to research focusing on the removal of MFGM components from whey. The application of salts to dairy by-products other than whey has yet to be investigated.

The β stream is a by-product of anhydrous milk fat production that has a composition similar to butter-milk and butter serum. The starting material for the β stream is cream, which is further processed into anhydrous milk fat. There are differences in the composition

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of the β stream compared with whey by-products, in that the β stream contains a higher protein content and a different protein profile. Casein is present in the β stream but not in whey by-products, as it is removed during the cheese making process. There is also less lactose in the β stream (43.6%). Working with the higher amount of protein present in the β stream will provide insight into how well the β stream proteins and lipid fractions are precipitated during the salt precipitation step of the extraction process.

Application of calcium chloride (Rombaut and Dewettinck, 2007) and zinc acetate (Damodaran, 2010) has shown a great potential to remove dairy PL and MFGM components from whey by-products. Both salts were used, in combination with temperature and pH adjustments, to precipitate the MFGM components present in whey by-products. However, an efficient and economical lipid extraction for the precipitated pellet has yet to be developed to obtain PL. Rombaut and Dewettinck (2007) attempted to use a water wash to remove lactose and other water-soluble components from the pellet, but PL losses ranging from 16.6 to 49.7% were observed. A novel approach to extract the PL in the pellet is to use ethanol for solubilizing the lipid and to precipitate the protein (Price et al., 2018). With such an ethanol extraction of the precipitate, it is important to understand the apparent solubility of each salt in ethanol. Calcium chloride has high ethanol solubility, whereas acetate salts have much lower ethanol solubility (Sheftel, 2000). Considering these factors, an investigation into the use of zinc acetate and calcium acetate on the β stream was performed to determine how efficiently the total lipid, total PL, and protein are precipitated, and then the effectiveness of PL extraction with ethanol.

The specific aim of this investigation was to determine the practical and effective salt concentration and pH for the precipitation of both PL and proteins. Each individual PL class was quantified to evaluate the effect of these treatments on their recovery. The apparent solubility of each salt under various ethanol concentrations and temperatures was also determined, to ascertain what ethanol extraction parameters are best suited to limit the amount of salt being solubilized during the extraction. Then the application of ethanol to remove the total lipid and PL from the precipitate was evaluated.

MATERIALS AND METHODS

The β stream powder used in this study was provided by Valley Queen Cheese (Milbank, SD). The extracted

lipid was quantified for PL using ^{31}P nuclear magnetic resonance (NMR; Wang et al., 2014; Price et al., 2018). Reagent-grade solvents and other chemicals were purchased from Thermo Fisher Scientific (Fair Lawn, NJ) and Sigma-Aldrich (St. Louis, MO).

Sample Preparation

The β stream powder was dispersed in deionized water (5% wt/wt) and allowed to hydrate for 4 h under constant agitation (450 rpm). The pH of the dispersion was 4.7, and 2 M sodium hydroxide solution was used to adjust the pH to a specific value (pH monitored using Thermo Fisher Scientific Orion Star A111 pH meter). After hydration, powdered zinc acetate or calcium acetate was added at the designed molar concentration (0, 25, 50, 75, 100, 150, and 200 mM). An exception was made for studying the effect of pH on the efficiency of lipid recovery, wherein the pH was adjusted after zinc acetate or calcium acetate addition, because these salts can lower the pH of the β stream dispersion (Figure 1). The samples were then placed in a hot water bath (Thermo Fisher Scientific, model 3582) at 30 or 60°C for 30 min. After this heat treatment, the samples were centrifuged (IEC Centra CL-2, Thermo Fisher Scientific) at $1,800 \times g$ for 5 min at ambient temperature. The samples were then allowed to cool overnight in the refrigerator (2 to 4°C), and the supernatant was then decanted and the lower pellet collected for total lipid extraction and PL quantification. The transmittance of the supernatant was measured at 500 nm using a Beckman Coulter (Brea, CA) DU 720 spectrophotometer as an indicator of effective MFGM precipitation.

Analytical Methods

Moisture and ash contents were determined by drying at 95°C for 24 h in a drying oven, and incineration in a muffle furnace (Barnstead Thermolyne 48000 Furnace model no. F48015, Waltham, MA) at 600°C for 24 h. The Dumas (CHN-2000; Leco, St. Joseph, MI) method was used to quantify protein content in the initial β stream powder and in the precipitated pellet. The protein concentration in the supernatant was assayed using the Bradford method (Bradford, 1976). Bovine serum albumin was used to establish a standard curve, and protein concentrations were reported as milligrams per milliliter. Protein distribution (initial β stream, zinc acetate and calcium acetate-precipitated pellets, and supernatant) were assessed qualitatively using SDS-PAGE under reducing conditions, as described by Primacella et al. (2018). Individual proteins

were identified using an SDS-PAGE molecular weight broad range standard (Bio-Rad laboratories, Hercules, CA), and protein bands were identified according to the profile reported by Mather (2000). The MFGM-salt complex would not dissolve in water, so a 0.2% EDTA solution was used to release the MFGM into the dispersion before SDS-PAGE. Total lipid extraction using the Folch method (Folch et al., 1957; Rodriguez-Alcalá and Fontecha, 2010) was performed to quantify the lipid content in the β stream and the precipitates. The PL content and the class composition of the lipid extracts were determined by using ^{31}P NMR, following a method reported by Price et al. (2018) and Wang et al. (2014).

Zinc Acetate and Calcium Acetate Apparent Solubility in Ethanol

The apparent solubility of each salt was determined by adding 10 mL of ethanol (70, 80, and 90% vol/vol) to 5 g of zinc acetate or calcium acetate. The sample vials were then capped and placed into a Thermo Fisher Scientific MaxQ 4450 heated shaker at 30, 50, and 70°C under constant agitation (200 rpm) for 24 h. The solubilized salts in ethanol were then decanted, and the ethanol was removed to determine how much salt was solubilized in the ethanol. The results were reported as grams of salt dissolved in 100 g of ethanol under the various ethanol concentrations and temperatures investigated.

Ethanol Extraction of Lipids from the Pellets

Once the pellet was recovered, the lipid was extracted using ethanol, according to Price et al. (2018). The optimum conditions for total lipid and PL recovery using a 1-step extraction were reported as 90% ethanol at 70°C, and these conditions were used to extract the lipid from the β stream pellet. The pellet (25 g, as is) was placed in 206 mL of neat ethanol for zinc acetate and 198 mL of 100% ethanol for calcium acetate. The difference in total solids percentage between the zinc acetate and calcium acetate pellets led to slightly different volumes of ethanol required to achieve a final ethanol concentration of 90%. After the pellet was in contact with the ethanol, incubation was allowed for 6 min before the ethanol with solubilized lipid was filtered to remove the residual pellet fraction. The ethanol was then rotary evaporated (Buchi Rotavapor R-205, New Castle, DE) to collect the lipid fraction. The residual pellet fraction was also recovered and saved for further analysis.

Statistical Analysis

All the treatments were randomized, with 3 replicates. The data were analyzed using SAS 9.1 software (SAS Institute Inc., Cary, NC), and the differences in treatment means and significance were evaluated at $P = 0.05$ among all the treatments analyzed. The standard deviation from the mean was calculated for

Figure 1. The pH of the β stream (5% dispersion) as affected by salt concentration before heat treatment.

Table 1. Total lipid recovery, phospholipid (PL) recovery, and PL class composition (% of total PL)¹ for zinc acetate at 30°C and calcium acetate at 30 and 60°C treatments under various salt concentrations

Treatment	Total lipid recovery (%)	PL recovery (%)	SM	PE	PS	PI	PC	PL (% of total lipid)
Control, 30°C	12.9 ^{ef}	10.0 ^{gh}	32.2 ^a	24.3 ^c	2.4 ^{cd}	6.9 ^a	34.3 ^a	12.3 ^{bc}
Zn 25 mM, 30°C	96.2 ^a	97.4 ^a	11.2 ^{de}	36.8 ^{ab}	8.6 ^{bcde}	9.8 ^a	33.5 ^b	31.3 ^a
Zn 50 mM, 30°C	94.5 ^a	97.3 ^a	11.7 ^{cde}	37.2 ^{ab}	8.0 ^{bcde}	9.4 ^a	33.7 ^b	31.9 ^a
Zn 75 mM, 30°C	97.3 ^a	97.5 ^a	10.8 ^e	40.5 ^a	7.1 ^{bcdef}	9.0 ^a	32.6 ^b	31.6 ^a
Zn 100 mM, 30°C	99.0 ^a	96.7 ^a	10.7 ^e	40.3 ^{ab}	7.5 ^{bcdeg}	8.7 ^a	32.8 ^b	30.6 ^a
Zn 150 mM, 30°C	98.9 ^a	93.8 ^{ab}	9.9 ^{cde}	41.9 ^a	1.5 ^{bcdef}	10.3 ^a	36.4 ^{ab}	34.4 ^a
Zn 200 mM, 30°C	96.1 ^a	91.2 ^{abc}	10.6 ^e	40.7 ^a	5.1 ^{bcdef}	9.3 ^a	34.4 ^b	29.8 ^a
Ca 25 mM, 30°C	41.6 ^{de}	36.5 ^{fg}	27.4 ^{abc}	29.2 ^{abc}	0.8 ^f	10.3 ^a	32.3 ^b	24.5 ^{abc}
Ca 50 mM, 30°C	51.4 ^{cd}	48.6 ^{ef}	27.7 ^{ab}	27.2 ^{bc}	0.6 ^f	10.7 ^a	33.4 ^b	27.0 ^{ab}
Ca 75 mM, 30°C	51.6 ^{cd}	56.6 ^{def}	27.8 ^{ab}	28.1 ^{abc}	1.1 ^{def}	10.3 ^a	32.7 ^b	30.4 ^a
Ca 100 mM, 30°C	61.8 ^{bcd}	65.8 ^{cde}	27.0 ^{abcd}	28.6 ^{abc}	1.8 ^{cdef}	10.0 ^a	32.6 ^b	31.1 ^a
Ca 150 mM, 30°C	73.5 ^{ab}	76.7 ^{abcd}	20.2 ^{abcde}	31.3 ^{abc}	6.9 ^{bcdef}	10.0 ^a	31.6 ^b	33.3 ^a
Ca 200 mM, 30°C	78.6 ^{ab}	80.3 ^{abc}	21.4 ^{abcde}	32.2 ^{abc}	3.3 ^{bcdef}	10.3 ^a	32.8 ^b	32.3 ^a
Control, 60°C	10.7 ^f	3.6 ^h	35.0 ^a	3.9 ^d	18.4 ^a	0.0 ^b	42.7 ^a	8.4 ^c
Ca 25 mM, 60°C	71.5 ^{abcd}	66.2 ^{bcd}	11.6 ^{bcde}	33.4 ^{abc}	7.6 ^{bcdef}	11.1 ^a	36.2 ^b	30.3 ^a
Ca 50 mM, 60°C	82.3 ^{ab}	78.8 ^{abcd}	9.3 ^{de}	35.9 ^{ab}	8.2 ^{bcdef}	11.0 ^a	35.6 ^b	31.9 ^a
Ca 75 mM, 60°C	88.4 ^{ab}	84.9 ^{abc}	10.1 ^{bcde}	36.9 ^{ab}	8.4 ^{bcdef}	10.7 ^a	34.0 ^b	32.3 ^a
Ca 100 mM, 60°C	90.9 ^{ab}	86.7 ^{abc}	9.9 ^{cde}	39.7 ^{ab}	9.0 ^{bcde}	9.7 ^a	32.3 ^b	31.3 ^a
Ca 150 mM, 60°C	91.2 ^{ab}	89.9 ^{abc}	11.2 ^{bcde}	36.1 ^{ab}	9.1 ^{bcde}	10.0 ^a	33.7 ^b	33.2 ^a
Ca 200 mM, 60°C	97.9 ^a	92.9 ^{abc}	11.2 ^{bcde}	37.3 ^{ab}	9.9 ^{ab}	9.6 ^a	31.9 ^b	32.1 ^a

^{a-h}Means with different superscript letters within the same column are significantly different at $P = 0.05$.

¹SM = sphingomyelin; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PC = phosphatidylcholine.

all treatments (presented as error bars in figures). The Tukey test was used to analyze statistical differences among means.

RESULTS AND DISCUSSION

The β stream powder contained 18.1% total lipid, of which 35.0% was PL (6.3% on product basis). It contained 29.1% protein, 6.1% ash, 43.6% lactose, and 3.1% moisture. The major PL classes found in the β stream were phosphatidylcholine (**PC**; 34.2% of total PL), phosphatidylethanolamine (**PE**; 28.0%), and SM (27.0%). The minor PL classes were PS (2.1%) and phosphatidylinositol (**PI**; 8.7%). The PL composition of the β stream was similar to that of native milk fat (Contarini and Povolio, 2013).

Effects of Calcium Acetate and Zinc Acetate Concentrations on Precipitation of Total Lipid, PL, and Protein

Total lipid recovery, PL recovery, PL content, and PL class composition for the zinc acetate- and calcium acetate-precipitated samples are shown in Table 1. The concentration and type of salt were both significant factors for total lipid and PL recoveries ($P < 0.05$). All concentration treatments resulted in significantly more total lipid and PL recoveries compared with the control treatments ($P < 0.05$). We found no significant

differences among the total lipid recoveries for the zinc acetate treatments ($P > 0.05$), which resulted in total lipid recovery ranging from 94.5 to 99.9% (25 to 200 mM). Treatment with calcium acetate (25 to 100 mM) at 30°C resulted in significantly less total lipid and PL recovery compared with the zinc acetate treatment, with total lipid recovery ranging from 41.6 to 61.8% and PL recoveries ranging from 36.5 to 65.8%. The concentration of 150 to 200 mM resulted in significantly higher total lipid and PL recovery than did the lower salt concentration. At 60°C, the 75 to 200 mM concentration range of calcium acetate led to similar total and PL recoveries compared with zinc acetate (88.4 to 97.9% total and 84.9 to 92.9% PL recoveries). The 30°C calcium acetate treatment resulted in significantly lower ($P < 0.05$) total and PL recoveries compared with the overall treatment means for zinc acetate or 60°C calcium acetate.

We discovered no significant difference in the PL contents of the precipitated total lipid fractions among any of the zinc acetate or calcium acetate treatments ($P > 0.05$). All the treatments resulted in significantly higher PL contents (24.5 to 34.4%) than did the control treatments (8.4 to 12.3%). The PL class compositions for all 3 types of treatment were similar, with a few significant variations in the content of SM, PS, and PE. The 30°C calcium acetate treatment as a whole resulted in significantly more SM recovery and less PS recovery than did the 60°C calcium acetate and zinc acetate treatments.

The 60°C calcium acetate treatment resulted in seemingly higher PS recovery compared with zinc acetate. Zinc acetate led to a slightly higher PE recovery than did either of the calcium acetate treatments. The PL classes of specific interest are SM and PS because of their health benefits, and enrichment of these PL during extraction is desired. Therefore, the 30°C calcium acetate treatment would be desired for good SM recovery, but the 60°C calcium acetate treatment would be desired for maximum PS recovery. The difference in recovery of different PL classes may be attributed to the location of these PL within the MFGM, which may lead to a preference for complexing with calcium compared with zinc. The most polar PL class, SM, is located on the outer bilayer of the MFGM, whereas PS, a more nonpolar PL class, is more closely associated with the inner membrane (Deeth, 1997; Dewettinck et al., 2008; Christie and Han, 2010). Zinc acetate may interact more with the integral hydrophobic proteins of the MFGM and have a preference to bind to the MFGM inner membrane, as more PE was recovered using zinc acetate. Due to variations in PL class recovery under the temperature treatments investigated, we speculate that calcium acetate may not have a preference for specific areas in the MFGM.

Supernatant transmittance and protein concentrations were analyzed to give an indication of how salt concentrations affect precipitation of protein, and the

results are summarized in Figures 2 and 3. Higher transmittance indicates that more protein or lipoprotein was precipitated into the pellet. As shown in Figure 4, a strong correlation exists between transmittance and protein concentration in the supernatant. As transmittance increased, we found less protein in the supernatant. Transmittance of all the supernatant from the zinc acetate treatments was significantly higher than that of the calcium acetate treatments, and the protein concentration in the supernatant was also significantly lower in calcium acetate treatments ($P < 0.05$). The zinc acetate treatment was more effective in removing lipoproteins from the supernatant and precipitating them into the pellet compared with the calcium acetate treatments. When the temperature of calcium acetate treatment was increased from 30 to 60°C, significantly higher transmittance and lower protein concentrations in the supernatant were observed ($P < 0.05$). Increasing the temperature resulted in a better binding affinity of calcium with protein and lipid, but not to the extent of zinc acetate. This indicates that zinc acetate had a stronger affinity toward protein, and calcium acetate had a lower affinity toward protein but stronger affinity toward lipid. These results may be due to how zinc and calcium interact with MFGM components. Zinc forms a tetracoordination complex with membrane phospholipids in a tetrahedral geometry, whereas calcium forms a hexacoordination complex in an octahedral geometry

Figure 2. The effect of salt concentration and temperature on transmittance of the supernatant of the 5% β stream dispersion. Error bars represent SD.

Figure 3. The effect of salt concentration and temperature treatment on the supernatant protein concentration (assayed with the Bradford method) of the 5% β stream dispersion. Error bars represent SD.

(Binder et al., 2001; D'Acapito et al., 2002; Damodaran, 2010). The differences in the geometry of the salt-MFGM complexes may lead to more opportunity for zinc to interact with the protein present in the β stream.

Effect of pH on the Efficiency of Total Lipid, PL, and Protein Precipitation

An investigation to determine which pH is ideal for the effective and most practical zinc acetate treatment was performed using the salt concentration of 25 mM at 30°C. At this concentration, a pH range of 5.0 to 8.0 was investigated, to determine whether the pH of the β stream had a significant effect on the recovery of total lipid, PL, and protein. We found no significant differences in the total lipid and PL recoveries among all the pH investigated for zinc acetate ($P > 0.05$). The numerically highest lipid recovery was seen for the 8.0 pH treatment, with 98.1% total lipid and 98.2% total PL recovery (Table 2). These results indicate that zinc acetate had no preference for neutral lipid or PL under all pH conditions investigated. This can also be observed in the PL contents of the overall lipid fraction, which were similar to those of the initial β stream (35.0% PL). The highest-PL fraction was the pH 7.5 treatment, with a PL content of 36.2%, but this was not statistically significant. Additionally, we discovered no significant differences among PL composition of classes under the various pH conditions ($P > 0.05$).

Results for supernatant transmittance and protein concentrations, as affected by pH, can be seen in Figure 5. As the pH increased, we found less protein in the supernatant, and a higher transmittance was observed. The pH of 6.5 and above yielded significantly higher transmittance than did the lower pH treatments ($P < 0.05$). The lower transmittance at pH below 6.5 indicates that proteins were not precipitated as effectively under lower-pH conditions. Similar results were observed by Damodaran (2010), who investigated only the pH range below 6.5 and concluded that charge variations of membrane proteins as a function of pH can contribute to the net charge of MFGM and its interaction with zinc. The isoelectric point of most milk proteins is about pH 4.6 to 5.2 (Fong et al., 2007), and at pH above this range (5.5 through 8.0) the charge on the proteins would be negative. The zinc ion has a positive charge; thus, better precipitation of protein and PL can be expected at pH above 5.2. The transmittance results observed in this study were higher than the results observed when the concentration of salt was studied. During this study, salt was added before pH adjustment (versus salt addition after pH adjustment during the salt concentration study) so there was a longer interaction time between the salt and MFGM components. This longer interaction time may have led to supernatant with increased transmittance and lower protein concentration.

For calcium acetate of 25 mM at 60°C, the pH significantly affected the total lipid and PL recoveries (Table 2). As the pH of the β stream increased, higher

Table 2. Total lipid recovery, phospholipid (PL) recovery, and PL composition (% of total PL)¹ from the 25 mM zinc acetate at 30°C and 25 mM calcium acetate at 60°C treatments under various pH levels

Treatment	Total lipid recovery (%)	PL recovery (%)	SM	PE	PS	PI	PC	PL (% of total lipid)
Zn 5.0	89.3 ^{abcd}	86.9 ^{abc}	11.7 ^{abcd}	32.6 ^a	7.5 ^a	11.6 ^{ab}	36.6 ^{ab}	34.9 ^a
Zn 5.5	92.7 ^{abc}	87.9 ^{abc}	10.6 ^{abcd}	34.2 ^a	7.5 ^a	12.0 ^a	35.7 ^{ab}	33.9 ^{ab}
Zn 6.0	93.9 ^{abc}	90.9 ^{ab}	12.6 ^{abcd}	34.6 ^a	8.1 ^a	10.4 ^{ab}	34.3 ^b	34.6 ^a
Zn 6.5	94.7 ^{ab}	93.1 ^a	12.3 ^{abcd}	34.7 ^a	6.8 ^a	10.6 ^{ab}	35.6 ^{ab}	35.2 ^a
Zn 7.0	96.6 ^{ab}	95.3 ^a	11.9 ^{abcd}	33.8 ^a	7.5 ^a	10.9 ^{ab}	35.9 ^{ab}	34.9 ^a
Zn 7.5	96.7 ^{ab}	96.9 ^a	11.5 ^{abcd}	33.7 ^a	7.6 ^a	11.3 ^{ab}	35.9 ^{ab}	36.2 ^a
Zn 8.0	98.1 ^a	98.2 ^a	10.5 ^{bcd}	34.0 ^a	7.0 ^a	11.1 ^{ab}	37.4 ^{ab}	34.6 ^a
Ca 5.0	53.1 ^e	27.0 ^e	16.0 ^{ab}	24.1 ^b	8.2 ^a	11.2 ^{ab}	40.5 ^a	19.6 ^{de}
Ca 5.5	50.3 ^e	24.4 ^e	17.0 ^a	24.5 ^b	6.5 ^a	10.7 ^{ab}	41.4 ^a	19.0 ^e
Ca 6.0	54.6 ^e	32.8 ^e	17.1 ^a	24.0 ^b	7.1 ^a	10.9 ^{ab}	40.8 ^a	22.4 ^d
Ca 6.5	80.0 ^d	63.9 ^d	12.0 ^{abcd}	34.0 ^a	7.4 ^a	10.5 ^{ab}	36.1 ^{ab}	28.5 ^c
Ca 7.0	81.9 ^d	75.3 ^{bcd}	14.5 ^{abc}	33.4 ^a	6.5 ^a	9.5 ^b	36.0 ^{ab}	31.2 ^{bc}
Ca 7.5	83.9 ^{cd}	73.6 ^{cd}	10.1 ^{cd}	36.3 ^a	6.7 ^a	9.7 ^b	37.2 ^{ab}	31.1 ^{bc}
Ca 8.0	84.3 ^{bcd}	68.8 ^d	7.9 ^d	35.5 ^a	7.0 ^a	10.9 ^{ab}	38.7 ^{ab}	28.4 ^c

^{a-e}Means with different superscript letters within the same column are significantly different at $P = 0.05$.

¹SM = sphingomyelin; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PC = phosphatidylcholine.

total lipid and PL recoveries were observed, and the pH range of 6.5 to 8.0 resulted in significantly higher total lipid, PL recovery, and PL content in the lipid fraction ($P < 0.05$). The PL content of calcium acetate treatments were significantly lower compared with the zinc acetate treatment ($P < 0.05$), indicating that calcium acetate did not bind PL as effectively as zinc acetate, or that binding was stronger to other non-PL components. With calcium acetate, pH also had a significant effect on PL class composition. It was observed that

significantly more ($P < 0.05$) SM was recovered in the pH range of 5.0 to 6.0, and significantly more ($P < 0.05$) PC was recovered at the pH range of 5.0 to 6.0. Significantly more ($P < 0.05$) PE was recovered at the pH range of 6.5 to 8.0.

The protein concentration and transmittance results for the calcium acetate treatment under various pH conditions can be seen in Figure 5. Transmittance of the calcium acetate supernatant was significantly higher in the pH range of 7.0 to 8.0 ($P < 0.05$). This

Figure 4. Power-Law correlation between protein concentration and transmittance of the supernatant from all treatments examined in this study.

pH range also resulted in significantly lower protein concentration in the supernatant ($P < 0.05$). The pH range of 5.0 to 6.0 resulted in a supernatant with significantly higher protein concentration ($P < 0.05$). The protein charge under the various pH treatments had a profound effect on how efficiently MFGM proteins were precipitated into the pellet.

Apparent Solubility of Zinc Acetate and Calcium Acetate in Ethanol for Extracting Lipids from the Pellet

The apparent solubility of zinc acetate and calcium acetate under 3 different ethanol concentrations (70, 80, and 90% vol/vol) and 3 different temperatures

Figure 5. The transmittance (a) and protein concentration (b) of the β stream supernatant treated with zinc acetate at 25 mM and 30°C and calcium acetate at 25 mM and 60°C under various pH conditions. Error bars represent SD.

(30, 50, and 70°C) were investigated, to determine the potential quantity of each salt that can be solubilized during ethanol lipid extractions. The results are shown in Table 3. It is apparent that the temperature and type of salt were significant factors for their solubility in ethanol ($P < 0.05$). Zinc acetate is significantly more soluble than calcium acetate in ethanol ($P < 0.05$). The temperature of 70°C solubilized significantly more salt compared with 30 and 50°C ($P < 0.05$), whereas the concentration of ethanol was not a significant factor. Because ethanol at higher concentration had a slightly lower salt solubility, an ethanol concentration of 90% was used for the ethanol extraction. Previous work with whey protein phospholipid concentrate showed that an ethanol concentration of 90% at 70°C was optimal for a 1-step total lipid extraction (Price et al., 2018). Under such conditions, 0.5 g of calcium acetate and 22.7 g of zinc acetate can be solubilized in 100 g of ethanol.

The precipitation treatments used for the ethanol extraction from the β stream pellets were 25 mM, pH 7.5, and 30°C for zinc acetate and 100 mM, pH 7.5, and 60°C for calcium acetate. The zinc acetate-precipitated pellet contained 0.9% total lipid [10.5% dry basis (DB)], 0.3% PL (3.6% DB), 3.2% protein (37.2% DB), 2.6% lactose (31.4% DB), and 1.8% ash (20.9% DB), with a total solids content of 8.6%. The PL content of the pellet was 36.2% of its total lipid, which was composed of 35.9% PC, 33.7% PE, 11.5% SM, 11.3% PI, and 7.6% PS. The calcium acetate-precipitated pellet contained 0.8% total lipid (6.6% DB), 0.3% PL (2.3% DB), 4.6% protein (38.0% DB), 2.8% lactose (23.1% DB), and 3.9% ash (32.2% DB), with a total solids content of 12.1%. The PL content of this pellet was 31.2% of its total lipid, which was composed of 38.3% PE, 34.2% PC, 10.0% SM, 9.7% PI, and 7.8% PS. The calcium acetate-precipitated pellet contained significantly higher ash and total solids content than did the zinc acetate pellet ($P < 0.05$). The usage of calcium acetate was 100 mM, which equates to 1.58% in the β stream dispersion, whereas zinc acetate usage was 25 mM and 0.46% in molar and weight concentration. This means that calcium acetate usage was 3.43 times higher than that of zinc acetate (in weight concentration), which may have resulted in a higher concentration of ash in the calcium acetate pellet.

The weight distribution of the pellet and supernatant was calculated from the weight of supernatant divided by the initial mass of the β stream. The higher total solids content seen in the calcium acetate treatment is due to the lower weight distribution to the pellet (23.8% of initial mass), which means there was a larger volume of supernatant (76.2%). The weight distribution for zinc acetate was 56.8% to the pellet and 43.2%

Table 3. The apparent solubility (g of salt/100 g of ethanol) of zinc acetate and calcium acetate under various ethanol concentrations (70, 80, and 90%) and temperatures (30, 50, and 70°C)

Salt	Ethanol concentration (%)		
	70	80	90
Calcium acetate 30°C	3.1 ^c	1.2 ^c	0.2 ^c
Zinc acetate 30°C	3.2 ^c	2.2 ^c	1.6 ^{bc}
Calcium acetate 50°C	3.2 ^c	1.6 ^c	0.4 ^c
Zinc acetate 50°C	6.9 ^b	6.2 ^b	3.4 ^b
Calcium acetate 70°C	3.3 ^c	1.8 ^c	0.5 ^c
Zinc acetate 70°C	23.6 ^a	23.4 ^a	22.7 ^a

^{a-c}Means with different superscript letters within the same column are significantly different at $P = 0.05$.

to supernatant. The calcium acetate-precipitated pellet contained 12.1% total solids, compared with 8.6% for zinc acetate. These results indicate that calcium acetate produced a denser pellet with significantly higher total solids ($P < 0.05$). These differences in the total solids content resulted in the zinc acetate-precipitated pellet requiring more ethanol for the ethanol extraction (8 mL more required for zinc acetate, as stated in Methods section). Also, the calcium acetate supernatant had a higher total solids (4.4%) content compared with the supernatant produced by zinc acetate (3.0%), mainly due to higher salt concentration usage. This could also indicate that more proteins were dissolved in the calcium acetate supernatant, which was confirmed by the protein concentration results.

Ethanol Extraction of Lipids from the Precipitated Pellets

Total lipid and PL recoveries of 96.7% and 97.7% were achieved from the zinc acetate-precipitated pellet, whereas 88.2% and 94.9 were achieved from the calcium acetate-precipitated pellet (Table 4). We found no significant difference in the total lipid recovery, PL recovery, or PL class composition between the lipid fractions extracted from the zinc acetate- and calcium acetate-precipitated pellets ($P > 0.05$). The PL percentage relative to the total lipid fraction was 38.7% for calcium acetate and 34.5% for zinc acetate, similar to the initial 35.0% of the β stream lipid fraction. The PL content in the pellet was 31.2% for calcium acetate and 36.2% for zinc acetate. A significant enrichment in PL content was found in the ethanol-extracted lipid from the calcium acetate lipid fraction compared with zinc acetate ($P < 0.05$). The binding geometry and spacing of the complex between zinc and calcium with the MFGM components may have affected how efficiently PL were extracted. Zinc binds with MFGM components in a tetrahedral geometry, leading to the closest

Table 4. Total lipid and phospholipid (PL) yield and class¹ distribution (% of total PL) from ethanol extraction (90% ethanol at 70°C)²

Salt	Total lipid recovery (%)	PL recovery (%)	SM	PE	PS	PI	PC	PL (% of total lipid)
Zinc acetate	96.7	97.7	11.8	36.2	2.6	11.4	37.9	34.5
Calcium acetate	88.2	94.9	11.7	40.5	4.0	8.0	35.8	38.7

¹SM = sphingomyelin; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PC = phosphatidylcholine.

²No significant difference between the two treatments, for all parameters.

distance (2.04 angstrom—smaller than the diameter of a water molecule) between the zinc MFGM complexes (Damodaran, 2010). This short distance would not easily allow for the diffusion of water or ethanol into the complex. In contrast, the calcium MFGM complex is about 3.43 angstrom (Damodaran, 2010), which may have provided more opportunity for ethanol to penetrate and extract the PL present in the calcium acetate pellet.

After the lipid was extracted from the pellets with ethanol, the ash content was quantified to determine the quantity of salt that was solubilized during the extraction. The lipid fraction from calcium acetate treatment had a significantly higher ash content (27.9%) than that from the zinc acetate treatment (18.5%). Under the extraction conditions (90% ethanol at 70°C), 206 mL of ethanol was used for the zinc acetate pellet and 198 mL of ethanol for the calcium acetate pellet. Such a volume of ethanol could potentially solubilize 36.8 g of zinc acetate and 0.8 g of calcium acetate. The amounts of ash in the zinc acetate- and calcium acetate-precipitated pellets were 1.8% and 3.9%, respectively, which equates to 0.05 and 0.10 g of ash in the 25 g of pellet, respectively. These quantities of salt are well below the apparent solubility limit for zinc acetate and calcium acetate, meaning that all the salt present in the pellet was dissolved in ethanol. With this current extraction procedure, reducing the amount of salt being dissolved in ethanol will be difficult, and additional processing to remove salt may be required.

Zinc acetate may have health concerns because intake of greater than 225 mg of zinc per day may suppress copper and iron absorption (Sheftel, 2000), but our newly identified calcium salt is safe and may provide additional benefit.

Protein Distribution Under Optimal Precipitation Conditions

The protein distributions of the β stream, supernatant, and pellet were investigated using SDS-PAGE (Figure 6). The major protein classes in the initial β stream were casein (28 to 35 kDa), whey (<25 kDa), and a small quantity of MFGM proteins (>50 kDa; Mather,

2000). The MFGM proteins represent only 1 to 2% of the total protein found in milk (Riccio, 2004), and the β stream seems to have a similar protein profile to that of native milk. The zinc acetate supernatant contained only whey proteins, which indicates that the casein and MFGM proteins were effectively precipitated into the pellet. The calcium acetate supernatant displayed all protein classes, which confirmed our supposition that calcium acetate has a lower binding affinity toward dairy proteins. The protein composition in the pellet was similar for zinc acetate and calcium acetate, with casein and whey being the predominant proteins. The concentrations of MFGM proteins in these protein fractions were very low compared with concentrations of casein and whey proteins, as shown on the SDS-PAGE gel. Nonetheless, 2 membrane proteins were visible in the samples (Figure 6).

CONCLUSIONS

Use of zinc acetate and calcium acetate was effective for precipitating the total lipid, PL, and some proteins found in the β stream. The lowest salt concentration treatment for zinc acetate was 25 mM, at pH above 6.5 at 30°C; for calcium acetate, the effective conditions were salt concentration of 75 to 200 mM and pH above 6.5 at 60°C. For better recovery of SM, the 30°C calcium acetate treatment would be desirable, whereas the 60°C calcium acetate treatment would be desirable for PS recovery. The aqueous ethanol extraction was an effective way to remove the lipid from the precipitated pellet, and PL recovery of as high as 98% can be achieved. Although high ash content was observed in the ethanol-extracted lipid fraction, a simple further processing step can remove the salt. This work demonstrates that calcium acetate precipitation has great potential for producing valuable lipid fractions from the β stream or from other similar dairy processing by-products.

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Figure 6. Sodium dodecyl sulfate-PAGE (SDS-PAGE) of the initial β stream and supernatant and pellet samples from zinc acetate and calcium acetate treatments. XO = xanthine oxidase; BTN = butyrophilin; ADPH = adipophilin.

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