Isolation of caseins from whey proteins by microfiltration modifying the mineral balance in skim milk

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ABSTRACT

The objective of this work was to study the effect of different salts and salt concentration on the isolation of casein micelles from bovine raw skim milk by tangential flow microfiltration. Tangential flow microfiltration (0.22 μm) was conducted in a continuous process adding a modified buffer to maintain a constant initial sample volume. This buffer contained calcium chloride (CaCl_2), sodium phosphate (Na_2HPO_4), or potassium citrate ($\text{K}_3\text{C}_6\text{H}_5\text{O}_7$) in concentrations ranging from 0 to 100 mM. The concentrations of caseins and whey proteins retained were determined by sodium dodecyl sulfate-PAGE and analyzed using the Scion Image software (Scion Corporation, Frederick, MD). A complete isolation of caseins from whey proteins was achieved using sodium phosphate in the range of 10 to 50 mM and 20 times the initial volume of buffer added. No whey proteins were detected at 50 mM but this was at the expense of low caseins being retained. When lower sodium phosphate concentrations were used, the amount of caseins retained was higher but a small amount of whey proteins were still detected by sodium dodecyl sulfate-PAGE. Among the salts tested, calcium chloride at 50 mM and all volumes of buffer showed the higher retention of casein proteins. The highest casein:whey protein ratio was found at 30 mM CaCl_2 , but no complete casein micelle isolation was achieved. Potassium citrate was the most ineffective salt because a rapid loss of caseins and whey proteins was observed at all concentrations and with low quantities of buffer added during the filtration process. Our results show the potential of altering the mineral balance in milk for isolation of casein micelles from whey proteins in a continuous tangential flow microfiltration system.

Key words: microfiltration, casein micelle, whey protein, mineral

INTRODUCTION

Milk proteins are well known for their nutritional value and for their potential application as functional ingredients to affect the physical and sensory attributes of a wide range of dairy food products. Caseins are one of the most important and complex proteins in bovine milk representing approximately 80% of the total protein fraction in milk. These proteins have excellent surfactant properties in emulsions and foams, gelling properties, and thermal resistance to denaturation (Fox and McSweeney, 1998) because of their lack of complex secondary and tertiary structure. However, they form colloidal particles (50–500 nm; the so-called casein micelle) composed of the proteins $\alpha_{\text{S1-}}$, $\alpha_{\text{S2-}}$, β -, and κ -caseins, and salts of Ca, P, Mg, and Zn (Fox, 2003). Casein isolates are mostly available as caseinates, derived from acid precipitation of milk and subsequent neutralization with NaOH, CaOH, or KOH, and commercialized as sodium, calcium, or potassium caseinates, respectively.

The wide particle size distribution of the various milk components (from nano- to micrometer scales) made separation based on size a convenient operation to obtain specific milk fractions (Korhonen and Pihlanto, 2007). Because of its relatively low operating cost, microfiltration using polymeric or ceramic membranes has been extensively used by the dairy industry to concentrate and isolate native caseins (Brans et al., 2004). However, electrophoresis (SDS-PAGE) of commercially available native casein isolates (e.g., micellar casein, American Casein Company, Burlington, NJ; micellnor, Kerry Dairy Ingredients, Kerry, Ireland) revealed that whey proteins remain in the final product and that complete isolation of the casein micelle remains a challenge (Figure 1). Methods were developed to obtain single fractions of casein (e.g., β -CN; Huppertz et al., 2006) but the effective isolation of the native casein micelle is still possible only at the laboratory scale (Rosenberg, 1995; Korhonen and Pihlanto, 2007). Membranes used in ultrafiltration usually have a pore size between 0.01 and 0.1 μm (or a molecular weight cutoff from 1 to 300 kDa; Ghosh, 2009) and those for microfiltration have a pore size from 0.1 to 1 μm . The ultrafiltration of skim

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milk using a molecular weight cutoff ≤ 300 kDa yields a permeate rich in water-soluble vitamins, minerals, lactose, and nonprotein nitrogen compounds and a retentate rich in colloidal minerals and proteins (caseins and whey; Rosenberg, 1995).

The mineral fraction of milk is relatively small (8–9 g/L; Gaucheron, 2005) and is mainly composed of the cations calcium, magnesium, sodium, and potassium, and the anions inorganic phosphate, citrate, and chloride. In milk, ions can be found free in the serum or in colloidal form associated with the caseins. Milk salts play an important role in the properties of dairy foods, because altering the balance of the mineral fraction in milk will affect the structure, stability, and functionality of casein micelles (Swaigood, 1996; Gaucheron, 2005).

An effective protocol for the isolation of the native casein micelles would be beneficial in the study of casein micelle structure-function properties and in the development of novel ingredients based on casein isolates (Brans et al., 2004; Mier et al., 2008). Because it is known that altering the mineral fraction of skim milk modifies the interactions between caseins and whey proteins, the purpose of this study was to evaluate the effect of ionic strength of milk relative to 3 salts and to establish the potential use of tangential flow microfiltration for the complete isolation of native casein micelles from bovine milk. Ionic strength was modified by changing the concentrations of calcium chloride (CaCl_2), sodium phosphate (Na_2HPO_4), and potassium citrate ($\text{K}_3\text{C}_6\text{H}_5\text{O}_7$), commonly used in the dairy industry or naturally present in milk.

MATERIALS AND METHODS

Raw milk was obtained from the dairy farm of The University of Tennessee (Knoxville) and skimmed by centrifugation ($1,500 \times g$, 15 min) using a Sorvall RC-5B Plus centrifuge (Thermo Scientific, Waltham, MA) equipped with a SLA-1500 rotor (Kendro, Newtown, CT). Sodium azide (0.02% wt/vol; Fisher Scientific, Fair Lawn, NJ) was added to the skim milk to prevent bacterial growth.

A 20 mM imidazole buffer solution was prepared and modified by adding CaCl_2 , Na_2HPO_4 , or $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$ at concentrations of 10, 30, 50, or 100 mM. The ionic strength for CaCl_2 and Na_2HPO_4 solutions ranged from 0.03 to 0.30 (10 to 100 mM), whereas that for $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$ solutions ranged from 0.06 to 0.60 (10 to 100 mM). The pH was adjusted to 6.8 with 1 N HCl and the solutions were stored at 4°C overnight to reach equilibrium. All chemicals were of analytical grade and purchased from Fisher Scientific.

Figure 1. Sodium dodecyl sulfate-PAGE of 2 commercial native casein isolates. Lane 1 = β -LG standard; lane 2 = β -CN and α -LA standards; lane 3 = commercial casein isolate (Micellnor, Kerry Dairy Ingredients, Kerry, Ireland); lane 4 = commercial casein isolate (Micellar Casein, American Casein Company, Burlington, NJ); and lane 5 = α_5 -CN standard.

Filtration was done using a 0.22- μm tangential flow microfiltration cartridge (Pellicon XL50, Millipore, Billerica, MA) with a filtration area of 50 cm^2 and a hydrophilic polyvinylidene fluoride membrane connected to a variable flow peristaltic pump (Vera, Barnat, Barrington, IL). Preliminary exploratory tests showed strong whey protein retention using filtration membranes with a small pore size (30, 100, and 300 kDa; 0.1 μm) and loss of caseins with larger pore size membranes (0.45 μm). Each modified buffer solution was initially added to skim milk (1:1, vol/vol), and then buffer solution was added during the filtration process to keep a constant volume of retentate. The retentate was recirculated during filtration and permeates were disposed. Experiments were conducted at room temperature and the microfiltration process was ended when 20 volumes of the modified buffer solution were circulated through the original skim milk sample (1:20, vol/vol) and the final and initial volumes of retentate were the same. Samples of retentates (0.5 mL) were collected at regular intervals during the microfiltration process and stored at -40°C until analyzed by SDS-PAGE.

For SDS-PAGE, the retentate samples were thawed at room temperature and diluted (1:4, vol/vol) in an SDS reducing buffer for SDS-PAGE according to Laemmli (1970). A vertical electrophoresis unit (Mini-Protean 3 Cell, Bio-Rad Laboratories, Hercules, CA), in conjunction with a power supply (PowerPack 300, Bio-Rad Laboratories), was used. The samples were loaded (20 μL) in precast gels (Ready gels, 15% Tris-HCl, Bio-Rad Laboratories) and run for 30 min at 200 V. The gels were then stained with a 0.25% (wt/vol) solution of Coomassie Brilliant Blue R-250 (Bio-Rad

Figure 2. Calibration curves obtained from SDS-PAGE and using the Scion Image software (Frederick, MD) used to measure the concentration of caseins (α_S -CN and β -CN; solid circles) and whey proteins (α -LA and β -LG; open circles). The solid and dashed lines correspond to the linear regression for the caseins and whey proteins, respectively.

Laboratories) and destained in a methanol (15%):acetic acid (10%) solution. For protein quantification, destained gels were scanned using a ScanMaker 4850 (Mikrotek International Inc., Hsinchu, Taiwan), and band density and dimensions analyzed using Scion Image software (version 4.0.3.2, Scion Corp., Frederick, MD). Calibration curves ($r > 0.98$; Figure 2) were created using SDS-PAGE of α_S -CN, β -CN, α -LA, and β -LG standards (Sigma-Aldrich, St. Louis, MO). Sodium dodecyl sulfate-PAGE has a relatively low resolution when separating individual caseins and, because our objective was to isolate casein micelles from whey

proteins, SDS-PAGE was used to quantify total caseins versus total whey proteins in retentates, rather than individual proteins. All experiments were conducted in duplicate and average values are presented.

RESULTS

The concentrations of caseins and whey proteins (α -LA and β -LG) in the retentate were monitored to determine the effect of the different salts, concentrations, and total volume of modified buffer circulated during tangential flow microfiltration processing of skim milk. Figure 3 shows the electrophoretic pattern of milk retentates obtained with the 3 salts tested (CaCl_2 , Na_2HPO_4 , and $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$) at 50 mM and increased total volume of buffer used during filtration. Similar SDS-PAGE runs were done for all the salt concentrations studied (10 to 100 mM). Our results demonstrate that only CaCl_2 allowed retention of caseins throughout the filtration process (Figure 3A) and that whey proteins remained in the retentates even after the initial samples were circulated through the tangential flow microfiltration system using 20 times their volume of buffer. The initial total milk protein content was considered constant at 3.4 g per 100 mL (Fox, 2003).

Retention Using Calcium Chloride

As buffer was continuously added to the system to maintain a constant volume during microfiltration, slow declines in casein and whey protein concentrations in the retentate were observed at all concentrations of CaCl_2 (Figure 4). Increasing the concentration of CaCl_2 up to 50 mM promoted an increased retention of pro-

Figure 3. Sodium dodecyl sulfate-PAGE of the UF retentates with the 3 salts tested: A) calcium chloride (CaCl_2), B) sodium phosphate (Na_2HPO_4), and C) potassium citrate ($\text{K}_3\text{C}_6\text{H}_5\text{O}_7$) at 50 mM and various initial skim milk sample volume:buffer circulated through the microfiltration system: lane 1 = 1:5; lane 2 = 1:10; lane 3 = 1:15; and lane 4 = 1:20.

Figure 4. Effect of concentration of calcium chloride (CaCl_2) and volumes of buffer added during microfiltration of skim milk in the concentration of casein from whey protein in the retentate.

teins (caseins and whey). However, a decrease in protein retention was observed with higher CaCl_2 concentrations at all volumes of buffer added. The reduction of whey proteins in the retentates was achieved early in the filtration processes and the caseins remained in the retentate even after 20 volumes of the initial buffer were added during filtration. All salt concentrations yielded total casein:total whey proteins >5 after 15 volumes of buffer were circulated through the microfiltration system. However, higher total casein retention was achieved when milk was suspended in buffer containing 50 mM CaCl_2 compared with all other concentrations tested. Almost complete removal of whey proteins was achieved with <40 mM CaCl_2 but at the expense of caseins also being removed from the retentate.

Retention Using Sodium Phosphate

A faster decrease in the concentrations of caseins and whey proteins in the retentate was observed when sodium phosphate buffer was used for microfiltration compared with CaCl_2 at all concentrations (Figure 5). Furthermore, the amount of casein proteins retained continuously decreased when the concentration of phosphates was increased at all levels of buffer added. The removal of whey proteins to nondetectable levels was achieved only after 20 volumes of buffer were circulated through the tangential flow microfiltration unit. However, the removal of whey proteins was achieved only at the expense of very little casein proteins remaining in the retentates. For the cases where whey proteins were

detected, the total casein:total whey protein remained <3.5 in all cases.

Retention Using Potassium Citrate

The use of potassium citrate resulted in the fastest lost of caseins and whey proteins during microfiltration of skim milk as increasing volumes of buffer were circulated during filtration (Figure 6). After 15 volumes of buffer containing >20 mM potassium citrate salt were circulated through the tangential flow microfiltration unit, no protein (casein or whey) was detected in the retentate samples by SDS-PAGE. The rapid loss of caseins was initially prevented when buffer containing 0 to 50 mM potassium citrate and <10 buffer volumes were circulated through the microfiltration unit. However, the separation of caseins from whey proteins in skim milk samples was not achieved for any of the potassium citrate concentrations or volumes of buffer circulated during microfiltration.

DISCUSSION

The addition of calcium ions to skim milk modifies the ionic balance between serum and micellar calcium as well as the hydrophobic and ionic interactions within the casein micelle (Philippe et al., 2005). Philippe et al. (2004) compared the physicochemical characteristics of skim milk with addition of 3 soluble calcium salts (Ca-gluconate, Ca-lactate, Ca-chloride) and found that, regardless of the specific salt added, the association of

Figure 5. Effect of concentration of sodium phosphate (Na_2HPO_4) and volumes of buffer added during microfiltration of skim milk in the concentration of casein from whey protein in the retentate.

calcium with casein micelles was similar as were the modifications promoted in the milk. They suggested that the addition of extra calcium (up to approximately 16 mM) leads to the formation of salt complexes (e.g., calcium-inorganic phosphate and calcium-citrate) that partially associate with casein micelles incorporating other casein proteins, releasing micellar water, and resulting in an increase in micellar density with no changes in the average hydrodynamic radius. Our results suggest that the CaCl_2 -induced proteinic reorganization in the casein micelle reported by Philippe et al. (2003) occurs with CaCl_2 concentrations up to approximately 50 mM has a protective effect on the casein micelle and leads to less permeation of casein during tangential flow microfiltration. However, this protective effect is rapidly lost when CaCl_2 concentrations exceed 50 mM.

Patocka and Jelen (1991) found a strong association of whey proteins to calcium at ionic strength of 0.04 (~13 mM CaCl_2), but minimal association after increasing the ionic strength up to 0.16 (~53 mM CaCl_2). This phenomenon could also explain the higher retention of caseins at 50 mM CaCl_2 , where more calcium would be available to interact with the casein micelles instead being chelated by the whey proteins. On the other hand, for lower CaCl_2 concentrations (e.g., 30 mM CaCl_2), less free calcium would be available because of the sequestration effect of whey proteins.

The loss of caseins during tangential flow microfiltration using increasing concentrations and volumes of Na-phosphate buffer in our experiments is in agreement with results reported by Gaucher et al. (2007). Those authors found that the addition of phosphates (as KH_2PO_4) to pH-adjusted skim milk induced formation of insoluble calcium phosphate salts and promoted partial micellar disintegration and liberation of individual casein proteins to the serum phase. Mizuno and Lucey (2005) also found evidence for micellar dissociation through a slow but continuous decrease in absorbance at 700 nm (from 0.7 to 0.5) when concentration of Na_2HPO_4 was elevated to approximately 100 mM in reconstituted skim milk. Mizuno and Lucey (2005) also found a sharp decrease in lightness, suggesting strong casein dissociation induced by micellar calcium depletion, when chelating polyphosphate buffers were used at similar concentrations. Our results were consistent with the loss in casein micelle integrity induced by the chelating effect of increasing concentrations of Na-phosphates in the serum.

Citrates have a strong chelating effect on calcium cations and are thus able to destabilize the casein micelle. Le Berre and Daufin (1998) found that 50 mM sodium citrate promoted a reduction in the casein micelle size to approximately 15 nm. Our experiments

Figure 6. Effect of concentration of potassium citrate ($\text{K}_3\text{C}_6\text{H}_5\text{O}_7$) and volumes of buffer added during microfiltration of skim milk in the concentration of casein from whey protein in the retentate.

suggest that even at very low concentrations of citrate, calcium chelation resulting in micellar dissociation results in little micellar casein remaining in the retentates after a sufficient volume has been circulated through the tangential flow microfiltration cartridge. On the other hand, the addition of calcium and phosphate ions ($>23 \text{ mM Ca}^{++}$) increased the average particle size of casein micelles to $>500 \text{ nm}$. These results support the difference in proteins retained when calcium chloride was used, because a greater particle size of casein would facilitate casein retention during tangential flow microfiltration. The fact that casein proteins remained in the retentate was clear evidence that these proteins were in micellar form. In concentrated systems, membrane fouling would constitute an additional barrier able to retain individual casein proteins. However, our experiments were done using a diluted system (1:1 milk:buffer), and negligible pressure changes were observed throughout the filtration process, indicating little fouling.

The effectiveness of ultra- or microfiltration processes for the concentration of casein proteins is dependent on the capacity of the process to avoid fouling by casein micelles by controlling shear stress and transmembrane pressure (Le Berre and Daufin, 1996; Gésan-Guiziou et al., 1999) and filter vibration (Al-Akoum et al., 2002) rather than the specific pore size (Le Berre and Daufin, 1998; Brans et al., 2004). Lawrence et al. (2008), using 0.3- and 0.5- μm polyvinylidene fluoride polymeric membranes, concluded that the protein layer formed on the surface of the membrane was a key factor in the performance of the filtration process and that

better separation was achieved when relatively lower processing pressures were used (≤ 50 Pa). As stated by Gésan-Guizieu et al. (2000), there exists a critical ratio (convection toward the membrane/erosion) in tangential flow microfiltration, below which there is no marked fouling by colloidal particles and above which performance is greatly altered by a sharp increase of fouling. Other factors that were not tested may affect the ability of a given membrane to efficiently retain casein micelles during filtration. Brans et al. (2004) pointed out the complexity of ultra- and microfiltration and their operation parameters when they reviewed different studies focusing on concentration of casein micelles.

The modification of skim milk during microfiltration by addition of a buffer with different salts has proven feasible for improved isolation of caseins from whey proteins as shown by our results. In addition, yield might be increased by controlling specific parameters (i.e., transmembrane pressures) during the microfiltration process.

CONCLUSIONS

Our results show that the isolation of casein micelles from bovine milk using tangential flow microfiltration of skim milk is dependent on ionic strength and the specific salt used. Calcium chloride at approximately 50 mM was the most effective treatment in retaining casein, but complete separation of casein micelles from whey proteins was not achieved. Sodium phosphate proved effective in isolating casein micelles from whey proteins, but at the expense of a very low casein yield. Further studies are required to improve the yield of casein micelle isolates obtained.

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