Investigation of Solubilization, Cold Gelation, and Rennet Coagulation Properties of Highly Concentrated Micellar Casein Concentrate for Use in Cheese Making

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INVESTIGATION OF SOLUBILIZATION, COLD GELATION, AND RENNET COAGULATION PROPERTIES OF HIGHLY CONCENTRATED MICELLAR CASEIN CONCENTRATE FOR USE IN CHEESE MAKING

by

Ying Lu

A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Nutrition and Food Sciences

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UTAH STATE UNIVERSITY
Logan, Utah

2016
ABSTRACT

Investigation of Solubilization, Cold Gelation, and Rennet Coagulation Properties of Highly Concentrated Micellar Casein Concentrate for Use in Cheese Making

by

Ying Lu, Doctor of Philosophy
Utah State University, 2016

Major Professor: Dr. Donald J. McMahon
Department: Nutrition, Dietetics, and Food Sciences (Specialization: Food Processing)

Highly concentrated micellar casein concentrate (HC-MCC), a potential ingredient for cheese making, containing ~20% casein with ~70% of serum proteins removed by microfiltration, and diafiltration of skim milk, and then further concentrated by vacuum evaporation. The objectives of this research were to investigate solubilization, cold gelation, rennet coagulation properties of recombined HC-MCC and cream for its use in cheese making.

In Chapter 3, either mixing thawed HC-MCC in water at high temperature (~50°C) or addition of trisodium citrate can achieve complete dispersion and more than 80% solubility of HC-MCC in water (3% protein). Overnight storage helps to fully disperse HC-MCC, but only reaches ~30% of solubility at 20°C. Cold-gelation of HC-MCC is thermally reversible and reducing protein levels in HC-MCC can decrease its CGT. The HC-MCC with less than 16% of protein does not gel at 5°C. We propose that
cold-gelation of HC-MCC occurs when the kinetic energy of the casein micelles is sufficiently reduced to inhibit their mobility in relation to adjacent casein micelles.

In Chapter 4, the recombined concentrated milk (RCM) by mixing thawed frozen HC-MCC and cream with 12% casein at pH 6.6 does not gel until cooled below 12°C. Addition of either sodium citrate or high levels of calcium increased CGT, although low levels of calcium did not impact CGT. Cold gelation of RCM was thermally reversible, even when citrate was added to partially chelate calcium. We propose that cold gelation of RCM occurs when protein strands that have been partially released from the casein micelles entangle, restrict their mobility and form a fine stranded gel network. The RCM at a casein level of 12% (wt/wt) has potential for use in cheese making.

In Chapter 5, reducing rennet level can increase coagulation time of RCM (11% casein) without impact on curd firmness or firming rate. Decreased coagulation temperature helps to increase coagulation time and decrease curd firmness rate, but also increases the initial viscosity of RCM. Pre-acidified RCM has no advantage in increasing coagulation time, decreasing curd firmness or firming rate. Microstructure of RCM and its coagulum indicates that the increased curd firmness probably results from the highly inter-linked and longer protein strands in RCM curd. Reducing rennet level can be applied to slow down rennet coagulation of RCM (11% casein) in cheese making.
PUBLIC ABSTRACT

Investigation of Solubilization, Cold Gelation, and Rennet Coagulation Properties of Highly Concentrated Micellar Casein Concentrate for Use in Cheese Making

Ying Lu

This work demonstrated potentials to use a microfiltrated, diafiltrated, and vacuum-evaporated milk protein concentrate-highly concentrated micellar casein concentrate (HC-MCC) for use in cheese making. Previously, ultrafiltrated milk concentrate has been used for cheese making to improve cheese yield and increase milk processing ability. However, ultrafiltrated milk contains high level of serum protein, which negatively impact cheese quality during aging. Microfiltrated milk is more suitable for cheese making with most serum proteins removed.

The project evaluated the potential of cheese making using recombined concentrated milk (RCM) by mixing HC-MCC and cream. We identify the method to solubilize and mix thawed frozen HC-MCC and the maximum protein level in RCM that is practical for cheese making. We further suggested modifications of cheese making procedure to retain quality of cheese made using RCM.
DEDICATION

My dissertation is dedicated to my parents Yiping Lu and Muhua Mao, who are willing to send their single child abroad to pursue her dream; my husband, Jianming Zhong, who helps and encourages me all through the process; my son: Luke Zhong, who brightens up my life.

Ying Lu
ACKNOWLEDGMENTS

I would like to express my deepest gratitude to Dr. Donald J. McMahon for providing me with such a valuable opportunity, encouraging, and guiding me through the whole program. He not only taught me how to be a good scientist but also how to be a good person. I also would like to thank my committee members, Dr. Marie Walsh, Dr. Silvana Martini, Dr. Craig Oberg, and Dr. David Britt, for their reviewing and guiding my research throughout the program. I also thank Dr. Silvana Martini for her guidance in rheology work, Dr. Almut Vollmer for her encouragement and assistance in microstructure work, and David Campbell and Doug Palmer for their assistance in my experiments. I am thankful for the +MD company and Western Dairy Center for funding my Ph.D. studies. Finally, I would like to thank my family for their support and encouragement; without them, this could not be successfully completed.

Ying Lu
# CONTENTS

ABSTRACT ........................................................................................................ iii
PUBLIC ABSTRACT ........................................................................................... v
DEDICATION ......................................................................................................... vi
ACKNOWLEDGMENTS ......................................................................................... vii
CONTENTS ........................................................................................................... viii
LIST OF TABLES ................................................................................................... x
LIST OF FIGURES ................................................................................................ xiv
LIST OF ABBREVIATIONS ................................................................................... xviii
CHAPTER ............................................................................................................... 1

1. GENERAL INTRODUCTION ............................................................................ 1

2. LITERATURE REVIEW .................................................................................... 3
   Casein Micelle ...................................................................................................... 3
   Solubilization of Dairy Proteins .......................................................................... 4
   Ways to Improve Solubilization of Dairy Proteins ............................................. 13
   Rennet Coagulation of Milk ............................................................................... 16
   Microfiltration of Milk ....................................................................................... 21
   Cheese Made from Ultrafiltrated or Microfiltrated Milk .................................... 25
   Rheological Properties of Food Gels .................................................................. 28
   References ......................................................................................................... 29

3. HYPOTHESIS AND OBJECTIVES .................................................................. 49

4. SOLUBILIZATION OF REHYDRATED FROZEN HIGHLY CONCENTRATED MICELLAR CASEIN FOR USE IN LIQUID APPLICATIONS .................. 50
   Abstract ........................................................................................................... 50
   Introduction ....................................................................................................... 51
   Materials and Methods ..................................................................................... 55
   Results .............................................................................................................. 64
   Discussion ....................................................................................................... 70
   Conclusions ..................................................................................................... 82
Acknowledgments .......................................................................................... 83
References ........................................................................................................... 83

5. INVESTIGATING COLD GELATION PROPERTIES OF
RECOMBINED HIGHLY CONCENTRATED MICELLAR
CASEIN CONCENTRATE AND CREAM FOR USE IN
CHEESE MAKING ......................................................................................... 92

Abstract .............................................................................................................. 92
Introduction ........................................................................................................ 93
Materials and Methods .................................................................................... 96
Results and Discussion .................................................................................... 99
Conclusions ....................................................................................................... 114
Acknowledgments ............................................................................................ 116
References .......................................................................................................... 116

6. INVESTIGATING RENNET COAGULATION PROPERTIES
OF RECOMBINED HIGHLY CONCENTRATED MICELLAR
CASEIN CONCENTRATE AND CREAM FOR USE IN
CHEESE MAKING .......................................................................................... 123

Abstract .............................................................................................................. 123
Introduction ........................................................................................................ 124
Materials and Methods .................................................................................... 126
Results and Discussion .................................................................................... 129
Conclusions ....................................................................................................... 140
Acknowledgments ............................................................................................ 141
References .......................................................................................................... 141

7. GENERAL SUMMARY .................................................................................... 145

APPENDICES ..................................................................................................... 148

APPENDIX A. STATISTICS FOR CHAPTER 3 .................................................. 149
APPENDIX B. STATISTICS FOR CHAPTER 5 .................................................. 152
APPENDIX C ....................................................................................................... 152
CURRICULUM VITA ............................................................................................ 158
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Manufactural process, composition, and state of casein micelle of caseinate, milk protein concentrate, micellar casein concentrate, and highly concentrated-micellar casein concentrate.</td>
</tr>
<tr>
<td>3.1</td>
<td>Composition of pasteurized skim milk and MF retentate</td>
</tr>
<tr>
<td>3.2</td>
<td>Composition of liquid highly concentrated micellar casein concentrate</td>
</tr>
<tr>
<td>3.3</td>
<td>Mean dispersibility(^1) of rehydrated HC-MCC, with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50°C, followed by optional overnight hydration (-O) at 4°C.</td>
</tr>
<tr>
<td>3.4</td>
<td>Mean suspendability(^1) of rehydrated HC-MCC, with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50°C, followed by optional overnight storage (-O) at 4°C.</td>
</tr>
<tr>
<td>3.5</td>
<td>Mean solubility(^{1}) of rehydrated HC-MCC, with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50°C, followed by optional overnight storage (-O) at 4°C.</td>
</tr>
<tr>
<td>3.6</td>
<td>Mean dispersibility, suspendability, and solubility of rehydrated HC-MCC, with pH adjusted to 7.2, 6.8 or 6.4, mixing using low shearing for 10 min, followed by high shearing for 1 min at 20°C.</td>
</tr>
<tr>
<td>3.7</td>
<td>Mean dispersibility, suspendability, and solubility of rehydrated HC-MCC in 60 or 120 mM trisodium citrate, with pH adjusted to 7.0, mixing using high shearing (HS) for 1 min at 4 or 20°C, followed by optional overnight storage (-O) at 4°C.</td>
</tr>
<tr>
<td>4.1</td>
<td>Composition of highly concentrated micellar casein concentrate (HC-MCC) made using microfiltration and vacuum evaporation.</td>
</tr>
<tr>
<td>4.2</td>
<td>Different protein to fat ratios (P:F), casein and fat levels, and casein levels in nonfat portion (casein% in NF) in recombined concentrated milk at pH 7.0, determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz.</td>
</tr>
</tbody>
</table>
5.1 Composition of highly concentrated micellar casein concentrate (HC-MCC) made using microfiltration and vacuum evaporation......................127

5.2 Mean rennet coagulation temperature (RCT, min), initial storage modulus ($G'_0$, Pa) and loss modulus ($G''_0$, Pa) within 4 min after rennet addition, storage modulus at 1, 1.5, and 2 times of rennet coagulation time ($G'_1$, $G'_{1.5}$, and $G'_2$) of recombined concentrated milk (11% casein and protein to fat ratio of 0.8) coagulated at different temperatures, under strain of 0.01, and frequency of 1.0 Hz. ..........................................................133

5.3 Mean rennet coagulation temperature (RCT, min), initial storage modulus ($G'_0$, Pa) and loss modulus ($G''_0$, Pa) within 4 min after rennet addition, storage modulus at 1, 1.5, and 2 times of rennet coagulation time ($G'_1$, $G'_{1.5}$, and $G'_2$) of recombined concentrated milk (11% casein and protein to fat ratio of 0.8) coagulated at different pH at 25°C, under strain of 0.01, and frequency of 1.0 Hz. ..............135

A.1 ANOVA of dependent variables for dispersibility of rehydrated highly concentrated micellar casein ..........................................................149

A.2 ANOVA of dependent variables for suspendability of rehydrated highly concentrated micellar casein after overnight storage .................149

A.3 ANOVA of dependent variables for suspendability of rehydrated highly concentrated micellar casein at 50°C ........................................149

A.4 ANOVA of dependent variables for solubility of rehydrated highly concentrated micellar casein after overnight storage .....................150

A.5 ANOVA of dependent variable for solubility of rehydrated highly concentrated micellar casein at 50°C ...........................................150

A.6 ANOVA of dispersibility of rehydrated highly concentrated micellar casein at different pH .................................................................150

A.7 ANOVA of suspendability of rehydrated highly concentrated micellar casein at different pH .................................................................150

A.8 ANOVA of solubility of rehydrated highly concentrated micellar casein at different pH .................................................................151

A.9 ANOVA of dispersibility of rehydrated highly concentrated micellar casein with addition of trisodium citrate ...........................................151
A.10 ANOVA of suspendability of rehydrated highly concentrated micellar casein with addition of trisodium citrate ............................................................. 151
A.11 ANOVA of solubility of rehydrated highly concentrated micellar casein with addition of trisodium citrate ............................................................. 151
B.1 ANOVA of rennet coagulation time of recombined concentrated milk at different casein levels ................................................................. 152
B.2 ANOVA of rennet coagulation time of recombined concentrated milk at different coagulation temperatures .............................................. 152
B.3 ANOVA of rennet coagulation time of recombined concentrated milk at different pH levels ................................................................. 152
B.4 ANOVA of initial $G'$ ($G'_0$) of recombined concentrated milk at different coagulation temperatures ......................................................... 152
B.5 ANOVA of initial $G''$ ($G''_0$) of recombined concentrated milk at different coagulation temperatures ......................................................... 153
B.6 ANOVA of $G'$ at rennet coagulation time ($G'_1$) of recombined concentrated milk under different coagulation temperatures ..................... 153
B.7 ANOVA of $G'$ at 1.5 times of rennet coagulation time ($G'_{1,5}$) of recombined concentrated milk under different coagulation temperatures .................................................................................................................. 153
B.8 ANOVA of $G'$ at 2 times of rennet coagulation time ($G'_{2}$) of recombined concentrated milk under different coagulation temperatures .................................................................................................................. 153
B.9 ANOVA of initial $G'$ ($G'_0$) of recombined concentrated milk at different pH .................................................................................................................. 154
B.10 ANOVA of initial $G''$ ($G''_0$) of recombined concentrated milk at different pH .................................................................................................................. 154
B.11 ANOVA of $G'$ at rennet coagulation time ($G'_1$) of recombined concentrated milk under different pH .................................................................................................................. 154
B.12 ANOVA of $G'$ at 1.5 times of rennet coagulation time ($G'_{1,5}$) of recombined concentrated milk under different pH .................................................................................................................. 154
B.13 ANOVA of $G'$ at 2 times of rennet coagulation time ($G'_{2}$) of recombined concentrated milk under different pH .................................................................................................................. 155
B.14 ANOVA of initial G’ (G’\(_0\)) of recombined concentrated milk with addition of different levels of rennet .................................................................155

B.15 ANOVA of initial G” (G”\(_0\)) of recombined concentrated milk with addition of different levels of rennet .................................................................155

B.16 ANOVA of G’ at rennet coagulation time (G’\(_1\)) of recombined concentrated milk with addition of different levels of rennet .........................155

B.17 ANOVA of G’ at 1.5 times of rennet coagulation time (G’\(_{1.5}\)) of recombined concentrated milk with addition of different levels of rennet ........................................................................156

B.18 ANOVA of G’ at 2 times of rennet coagulation time (G’\(_2\)) of recombined concentrated milk with addition of different levels of rennet ........................................................................156

B.19 ANOVA of G’ at 3 times of rennet coagulation time (G’\(_3\)) of recombined concentrated milk with addition of different levels of rennet ........................................................................156
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic diagram of an interlocking lattice model of the casein micelle with casein-calcium phosphate aggregates throughout the entire supramolecule and chains of proteins extending between them. Drawn as cross-sectional scaled views of (A) the complete supramolecule, and (B) a portion of the supramolecule periphery. Calcium phosphate nanoclusters are shown with a diameter of 4.8 nm and approximately 18 nm apart, and caseins are shown with a hydrodynamic diameter of 8 nm. (Reprinted from McMahon and Oommen (2008), with permission from Elsevier.)</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic descriptions of different steps in manufacture of caseinate powder, micellar casein concentrate powder, and milk protein concentrate (MPC) powder. Brackets show material concentrated by vacuum evaporation (Adopted from Schuck (2002)).</td>
</tr>
<tr>
<td>1.3</td>
<td>Effect of chymosin concentration, temperature, pH and Ca(^{2+}) concentration on the rates of chymosin inactivation, splitting of κ-casein (in milk), flocculation of paracasein micelles, and the clotting time of milk. Meant to illustrate trends, (a) at pH 3.5; (b) at pH 7; a blank space implies no or little effect, a broken line a rough estimate; arrows indicate the conditions as often used in making cheese from fresh milk (Reprinted from Walstra, P. et al.,(2014)).</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic diagram of four-vessel, polymeric spiral-wound microfiltration unit.</td>
</tr>
<tr>
<td>3.2</td>
<td>Cold-gelling temperature of micellar casein concentrate (23% protein and lower protein levels upon dilution with water) determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1.0 Hz. (R^2), correlation coefficient. Dash line shows extrapolated trendline.</td>
</tr>
<tr>
<td>3.3</td>
<td>Storage modulus (G’) (open square, left axis) of highly concentrated micellar casein concentrate during temperature cycling (solid line, right axis) at 1°C/min from 50°C to 5°C measured using 0.5% strain applied at 1.0 Hz frequency.</td>
</tr>
<tr>
<td>3.4</td>
<td>Transmission electron micrographs of thin sections of cold-gelled highly concentrated micellar casein concentrate showing casein</td>
</tr>
</tbody>
</table>
micelles (grey-black) of various sizes, including chains of protein extending out from casein micelles (black arrows), some casein micelles that appear aggregated (asterisks), as well as some very small milkfat globules (white arrows).

3.5 Approximate size distribution of protein particles in HC-MCC and water mixture containing 3% protein. Black bars: mixing with low shear for 10 min and subsequent high shear for 1 min at 20°C. Gray bars: mixing with high shear for 1 min and subsequent overnight storage at 4°C. Bars with diagonal strips: mixing with high shear for 1 min at 4°C with addition of 60 mM of trisodium citrate. White bars: mixing with high shear for 1 min at 50°C. Size distribution was calculated as: > 250 μm = 1– dispersibility; 1-250 μm = suspendability – dispersibility; 0.5-1 μm = solubility – suspendability; < 0.5 μm = solubility. Bars=SE, n=3.

3.6 Schematic representation of the spatial arrangement of individual casein micelles in relation to casein concentration. (A) Single casein micelle with corresponding hydration sphere. (B) Casein micelles in milk (~2.6% casein) with ample of free space around the micelles. (C) Casein micelles in MCC (~9% casein) have less space between each but hydration spheres are still separated. (D) Casein micelles in HC-MCC are packed closely with overlapping spheres. Black circles represent colloidal calcium phosphates, grey circle represent individual caseins in casein micelle, and dash line represents surface of hydration sphere of an individual casein micelle.

3.7 Schematic representation of two adjacent casein micelles with overlapping surface casein protuberances which are connected by free calcium-ion bridges. Black circles represent caseins with associated hydrodynamic spheres (grey), and dash line represents surface of casein micelles.

4.1 Transmission electron micrographs of cold-gelled (at ~21°C) recombined concentrated milk (12% casein, casein to fat ratio of 0.8, pH of 7.0) with (B, D, and F) or without (A, C, and E) addition of 0.21 mmol/g casein of calcium chloride (f = fat globules, e = loosely entangled protein, white arrows = chains of protein located between protein stains, asterisks = aggregated casein micelles).

4.2 Transmission electron micrographs of cold-gelled micellar casein concentrate showing casein micelles (grey-black) of various sizes, including chains of protein extending out from casein micelles (black arrows), some casein micelles that appear aggregated.
(asterisks), as well as some very small milkfat globules (white arrows). (Reprinted with permission from Lu et al. (2015)).

4.3 Transmission electron micrographs of thin sections of milk gel coagulating for 30 min after rennet addition at 31°C.

4.4 Transmission electron micrographs of acid milk gels at pH 4.8 formed after acidification of skim milk by glucono-δ-lactone at (A) 40°C, (B) 30°C, (C) 20°C, and (D) 10°C (bar = 1 μm) (Reprinted with permission from McMahon et al. (2009)).

4.5 Cold-gelling temperature (CGT, °C) of recombined concentrated milk (pH 7.0 and protein to fat ratio of 0.8) at different casein levels, determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz.

4.6 Cold-gelling temperature (CGT, °C) of recombined concentrated milk (12% casein, protein to fat ratio of 1.2) at pH 6.6, 6.8, and 7.0, determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz. Dashed line shows extrapolated trend line.

4.7 Cold-gelling temperature (CGT, °C) of recombined concentrated milk (12% casein, casein to fat ratio of 0.8, and pH 6.6) with addition of trisodium citrate (TSC, mmol/g casein) determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz.

4.8 Cold-gelling temperature (CGT, °C) of recombined concentrated milk (12% casein, casein to fat ratio of 0.8, and pH 6.6) with addition of calcium chloride (mmol/g casein) determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz.

4.9 Storage modulus (G’, kPa) of recombined concentrated milk (10% protein, protein to fat ratio of 0.8, pH of 7.0) during temperature cycling (solid line, right axis, °C) with (black open square, left axis) or without (grey open triangle, left axis) addition of 0.08 mmol/g casein of trisodium citrate during temperature cycling (solid line, right axis) at 1°C/min from 50°C to 5°C measured using 0.5% strain applied at 1 Hz.

5.1 Storage modulus (G’, Pa) of rennet gel of recombined concentrated milk (pH 6.4 and protein to fat ratio of 0.8) at different casein levels: 3.2% (□), 5.7% (○), 8.4% (△), and 10.9% (◇), as well as gel of
whole milk (+), coagulated at 31°C, under strain of 0.01, and frequency of 1.0 Hz. Curves represent means of three replications.

5.2 Linear relationship between logarithm of storage modulus of recombined concentrated milk (pH 6.4 and protein to fat ratio of 0.8) at rennet coagulation time (●), at 1.5 times of rennet coagulation time (■), and at 2 times of rennet coagulation time (▲), under different casein levels, strain of 0.01, and frequency of 1.0 Hz.

5.3 Storage modulus (G', Pa) of rennet gel of recombined concentrated milk (11% casein and protein to fat ratio of 0.8) coagulated at different temperatures: 31(Δ), 28 (○), and 25°C (□), as well as gel of whole milk (+) coagulated at 31°C, under strain of 0.01, and frequency of 1.0 Hz. Curves represent means of three replications.

5.4 Storage modulus (G', Pa) of rennet gel of recombined concentrated milk (11% protein and protein to fat ratio of 0.8) at different pH: 6.6 (□), 6.4 (○), and 6.2 (Δ), coagulated at 25°C, as well as gel of whole milk (+) coagulated at 31°C, under strain of 0.01, and frequency of 1.0 Hz. Curves represent means of three replications.

5.5 Linear relationship between inverse of rennet level (1/R, 1/µL) and rennet coagulation temperature (RCT, min) in recombined concentrated milk (11% casein and protein to fat ratio of 0.8) coagulated at 31°C, under strain of 0.01, and frequency of 1.0 Hz.

5.6 Transmission electron micrographs of recombined concentrated milk, glutaraldehyde-fixed at 31°C and agarose-solidified at 22°C (~11% casein, casein to fat ratio of 0.8) (f = fat globules, asterisks = aggregated casein micelles, white arrows = chains of protein located between protein stains, and black arrows = agarose fibers).

5.7 Transmission electron micrographs of rennet coagulum of recombined concentrated milk at ~31°C (~11% casein, casein to fat ratio of 0.8) at rennet coagulation time (A, C, and E) or 3 times of rennet coagulation time (B, D, and F) (f = fat globules).
LIST OF ABBREVIATIONS

ANOVA = Analysis of variance
Casein N/total N = Ratio between casein nitrogen and total nitrogen
CGT = Cold-gelling temperature
DF = Diafiltration
EDTA = Ethylenediaminetetraacetic acid
FDB = Fat on a dry basis
G’ = Storage modulus
G’₀ = Initial storage modulus
G’₁ = Storage modulus at rennet coagulation time
G’₁.₅ = Storage modulus at 1.5 times of rennet coagulation time
G’₂ = Storage modulus at 2 times of rennet coagulation time
G’’ = Loss modulus
G’’₀ = Initial loss modulus
HC-MCC = Highly concentrated - micellar casein concentrate
HS = High shearing speed
HS-O = High shearing speed followed by overnight hydration at 4°C
LS = Low shearing speed
LS-O = Low shearing speed followed by overnight hydration at 4°C
MCC = Micellar casein concentrate
MF = Microfiltration
MNFS = Moisture in the nonfat substance
MPC = Milk protein concentrate
Noncasein N = Noncasein nitrogen
NPN = Nonprotein nitrogen
1/R = Inverse of rennet level
RCM = Recombined concentrated milk
RCT = Rennet coagulation time
S/M = Salt-in-moisture level
SP = Serum protein
TS = Total solids
TSC = Trisodium citrate
Total N = Total nitrogen
TEM = Transmission electron microscopy
UF = Ultrafiltration
CHAPTER 1
GENERAL INTRODUCTION

Bovine milk contains about 3% of protein, out of which 80% is casein with the rest being whey protein. Casein is the main protein in cheese, and also a food ingredient widely used in dairy, bakery, meat, beverage and nutraceutical industries for its functions such as emulsifying, foaming, whipping, water-binding, cheese making, as well as for its texture properties and nutritional values. Microfiltration (MF) of skim milk has been used to concentrate casein micelles since the 1990s. It is easy to reach ~8% casein using MF and up to 95% of serum protein can be removed when combined with diafiltration. Higher casein levels (~20%) can be achieved using MF and diafiltration, followed by vacuum evaporation. A highly concentrated micellar casein concentrate (HC-MCC) has been made at South Dakota State University using this method.

The use of ultrafiltration (UF) to concentrate milk is well established and has benefits of increasing cheese yield and milk processing capacity. However, when making cheese using UF retentate of total solids ≥ 30%, high level of whey protein slows down flavor development during aging, as whey protein inhibits some enzyme activity and reducing proteolysis of the caseins. The milk concentrated by MF such as HC-MCC is potentially more suitable for cheese making compared to UF milk since it contains less whey protein. Therefore, this study focuses on the potential application of HC-MCC for cheese making.

The HC-MCC forms into a gel when it is cooled. Therefore, it is crucial to disrupt the gel structure to fully disperse the casein micelles. Methods were adapted from dairy powder studies to determine dispersibility, suspendability, and solubility of HC-MCC in
water (Chapter 4). Experiments were then performed to identify the effects of agitation, temperature, time, and calcium chelation on solubilization. A rheometer was used to study the effects of protein level on cold gelling temperature of HC-MCC (Chapter 4).

In order to use HC-MCC for cheese manufacture it first must be combined with cream so that it has the necessary casein-to-fat ratio. Such a recombined concentrated milk (RCM) also undergoes cold gelation and this was also studied to determine it did not interfere with cheese making (Chapter 5). This was also studied using a rheometer to determine effects of protein level, pH, and calcium chelation on cold gelling temperature of RCM. The information obtained on solubilization and cold gelation of HC-MCC and RCM was then applied to study rennet coagulation properties of RCM (Chapter 6).

In each of these chapters (4, 5 and 6), samples of HC-MCC or RCM were prepared for study using transmission electron microscopy. Further sample preparation was performed at the University of Utah, with imaging performed by Dr. Almut Vollmer. This microstructural work provided insights into the cold gelation of HC-MCC and RCM, and the rennet coagulation of RCM.
CHAPTER 2
LITERATURE REVIEW

Casein Micelle

General Introduction. Casein is a food ingredient widely used in dairy, bakery, meat, beverage and nutraceutical industries for its functions such as emulsifying, foaming, whipping, water-binding, cheese making, as well as for its texture properties and nutritional values (Fox, 2001, Fox and Kelly, 2004, Séverin and Wenshui, 2005). Bovine milk contains about 3% of protein, out of which 80% is casein with the rest being whey protein. There are four types of casein: $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$-, and $\kappa$-caseins with the approximate ratio of 4:1:3.5:1.5, respectively. In milk, caseins arrange in a colloidal complex form called casein micelle, which consists of casein polymers interlocked by calcium phosphate nanoclusters (McMahon and Oommen, 2013). Casein micelles have an open, porous, and highly hydrated structure with 1 to 8 g water/g of protein (Kumosinski et al., 1988). The size of casein micelle ranges from 20 to 600 nm, with the median size between 100 and 200 nm (Bloomfield and Morr, 1973, McMahon and Oommen, 2008).

Surface Structure of Casein Micelle. Solubilization of casein micelle is highly related to its surface structure. Over 50% of $\kappa$-caseins are present on surface of casein micelle, with its macropetide portion could extend from the micellar surface and form a hairy layer of 5-10 nm presumably (Heth and Swaisgood, 1982, Holt and Horne, 1996). When two casein micelles approaching each other, those hairy layers could cause steric repulsion between casein micelles and prevent their aggregation (Holt and Horne, 1996).
Recently, McMahon and Oommen (2008) proposed an interlocking lattice model of casein micelle, according to which, chains of caseins can extend from core of casein micelle and end with κ-caseins (Figure 1.1). These protein chains were observed with an approximate length of 30 nm and longer than previously assumed 5-10 nm κ-casein hairy layer. Similar protuberances on surface of casein micelle were observed before by scanning electron microscopy (Dalgleish et al., 2004).

**Solubilization of Dairy Proteins**

Solubilization of dairy protein powders generally takes four steps: wetting, sinking, dispersing, and dissolving, which were evaluated by wettability, sinkability, dispersibility, and solubility, respectively (Fang et al., 2007). Wetting is the step that powder particle to overcome surface tension and absorb water. After wetting, water takes place of the gaseous phase surrounding the particles, leading to sinking of particles. Once wet and sank, powder particles would immediately start to disperse into solution as less aggregated particles. Finally, less aggregate particles would separate into individual molecules and dissolve (Fang et al., 2007, Hussain et al., 2012). The four stages happen in the sequence listed above with some overlapping between adjacent steps (Fang et al., 2007).

**Dispersibility and Solubility Measurement.** Most dispersion and solubility tests are mainly based on a similar principle. Firstly, powder of a specific amount is mixed with solvent, and the mixture is sieved or centrifuged. Then either amount of sedimentation on the mesh or protein level in the supernatant of centrifugation was
Figure 1.1 Schematic diagram of an interlocking lattice model of the casein micelle with casein-calcium phosphate aggregates throughout the entire supramolecule and chains of proteins extending between them. Drawn as cross-sectional scaled views of (A) the complete supramolecule, and (B) a portion of the supramolecule periphery. Calcium phosphate nanoclusters are shown with a diameter of 4.8 nm and approximately 18 nm apart, and caseins are shown with a hydrodynamic diameter of 8 nm. (Reprinted from McMahon and Oommen (2008), with permission from Elsevier.)
measured and compared to the amount of powder added for calculation of dispersibility and solubility (Thomas et al., 2004). Protein dispersibility index is commonly used to express solubility of protein, which is calculated as the percentage of protein concentration in the supernatant after powder mixing with solvent and centrifugation under well-defined conditions, compared to total protein level in the mixture (AOCS, 1989, Hall, 1996). Nitrogen solubility index evaluates powder solubility based on percentage of nitrogen content in supernatant compared to total nitrogen level of powder (AOCS, 1989, Hall, 1996). Another widely used indicator of solubility, especially in industry, is solubility index (also referred as insolubility index), which is measured by volume of sediment in terms of milliliters after powder dispersing and centrifugation under well-defined conditions. Thus, solubility is inversely correlated with the value of solubility index. The results from different measuring method are usually hard to compare. Even when using similar methods, various parameters such as protein concentration in dispersion, temperature of solvent, time or speed of centrifugation, are usually different in various studies, resulting in difficulty of comparison (Thomas et al., 2004, Fang et al., 2007).

Recently, static light scattering was applied to evaluate extent of hydration by monitoring changes of particle size and its distribution in the dispersed system using a Malvern master sizer (Regnault et al., 2004, Gaiani et al., 2007, Mimouni et al., 2009, Richard et al., 2013, Chandrapala et al., 2014, Crowley et al., 2015). Change of turbidity, measured by a turbidity sensor, is also an indicator of rehydration extent (Gaiani et al., 2007). Based on results from these tests, complete solubilization was defined as the state
of hydration when particle size distribution fell into a defined size range or turbidity in
dispersion was stable (Regnault et al., 2004, Gaiani et al., 2005, Gaiani et al., 2007,
Mimouni et al., 2009). However, these measurements may cause biased results because
particle size measurement using static light scattering requires dilution of samples, and
stable turbidity does not reflect particle size or the disappearance of aggregates.
Microstructure of dispersed powders was observed by scanning electron microscopy or
transmission electron microscopy to better understand solubilization process and factors
affecting solubility (Oommen, 2004, Gaiani et al., 2007, Mimouni et al., 2009, Mimouni
et al., 2010a, Hussain et al., 2011a)

Dairy Proteins. Various forms of caseins or casein-dominant products are
available, such as sodium caseinate, calcium caseinate, milk protein concentrates, and
micellar casein concentrate (McCarthy et al., 2014) (Table 1.1). Manufacturing processes
of these products are shown in Figure 1.2. These dairy proteins are typically spray dried
for convenience of storage and transportation. Complete solubilization of these functional
proteins is desirable for their usage as food ingredients in liquid food systems.
Caseinates are defined as acid-precipitated casein that dissolved in alkali and followed by
spray drying into powders (Walstra et al., 2014). The appearance and viscosity of calcium
and sodium caseinate solutions are different. Calcium caseinate dispersion has a white
and opaque color, and relatively lower viscosity than other caseinates, whereas sodium
caseinate solution is translucent with a straw-like color, and more viscous than calcium
caseinate (Southward, 1985).
Table 1.1 Manufactural process, composition, and state of casein micelle of caseinate, milk protein concentrate, micellar casein concentrate, and highly concentrated-micellar casein concentrate.

<table>
<thead>
<tr>
<th>Dairy protein products</th>
<th>Manufactural process</th>
<th>Composition</th>
<th>State of casein micelles</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Caseinate</td>
<td>Isoelectric precipitation, alkali neutralization, and spray drying</td>
<td>Caseinate has weight percentage of total solids ~96%, casein ~91%, ash ~4%, and fat ~1%.</td>
<td>Casein micelle structure has changed substantially, by dissociation or binding with excessive metal ions.</td>
<td>Huffman and James Harper, 1999, Southward, 1985</td>
</tr>
<tr>
<td>Milk Protein Concentrate (MPC)</td>
<td>Ultrafiltration, diafiltration, and spray drying</td>
<td>MPC powder consists of ~95% total solids, 1% to 2% of fat, and 7% to 8% of ash. Based on different degree of ultrafiltration and diafiltration, MPC powder has different protein level from 35% to 86%, and lactose from 0.4% to 50%. The ratio of caseins to whey proteins are the same in MPC as in milk, of about 4 to 1.</td>
<td>native without substantial change</td>
<td>Morr and Foegeding, 1990, Zwijgers, 1992, Huffman and James Harper, 1999, Augustin et al., 2011, Crowley et al., 2015</td>
</tr>
<tr>
<td>Micellar Casein Concentrate (MCC)</td>
<td>Microfiltration, diafiltration, optional spray drying</td>
<td>MCC typically has weight percentage of total solids from 7% to 14%, casein from 5% to 9%, and whey protein less than 1%. It can also be spray-dried into powder, with over 95% of total solids and 86% of total protein.</td>
<td>native without substantial change</td>
<td>Gaiani et al., 2005, Nelson and Barbano, 2005, Beckman et al., 2010, Hurt et al., 2010, Beckman and Barbano, 2013, Zulewska and Barbano, 2013</td>
</tr>
<tr>
<td>Highly Concentrated - Micellar Casein Concentrate (HC-MCC)</td>
<td>Microfiltration, diafiltration, combined with pre-acidification, gravity separation, or vacuum evaporation</td>
<td>HC-MCC has weight percentage of total solids from 22 to 28%, total protein from 18% to 20%, casein ~18%, and whey ~2%.</td>
<td>native without substantial change</td>
<td>Jost et al., 1999, Amelia and Barbano, 2013, Metzger and McMahon, 2013</td>
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Figure 1.2 Schematic descriptions of different steps in manufacture of caseinate powder, micellar casein concentrate powder, and milk protein concentrate (MPC) powder. Brackets show material concentrated by vacuum evaporation (Adopted from Schuck (2002)).
During manufacturing of caseinate, steps of precipitation or coagulation of milk have irreversibly changed native structure of casein micelles (Farrell Jr et al., 1988, Oommen, 2004, McMahon and Oommen, 2013). No colloidal structure except small strains or aggregates of proteins were found in hydrated sodium caseinate (Oommen, 2004, McMahon and Oommen, 2013). In completely hydrated calcium caseinate, colloidal casein was observed to be more electron dense and darker after staining by heavy metals, indicating a more concentrated protein structure with more calcium binding compared to native casein micelle (Oommen, 2004, McMahon and Oommen, 2013). Aggregated casein particles were observed in calcium and sodium caseinate dispersion mixing for 4h using low shear, indicating incomplete solubilization of casein micelles (Oommen, 2004). Full solubilization of caseinate powder into a typical size range may take 6h with moderate agitation (Moughal et al., 2000). High shear is usually more effective than low shear in reducing particle size and rehydration time during solubilization of dairy powder (Hixson and Crowell, 1931b, Oommen, 2004, Bock et al., 2008, Jeantet et al., 2010, Richard et al., 2013, Chandrapala et al., 2014). More aggregates with larger sizes were observed in sodium caseinate dispersion hydrated using low shear for 10h than using high shear for 10 min followed by hydration for 1h (Oommen, 2004).

Milk protein concentrate (MPC) is manufactured by UF and DF of skim milk followed by spray drying into powder. Depending on the removal rate of lactose and salts by DF, MPC has a range of protein levels from 35 to 90%. Solubilization of MPC is generally poor, and complete solubilization of dry milk proteins takes 6 to 10h with low-speed shearing. (Moughal et al., 2000, Oommen, 2004, Gaiani et al., 2005, Mimouni et
al., 2009). Wetting of MPC powder is fast, whereas dispersing of casein micelles from MPC particles is very slow, the latter of which is the rate-limiting step in MPC solubilization (Mimouni et al., 2010b).

Mimouni et al. (2009) monitored the rehydration process of MPC85 powder using static light scattering, and hypothesized the process consisted of two overlapping stages: breaking-down of agglomerated particles (60 to 500 µm) into primary particles with an average size of 35 µm, and dissolving of primary particles into particles of size smaller than 1.6 µm. The second stage was slower and accelerated by increasing hydration temperature. Actually, this is not the end of hydration and a third step is needed for these particles to release the individual casein micelles that have an average size of 0.1 to 0.2 µm.

Reduced solubilization of dairy powders was associated with heat treatment during drying process, protein and mineral levels, or storage condition (Baldwin and Truong, 2007, Mimouni et al., 2010a, Sikand et al., 2011). Mimouni et al. (2010a) compared field emission scanning electron micrographs of rehydrated fresh and 2-month aged MPC and observed a more compact structure, as well as the presence of an extra layer skin of casein micelles on the surface of aged MPC particles. These structural changes were suspected of delaying solubilization of stored MPC.

From the 1990s, concentration of skim milk using microfiltration produces a novel dairy product that has been called micellar casein concentrate (MCC) (Saboyainsta and Maubois, 2000), also known as native phosphocaseinate (Pierre et al., 1992). This product has two features that distinguishing it from other dairy proteins. Firstly, casein micelles are still in original state as in milk without substantial change. Secondly, only
micelle casein is concentrated, whereas whey proteins are removed. The name of micellar casein concentrate is preferred as it describes both features, whereas caseinate is defined as acid casein curd dissolved in alkali (Fox, 2003, Walstra et al., 2014).

Micellar casein concentrate can be spray-dried into powder containing over 90% of total solids and over 80% of total protein, with low levels of lactose, ash and whey protein (Schuck et al., 2002, Gaiani et al., 2005). It is often used as a model of casein micelles because of its high level of micellar casein and extremely low level of whey protein (Famelart et al., 1999, de Kort et al., 2011, Ye and Harte, 2013). The MCC solution exhibits better rennet coagulation properties by more than half reduction in coagulation time and ~57% increase in gel strength than milk, as well as ability to coagulation even after high heat treatment at 100°C (Pierre et al., 1992).

Micellar casein is very heat stable and coagulate after heating at 140°C for 15 to 20 min at pH of 6.7, whereas whey protein is heat sensitive and denatures at temperatures over 70°C (Fox, 2003). When heated over 70°C, whey protein is denatured and bound covalently to κ-casein of casein micelle (Walstra et al., 2014). Decreased ash content of ultrafiltrated milk concentrate was associated with higher heat stability at 140°C (Hinrichs, 2000). Consequently, MCC may have increased heat stability than MPC because of low levels of whey protein and ash. Moreover, low level of lactose may improve the storage stability of MCC by limiting maillard browning (O'brien, 1995).

However, poor solubility and prolonged rehydration time of MCC limits its usage as a food ingredient in industry (Schuck et al., 1999, Gaiani et al., 2005, Gaiani et al., 2007). Schuck et al. (1999) reported a solubility index of 15 mL of MCC at 24°C, indicating an extremely low solubility compared to low heat powder with SI < 0.5 mL.
Gaiani et al. (2007) observed that only after 9 to 13h of mixing using low shear, stable particle size of ~ 360 nm and stable turbidity were reached in MCC dispersion. The MCC powder has a fast wetting stage, whereas the dispersing stage is very slow, resulting in a long rehydration time (Gaiani et al., 2005, Hussain et al., 2011b).

Recently, several studies were carried out to improve solubility of MCC by addition of different agents including mineral salts, skim milk ultrafiltrate, sodium caseinate, whey, or calcium chelators (Schuck et al., 2002, Gaiani et al., 2005, Gaiani et al., 2007, Beckman et al., 2010, Hussain et al., 2011b, Schokker et al., 2011). However, micellar casein structure could change or dissociation of casein micelles could occur under various ionic environments (Schuck, 2002, Schucka et al., 2007). Instead, addition of CaCl₂, or application of agglomeration could increase rehydration time and retard the solubilization process (Gaiani et al., 2007).

**Ways to Improve Solubilization of Dairy Proteins**

*Temperature, Time, and Shearing.* Increasing hydration temperature, time and shearing are commonly used to increase solubility of dairy proteins in industry. Increasing mixing temperature and time were associated with decrease in amount of sediment or rehydration time of dairy powders (Hixson and Crowell, 1931a, Mimouni et al., 2009, Jeantet et al., 2010, Schokker et al., 2011, Richard et al., 2013, Crowley et al., 2015). Increase in solvent temperature accelerates the releasing of material from powder particle into solution, which appears to be the rate-limiting step in solubilization of MPC (Mimouni et al., 2009).

Increasing shearing rate was associated with reduced rehydration time or smaller particle sizes of dairy powder, presumably by physically breaking particles apart,
increasing turbulence, accelerating mass transfer and releasing of casein micelles into solution (Hixson and Crowell, 1931b, Oommen, 2004, Bock et al., 2008, Jeantet et al., 2010, Richard et al., 2013, Chandrapala et al., 2014). Jeantet et al. (2010) found that increase of mixing temperature of 4°C is as effective as doubling stirring rate in MCC rehydration, indicating rehydration is more sensitive to mixing temperature than shearing speed. This result agrees with Richard et al. (2013), who also found that constant agitator revolutions were associated with the same level of rehydration regardless of stirring speed. Usage of ultrasonication and high-pressure homogenization were reported to improve solubilization of dairy powder (Chandrapala et al., 2014), but their effect on structure and function of dairy proteins was not fully elucidated.

**Ionic Strength.** Generally, addition of NaCl (≤ 0.4 M) to MCC before spray drying or addition to MCC powder during rehydration increases water hydration and reduces rehydration time of casein suspension (Famelart et al., 1999, Schuck et al., 2002, Schucka et al., 2007). It is known that addition of NaCl was associated with increased hydration ability of protein, such as in renneted milk (Creamer, 1985). Moreover, improved hydration of MCC powder by NaCl addition was correlated with increasing solubilization of calcium and phosphate from casein micelles, implying dissociation of casein micelles in the dispersion (Famelart et al., 1999). Hussain et al. (2011a) observed casein micelle dispersion with addition of 0-12% NaCl under transmission electron microscopy and found that casein micelle of spherical shape with size ranging from 100 to 200 nm changed into lumpy and disintegrated protein aggregates with size ~ 20 nm. It implies dissociation of casein micelle into protein aggregates, which is similar to small protein particles with size from 2 to 5 nm in rehydrated sodium caseinate solution.
Changes in secondary structure of casein micelles upon addition of NaCl up to 6% were also reported (Hussain et al., 2011a, Hussain et al., 2012).

Addition of calcium-chelating agents to MCC either before spray drying or during powder hydration results in significantly increased hydration rate and reduced reconstitution time, which were probably caused by solubilization of colloidal calcium phosphate and destruction of casein micelle structure (Schuck et al., 2002, Schokker et al., 2011). De Kort et al. (2011) added different calcium chelators to a complete solubilized micellar casein concentrate solution, and detected increased viscosity, voluminosity, and decreased turbidity. These observations were attributed to releasing of calcium and phosphate from casein micelles, as well as swelling and dissociation of casein micelles. Similar results were also found in MPC solution (Kaliappan and Lucey, 2011).

On the other hand, addition of CaCl₂ causes little or negative effect on MCC hydration (Famelart et al., 1999, Schuck et al., 2002). Schuck et al. (2002) added 0.22M of CaCl₂ to MCC either before spray drying or during MCC powder hydration causes binding of calcium to casein micelles, formation of insoluble aggregates, resulting in increasing amount of sediment in MCC dispersion. However, addition of CaCl₂ at a much lower level of 0.12 M did not significantly affect MCC powder hydration (Famelart et al., 1999). Currently, no method is available for increasing solubility of MCC while still maintaining concentrated and unmodified casein micelles without calcium depletion or introduction of other substances.

**Presence of other components.** Addition of UF filtrate, whey protein isolate, or sodium caseinate to MCC before spray drying greatly increased solubility and reduced
rehydration time of MCC powder (Gaiani et al., 2005, Gaiani et al., 2007, Schokker et al., 2011). Schokker et al. (2011) reported ~20% increase in micellar casein powder after mixing MCC with 1.5% of sodium caseinate before diafiltration and spray drying. The increased solubilization was attributed to 2 possible mechanisms both resulting in fewer chances of casein micelle aggregation: decreased number of casein micelles absorbed to air-water interface because of favorable attachment of non-micellar casein to the latter; or increased distance between casein micelles caused by non-micellar casein in-between.

Gaiani et al. (2005) monitored turbidity and static light scattering of rehydrated casein powder and detected more than 93% reduction in rehydration time when UF filtrate was mixed with micellar casein before spray drying. Presumably, UF filtrate rehydrated faster and followed by solubilization of casein micelle. No improvement in rehydration time of micellar casein powder if UF filtrate powder was mixed with casein powder after spray drying or casein powder was resuspended in UF filtrate. Similar results in reduction of rehydration time were found when mixing MCC and whey protein isolate before drying (Gaiani et al., 2007).

**Rennet Coagulation of Milk**

Rennet enzymes specifically hydrolyze the peptide bond between amino acid 105 phenylalanine and 106 methionine of κ-casein in casein micelles into two parts: para-κ-casein (amino acids 1-105) and macropeptide (amino acids 106-169). The macropeptide is negatively charged, hydrophilic, and dissolved in whey during cheese manufacture, whereas para-κ-casein is positively charged, hydrophobic, and starts to aggregate into clusters, eventually forming a protein gel at a temperature over 5°C.
Generally, rennet curd formation includes following steps. Firstly, $\kappa$-casein is hydrolyzed and casein micelles are destabilized. Then macropeptide aggregates into clusters. These clusters gradually fused into a three-dimensional gel network. At the same time, fat molecules are trapped, whereas whey is expelled from the network. These steps may happen overlapping or even simultaneously.

Rennet coagulation strongly affects cheese quality and yield in cheese making. Therefore, understanding effect of factors such as pH, calcium, temperature, rennet level, and protein concentration to coagulation process is important.

**Effect of pH.** Rennet coagulation time is defined as the time taken from addition of rennet to the appearance of flocculation in milk. Optimum pH for rennet to hydrolyze $\kappa$-casein is $\sim 5.3$, whereas, at pH higher or lower than this value, speed of rennet coagulation is decreasing (Humme, 1972, Kowalchyk and Olson, 1977, Visser et al., 1980, Carlson et al., 1986). Reduction of pH from 6.8 to 5.6 accelerated the aggregation of destabilized casein micelle after hydrolysis of $\kappa$-casein and decreased coagulation time (Cheryan et al., 1975, Nájera et al., 2003). Decreasing pH from 6.8 to 6.0 was also associated with increased curd firming rate (Kowalchyk and Olson, 1977, Daviau et al., 2000, Nájera et al., 2003). On the other hand, reducing pH of milk also causes solubilization of colloidal calcium phosphate and increasing Ca$^{2+}$ level, both of which are associated with increasing coagulation time (Shalabi and Fox, 1982).

**Effect of Temperature.** Increasing temperature is correlated with decreasing coagulation time and increasing rate of gel firmness (Cheryan et al., 1975, McMahon and Brown, 1984a, Carlson et al., 1986, Nájera et al., 2003). At higher temperature, both the rate of $\kappa$-casein hydrolysis and gel aggregation accelerate, with the latter of which having
a larger effect (McMahon and Brown, 1984a, Carlson et al., 1986). Temperature coefficient ($Q_{10}$) represents the number of times a reaction rate changes when temperature is increased by 10°C. Between 20 and 50°C, casein micelle aggregation step has a $Q_{10}$ value from 11 to 12, whereas hydrolysis of κ-casein only has $Q_{10}$ of 2 (Cheryan et al., 1975). In occurrence of κ-casein hydrolysis, milk does not coagulate at temperature < 8°C, whereas resumes coagulation on subsequent warming. Approximately 65% hydrolysis of κ-casein is required for milk aggregation at 35°C, whereas 95% hydrolysis is needed at 12°C (Carlson et al., 1986). The strong effect of temperature on aggregation may attribute to hydrophobic interaction, which increases and accelerates coagulation at higher temperature (Kowalchyk and Olson, 1977, McMahon and Brown, 1984a). Another explanation is that higher temperature, reduced calcium solubility and increased level of colloidal calcium phosphate, which may accelerate gel coagulation (Carlson et al., 1986).

**Effect of Calcium.** Generally, addition of calcium is correlated with reduced coagulation time and increased curd firming rate (McMahon et al., 1984, Carlson et al., 1986, Nájera et al., 2003). McMahon et al. (1984) measured rennet coagulation time using the formagraph, which evaluates extent of milk coagulation by measuring movement of small pendulums immersing in linearly oscillating rennet milk. They found that at the level of 50mM calcium, curd coagulation time reached the minimum, and at level of 10mM curd firming rate reached the maximum. Moreover, at high level of calcium (~400mM), curd coagulation was delayed with a weak body. Similarly, Udabage et al. (2001) also reported an optimum calcium addition level of 10 mM for maximum curd firming rate by dynamic rheology, which reflects the transition of milk gel from a viscous state to a viscoelastic state by measuring the gel response under an oscillatory
Figure 1.3 Effect of chymosin concentration, temperature, pH and Ca\textsuperscript{2+} concentration on the rates of chymosin inactivation, splitting of κ-casein (in milk), flocculation of paracasein micelles, and the clotting time of milk. Meant to illustrate trends, (a) at pH 3.5; (b) at pH 7; a blank space implies no or little effect, a broken line a rough estimate; arrows indicate the conditions as often used in making cheese from fresh milk (Reprinted from Walstra, P. et al., (2014)).
shear strain.

Initially, reduction of milk coagulation time was attributed solely to increased colloidal calcium phosphate, which is insoluble calcium interlocked in casein micelle for stability. Decreasing pH of milk using mixture of trisodium citrate and citric acid increased rennet coagulation time of milk by decreasing insoluble calcium while maintaining constant Ca\(^{2+}\) concentration (Pearce, 1976, Shalabi and Fox, 1982). Later, Shalabi and Fox (1982) pointed out that increasing Ca\(^{2+}\) concentration could offset the impact of reduction in colloidal calcium phosphate and found decreased coagulation time in direct-acidified milk with non-chelating acids. Therefore, increased milk coagulation rate can be attributed to increasing level of either Ca\(^{2+}\) or colloidal calcium phosphate. Addition of CaCl\(_2\) (up to 0.02% (wt/wt)) to cheese milk is permitted in the US, for reduction in amount of rennet needed, and thus, lower production cost of cheese making (McMahon et al., 1984, FDA-DHHS, 2012).

**Effect of Rennet Level.** Generally, increasing rennet level is associated with higher level of hydrolysis of \(\kappa\)-casein, and therefore, decreasing coagulation time (Foltmann, 1959, McMahon and Brown, 1983, Brown and Collinge, 1986, Nájera et al., 2003). Linear relationship between inverse of rennet level and milk coagulation time was first suggested by Storch and Segelcke (1874) that the product of coagulation time and rennet concentration is a constant. Foltmann (1959) rearranged the equation:

\[
CT = \frac{k}{E} + A
\]

where CT is rennet coagulation time, E is rennet concentration, k and A being constants. The constant A was added to account for other variations for fitting a wider range of rennet concentrations (Holter, 1932). McMahon and Brown (1983) measured rennet
coagulation time at 35°C using the formagraph, and found that coagulation time (min) and rennet activity (RU/mL) fitting Foltmann’s equation (1):

\[ CT = 0.0754/E + 0.685 \]  

They noted that this linear relationship is affected by milk composition. Additionally, using different methods to measure coagulation time may reach different values of k and A. Similar linear relationship was obtained by measuring coagulation time using a spectrophotometer (Brown and Collinge, 1986).

**Effect of Protein Concentration.** Generally, increasing protein level in concentrated or ultrafiltrated milk causes increasing hydrolysis rate of κ-casein, curd firming rate and maximum curd firmness, as well as decreasing coagulation time and percentage of κ-casein being hydrolyzed (Dalgleish, 1980, Garnot and Corre, 1980, Dalgleish, 1981, Guinee et al., 1996, Daviau et al., 2000, Sandra et al., 2011). At low concentration factor, coagulation time is dominated by casein aggregation step, whereas at high concentration factor, rapid increase in aggregation results in enzymatic hydrolysis of κ-casein as the rate limiting step (Dalgleish, 1980, Orme, 1998). Orme (1998) manufactured cheese using 5× UF concentrate and observed that the rate of casein aggregation increased by 25 times at pH of 6.7. He also found that increasing UF concentration is associated with reduced percentage of hydrolyzed κ-casein and formation of small clusters with less reactivity, resulting in reduced whey expulsion from curd and rough texture of cheese.

**Microfiltration of Milk**

Microfiltration (MF) technology is a membrane separation process based on different size and shape of components. It has been used for bacteria and spores removal
and fractionation of milk fat in dairy industry for about 30 years attribute to the development of ceramic membrane and uniform transmembrane pressure process (Sandblom, 1978, Saboyainsta and Maubois, 2000). Since the 1990s, MF started to be used for casein micelle separation from milk (Saboyainsta and Maubois, 2000, Pouliot, 2008).

Most research was carried out using ceramic membrane with pore size diameters from 0.1 to 0.2 μm (Marella et al., 2013, Zulewska and Barbano, 2014). Microfiltration using ceramic membrane with multiple stages and maintaining uniform transmembrane pressure can lead to more than 95% removal of serum protein (SP, also referred as whey protein) (Fox, 2003), and produce a micellar casein concentrate of about 9% (wt/wt) of casein, 1.4% of SP and 0.4% of lactose (Hurt et al., 2010).

Since the 1990s, polymeric spiral-wound membranes attracts more attention because of inexpensive of membrane material and process operation (Cheryan, 1998). However, low rate of SP removal and low permeate flux limited its usage in MF in dairy industry (Beckman and Barbano, 2013). Diafiltration (DF), the process of diluting MF retentate using water followed by further concentration, was used to increase removal of SP, lactose, and control membrane polarization during MF (Govindasamy-Lucey et al., 2007, Marella et al., 2013). The level of 200% DF at 15 psi was reported to increase SP removal rate from 35% to 85% during spiral-wound MF of skim milk (Marella et al., 2013). Applying a 3-stage 3×concentration MF using a 0.3 μm spiral-wound membrane at 50°C and combined with DF can remove about 70% of SP (Beckman et al., 2010). However, high process temperature (i.e., 50°C) in this experiment may increase energy expenses, SP denaturation, and bacterial contamination. At the temperature of 18°C,
using base and boost pressure of 5 and 15 psi in MF operation with polymeric spiral-wound membrane was reported to maximum efficiency of SP removal (Marella et al., 2013). Low operating temperature less than 7°C was also used in MF of skim milk using polymeric spiral-wound membrane (Govindasamy-Lucey et al., 2007). Low efficiency of whey protein removal was also attributed to fouling of polymeric membrane, with casein micelle being the predominant foulant (Beckman et al., 2010, Zulewska and Barbano, 2013).

Micellar casein concentrate manufactured using MF typically contain 7 to 10% casein with up to 3× to 4× concentration (St-Gelais et al., 1995, Jost et al., 1999, Nelson and Barbano, 2005, Beckman et al., 2010, Hurt et al., 2010, Amelia et al., 2013, Beckman and Barbano, 2013, Hurt et al., 2014). Higher concentrations of 7× to 8× are achievable when milk is acidified (Brandsma and Rizvi, 1999, Brandsma and Rizvi, 2001) or if concentrated further using gravity separation or vacuum evaporation (Amelia and Barbano, 2013, Metzger and McMahon, 2013). Such highly concentrated MCC (HC-MCC) has many potential applications in food industry and offers many potential advantages over caseinate or MPC. The HC-MCC (1) is still in native casein micelle structure, which is closer to its structure in milk (Saboyainsta and Maubois, 2000); (2) has low levels of whey protein and lactose, may lead to improved heat stability or storage stability by reducing binding of denatured whey protein to casein and maillard browning; (3) is still hydrated with water, therefore may show improved rehydration ability than dried dairy protein ingredients such as caseinate, MPC, or MCC powder; (4) can be used in various forms such as refrigeration, freezing, or drying (Schokker et al., 2011, Sauer et
The bacteria count of HC-MCC stayed under 20,000 cfu/mL for 16 wk at 4°C (Amelia and Barbano, 2013).

The HC-MCC can be used as a protein-fortification ingredient in UHT or retort liquid food system of neutral pH (Sauer and Moraru, 2012), or for making cheese with no standard of identity in the US (Govindasamy-Lucey et al., 2007). Currently, US Food and Drug Administration permitted the use of UF retentate in manufacture of standardized cheeses, although use of MF retentate has not been approved (Cessna, 2004, FDA-DHHS, 2012). The HC-MCC is very suitable for cheese milk standardization because of its high casein content (91.5% wt/wt). Using cold MF retentate in standardization of cheese milk produced cheese with higher yield without sacrifice in quality (Govindasamy-Lucey et al., 2007). Manufacture of low-fat cheddar cheese and Greek-style yogurt using micellar casein concentrate has also been studied (Amelia et al., 2013, Bong and Moraru, 2014).

Besides HC-MCC, MF of skim milk can produce whey protein concentrate from permeate. Over the last decade, price of whey protein concentrate tripled, probably caused by constant cheese production whereas increasing demand of whey protein (USDA, 2014, 2015). In composition, a protein concentrate (80%) prepared from the permeate of microfiltered milk has less fat and none of glycomacropeptides compared to a protein concentrated from cheese whey (Evans et al., 2009, Evans et al., 2010). In functionality, milk-derived whey protein is considered to have stronger foaming ability, gel strength, solubility, and emulsifying ability (Burrington, 2013). Whey protein produced by MF also has a clean flavor and clear appearance in solution, leading to its potential usage in clear beverages (Heino et al., 2007, Burrington, 2013). Therefore,
microfiltration of skim milk can increase production of whey protein to satisfy a fast growing market.

**Cheese Made from Ultrafiltrated or Microfiltrated Milk**

Ultrafiltration (UF) is widely used in dairy industry for production of whey protein concentrate. The UF retentate of skim milk with low concentration levels of 1.2 to 1.5× is used to increase cheese yield without requiring specialized equipment or large changes in cheese making procedures (Govindasamy-Lucey et al., 2004). Diafiltration and pre-acidification of milk were combined with UF to reduce lactose and milk minerals to normal level in UF cheese (Ernstrom et al., 1980, Sutherland and Jameson, 1981). Cheddar cheese made from UF milk retentate over 2× to 4× was reported to have a higher pH and moisture level, fast curd formation, reduced fat recovery, less aggregated protein network, lumpy texture, and lack of Cheddar flavor (Green et al., 1981).

Ernstrom et al. (1980) blended cheese base made from fermented and diafiltered 5×UF whole milk retentate with aged Cheddar cheese, and produced process cheese food of satisfying flavor and quality. Cheese base, made from fermented UF retentate, shared same pH and gross composition with Cheddar cheese, with satisfying flavor and stability and 16 to 18% higher yield than expected. However, cheese base does not have characteristic cheese body and texture and is used only for manufacture of processed cheese.

Creamer et al. (1987) manufactured Cheddar cheese from 5× whole milk UF concentrate or whole milk (control). Higher pH and calcium level were found in UF cheese, whereas no significant difference in fat on a dry basis (FDB), moisture in the nonfat substance (MNFS), or salt-in-moisture level (S/M). Increased smoothness and
decreased crumbliness were also observed in UF cheese. Cheese made from UF milk had
3 times of residual rennet concentration, yet similar ripening rate compared to control. It
indicates that UF cheese would probably ripen slower than normal cheese if retaining the
same rennet activity. It was suspected that increasing level of whey protein in UF cheese
may inhibit rennet activity, resulting in slow ripening of cheese. Lelievre et al. (1990)
further confirmed this assumption and found this inhibition effect on casein coagulation
and hydrolysis was more pronounced in high-molar-mass whey protein fraction, which
mainly consisted of immunoglobulins.

Microfiltration addressed the problem of UF milk in cheese making by allowing
whey protein passing through the membrane, and only concentrating casein micelles
without substantial change. Limited research has been done using MF retentate (i.e.,
micellar casein concentrate) to make Cheddar (St-Gelais et al., 1995, Neocleous et al.,
2002a, b), Mozzarella (Garem et al., 2000, Brandsma and Rizvi, 2001), or cheese without
standard of identity, such as pizza cheese (Govindasamy-Lucey et al., 2007). Cheese
made from MF retentate typically has lower moisture level, higher pH and total calcium
level, increased hardness, and reduced proteolysis during aging (St-Gelais et al., 1995,
Brandsma and Rizvi, 1999, Neocleous et al., 2002b, Govindasamy-Lucey et al., 2007).

St-Gelais et al. (1995) made Cheddar cheese from mixture of cream and 1.22×,
1.43×, 1.66× MF concentrates, or skim milk (1×, control) with constant protein to fat
ratio. As concentrating factor increasing, calcium, protein and pH level in cheese
significantly increased, whereas moisture, MNFS, and FDB level significantly reduced.
Higher protein recovery and cheese yields were found in cheeses made from MF retentate
of higher concentrating factors, whereas no significant difference in fat recovery.
Increase in hardness and stickiness, as well as reduced springiness and proteolysis, were observed in MF cheese with increasing concentrating factor during aging of 12 wk.

Brandsma and Rizvi (1999, 2001) modified cheese-making procedure and utilized 7× skim milk MF retentate and butter oil to produce low moisture part skim Mozzarella Cheese of composition fitting legal and commercial range. To achieve effective acidification and reduce total calcium level in MF cheese, skim milk MF retentate was pre-acidified to pH 6.0 using glucono-δ-lactone. Decreased cutting time of 15 to 20 min after coagulation, single-strength rennet addition of 80 to 100 µL/kg MF cheese milk, and coagulation temperature of 32 to 36°C during cheese-making process were suggested to optimize cheese quality.

Neocleous et al. (2002a, b) manufactured Cheddar cheese by combining cream with 1×, 1.26×, 1.51×, or 1.82× MF at a constant casein-to-fat ratio. As concentrating factor increasing, protein, calcium level, and calcium as a percentage of protein in cheese increased, moisture level and MNFS decreased, whereas no significant difference on FDB, pH, salt, or S/M. Both actual yield and salt and moisture adjusted yield increased with higher concentrating factors, whereas no significant difference in cheese yield efficiency (i.e., percentage of composition adjusted yield over theoretical yield). It indicates that cheese making using MF retentate can process larger amount of cheese per day using equipment of original capacity, given that more milk is purchased. Cheeses were aged 6 months and tested proteolysis, hardness, and flavor. Decreased proteolysis and cheese flavor, and increased hardness were observed in aged cheese with higher concentrating factor. Another trial of Cheddar cheese was made from skim milk (1×, control) and 1.8× MF retentate, the latter of which using modified procedure including
adding rennet at higher temperature and higher level (compared to the first trial), adding rennet at a set pH based on control, reducing cooking time and temperature gradient. Result MF cheese had similar composition as control except higher calcium level. No significant difference in proteolysis or hardness during 6-month aging, or in taste by a triangle test after aging. Interestingly, hardness of control and MF cheese were both increased compared to the first trial, indicating no actual improvement after modifying cheese making procedure.

Govindasamy-Lucey et al. (2007) standardized 4.6× MF retentate with whole milk to casein to fat ratio of 1.0 to manufacture pizza cheese. Cheese made from MF retentate had 2 to 3% less moisture content compared to control cheese made from part-skim milk. Increased fat recovery and satisfying cheese quality was obtained in MF standardized cheese after modifying cheese-making procedures, including pre-acidifying cheese milk to pH of 6.3, reducing setting temperature from 34 to 31°C, increasing curd sizes, and decreasing washing temperature from 26 to 24°C.

**Rheological Properties of Food Gels**

Rheology investigates how material responds to applied forces or deformation. Oscillatory testing is often used to monitor viscoelastic properties in various food applications, such as gel strength evaluation, protein coagulation or denaturation, acid or rennet milk coagulation, cheese texture and melting properties (Tunick, 2000). In an oscillatory test, the sample is placed between two parallel plates and subjected to a harmonically varying stress or strain. Food gels are viscoelastic fluids that exhibit both
fluid-like and solid-like properties simultaneously (Tabilo-Munizaga and Barbosa-Cánovas, 2005).

Within the linear viscoelastic range, time development of storage modulus (G’), loss modulus (G’’), and shift angle (\( \delta \)) can be monitored under different conditions. The G’ value measures energy stored in the sample during deformation, indicating the elastic (solid-like) behavior. The G’’ value measures energy lost during deformation, indicating viscous (liquid-like) behavior. In food systems, G’ is often used to imply gel strength, whereas G’’ is an indicator of viscosity in liquid (Tabilo-Munizaga and Barbosa-Cánovas, 2005). The slope of the G’ curve over time implies the rate of change in gel firmness (Sandra et al., 2011).

Measurement of G’ and G’’ to follow gelation during acid and rennet coagulation of milk and UF retentate, with coagulation time being designated as the time when G’ equals G’’ (Kristo et al., 2011, Sandra et al., 2011). The same approach has been used to study gelling temperature for whey protein isolate gel (Puyol et al., 2001). Other researchers have used the time when G’ increases appreciably as a measure of gelling temperature (Gezimati et al., 1997, Jorge et al., 2014). Temperature reversibility of viscoelastic gel can be determined based on similarity of moduli change in gel samples when subjected to thermal cooling and heating cycles (Chen and Dickinson, 2000)

References


CHAPTER 3

HYPOTHESIS AND OBJECTIVES

I hypothesize that highly concentrated – micellar casein concentrate (HC-MCC) is potential for use in cheese making through understanding of its solubilization, cold gelation, and rennet coagulation properties.

Efforts to explore this hypothesis focused on the following objectives:

**Objective 1.** Determine how to convert cold-gelled HC-MCC into a soluble casein micelle dispersion as a function of temperature, time, shear, pH, calcium chelation, and protein level.

**Objective 2.** Determine the protein level to which cold-gelled HC-MCC can be recombined with cream so as to be suitable for cheese manufacture.

**Objective 3.** Determine the rennet coagulation properties of recombined HC-MCC and cream as a function of casein level, temperature, pH, and calcium chelation.
CHAPTER 4

SOLUBILIZATION OF REHYDRATED FROZEN HIGHLY CONCENTRATED MICELLAR CASEIN FOR USE IN LIQUID APPLICATIONS ¹

ABSTRACT

Highly concentrated micellar casein concentrate (HC-MCC), a potential ingredient of protein-fortified food, is a gel at cold temperature. It contains ~17% to 21% casein with most serum proteins and lactose removed by microfiltration and diafiltration, and it is then further concentrated using vacuum evaporation. The HC-MCC can be stored frozen, and our objective was to determine the conditions needed to obtain complete solubility of thawed HC-MCC in water and to understand its gelation upon cooling. Dispersibility (ability to pass through a 250-µm mesh sieve), suspendability (percentage of protein not sedimented at 80 g within 5 min), and solubility (percentage of protein not sedimented at 20,000 g within 5 min) were measured at various mixing conditions. Gelation upon cooling from 50°C to 5°C was monitored based on storage (G’) and loss (G”) modulus. The gelled HC-MCC was also examined by transmission electron microscopy.

Thawed HC-MCC was added to water to reach a protein concentration of 3% and mixed using high shear² (7,500 rpm) for 1 min or low shear (800 rpm) for 30 min at 4, 12, 20, or 50°C and at pH 6.4 to 7.2. The HC-MCC completely dispersed at 50°C, or at ≤

² Different agitation speeds were used to provide different shearing rates.
20°C followed by overnight storage at 4°C. Suspendability at 50°C was ~90% while mixing at ≤ 20°C followed by overnight storage resulted in only ~57% suspendability. Solubility followed a similar trend with ~83% at 50°C and only ~29% at ≤ 20°C. Mixing HC-MCC with 60 mM trisodium citrate increased dispersibility to 99%, and suspendability and solubility to 81% at 20°C. Cold-gelling temperature, defined as the temperature at which $G' = G''$ when cooling from 50°C to 5°C, was positively correlated ($R^2 = 0.97$) with protein level in HC-MCC. Gelation occurred at 38°C, 28°C and 7°C with 23%, 20% and 17% of protein, respectively. Gelation was reversible upon heating although after a second cooling cycle the HC-MCC gel had lower $G'$. In micrographs of gelled HC-MCC, the casein micelles were observed to be within the normal size range but packed very closely together with only ~20 to 50 nm space between them. We propose that cold-gelation of HC-MCC occurs when the kinetic energy of the casein micelles is sufficiently reduced to inhibit their mobility in relation to adjacent casein micelles. Understanding solubilization of rehydrated frozen HC-MCC and its rheological properties can help in designing process systems for using HC-MCC as a potential ingredient in liquid food.

Keywords: solubility, micellar casein, microfiltration,

INTRODUCTION

Casein is a food ingredient that is widely used in dairy, bakery, meat, beverage, and nutraceutical industry based on its diverse functions in emulsifying, foaming, whipping, water-binding, and cheese making. Texture properties and nutritional value of casein further support its application as a food additive (Fox, 2001, Fox and Kelly, 2004, 2004).
Séverin and Wenshui, 2005). Traditionally, casein or caseinate was manufactured in industry by either isoelectric precipitation or by chymosin coagulation (Fox, 2001). Through these processes, casein micelles have irreversibly changed their native colloidal structure into spherical or linear aggregates (Farrell Jr et al., 1988, Oommen, 2004, McMahon and Oommen, 2013).

Since the 1990s, microfiltration (MF) of skim milk has been applied to produce micellar casein concentrate (MCC) with casein levels ranging from 7 to 20% and concomitant serum protein removal ranging from 46 to 79% based on the composition of MCC, or 60 to 95% based on the serum protein level in MF permeate (Pierre et al., 1992, Garem et al., 2000, Brandsma and Rizvi, 2001, Schuck et al., 2002, Fox and Kelly, 2004, Nelson and Barbano, 2005, Hurt et al., 2010, Marella et al., 2013). Typically, MCC manufactured using MF contains only 7 to 10% casein which translates into a 3 to 4-fold concentration (St-Gelais et al., 1995, Jost et al., 1999, Nelson and Barbano, 2005, Beckman et al., 2010, Hurt et al., 2010, Amelia et al., 2013, Beckman and Barbano, 2013, Hurt et al., 2015). Higher concentrations of 7 to 8-fold are achievable when milk is acidified (Brandsma and Rizvi, 1999, Brandsma and Rizvi, 2001) or when concentrated further using ultrafiltration or vacuum evaporation (Amelia and Barbano, 2013).

Such highly concentrated MCC (HC-MCC) has many prospective applications in food industry since it offers several potential advantages over caseinate or milk protein concentrate (MPC) made using ultrafiltration. (1) Compared to caseinate, casein micelles in HC-MCC still exhibit their native structure (Saboyainsta and Maubois, 2000); (2) HC-MCC has lower levels of serum protein compared to MPC, which in turn may lead to improved heat stability or storage stability by reducing binding of denatured serum.
protein to casein; (3) HC-MCC is still hydrated with water. Hence, it may show improved functionality compared to milk protein powders that can lose solubility because of heat exposure during drying as well as during storage (Baldwin and Truong, 2007, Mimouni et al., 2010a, Sikand et al., 2011). (4) HC-MCC can be stored under refrigeration or frozen (Schokker et al., 2011, Sauer et al., 2012). This is beneficial since bacterial growth is repressed during refrigerated storage at 4°C. For example, Amelia and Barbano (2013) reported that the bacterial count of refrigerated HC-MCC stayed below 20,000 cfu/mL for 16 wk.

It is very difficult to re-solubilize MCC that has been spray-dried (Schuck et al., 1999, Schuck et al., 2002). Solubility index has been measured by volume of sediment after dispersing a specified amount of powder and centrifugation under well-defined conditions. Thus, solubility is inversely correlated with solubility index. Schuck et al. (1999) reported a solubility index of 15 mL of MCC powder at 24°C, indicating an extremely low solubility compared to low-heat NDM powder with a solubility index of < 0.5 mL. Having a non-dried form of HC-MCC would be advantageous for use in liquid food systems where high solubility is needed. However, HC-MCC forms into a gel when it is cooled and it is then crucial for disrupting the gel structure to fully disperse the casein micelles.

To test the extent of disruption of the HC-MCC gel, it was necessary to adapt tests used for measuring solubility of milk powders. Many reports have been published on the solubility of dairy protein powders, such as caseinate, MPC, or MCC powder (Schuck et al., 2002, Gaiani et al., 2005, Fang et al., 2007, Gaiani et al., 2007, Schokker et al., 2011, Hussain et al., 2012, Richard et al., 2013, Chandrapala et al., 2014, Crowley et al., 2015).
The International Dairy Federation standard dispersibility test involves pouring reconstituted milk powder through a sieve with a mesh size of 250 µm (Westergaard, 2004). This dispersibility test is used to determine if any of the HC-MCC gel remains in relatively large pieces when dispersed in water. Such macrogel pieces (≥ 250 µm) would be too big to remain dispersed and would rapidly sediment. Smaller microgel pieces (containing aggregates of casein micelles) could be dispersed but probably not visibly observable. However, and residual small microgel pieces would sediment at centrifugation speeds used by researchers who have studied dispersibility of milk powders, such as 700 g for 10 min (Moughal et al., 2000, Havea, 2006), 750 g for 15 min (Schokker et al., 2011), or 36 g for 10 min followed by 168 g for 10 min (Crowley et al., 2015). In preliminary studies, we observed that there was some sedimentation from pasteurized skim milk when centrifuging conditions were greater than 80 g for 10 min.

The final step in fully solubilizing a dried or a cold-gelled HC-MCC includes disruption of any remaining aggregates into individual casein micelles. This can be measured by centrifuging at a speed at which any particles larger than individual casein micelles would sediment, such as centrifuging at 20,000 g for 5 min. Our objective was to determine the best way to disperse and solubilize cold-gelled HC-MCC for its use in liquid food applications as a function of shear speed and time combinations, mixing temperatures, pH, and extended time. We further investigated the effect of citrate addition on dispersibility, suspendability, and solubility of HC-MCC. To better understand the factors affecting solvation of HC-MCC we also studied rheological properties and microstructure of HC-MCC gel.
MATERIALS AND METHODS

**HC-MCC Manufacture**

*Microfiltration.* Pasteurized skim milk (72°C for 20 s) was processed into micellar casein concentrate (MCC) in a four-vessel, continuous MF unit (Filtration Engineering Inc., Champlin, MN) (Figure 3.1) at the Institute for Dairy Ingredient Processing, South Dakota State University, Brookings, SD. The four vessels were 161 mm in diameter and 965 mm in length and were fitted with polyvinylidene fluoride membranes in spiral wound configuration. The four membranes utilized were FH6438-OS03S, FH6430-OS03S, FH6430-OS03S, and FH6430-OS03S (Parker Process Advanced Filtration Division, Oxnard, CA), respectively, for vessel one, two, three, and four. The total surface area of the four membranes was 57.4 m². Immediately prior to processing skim milk, membranes were subjected to a short clean and sanitization. The short clean consisted of a water rinse to neutral pH, followed by a 30-min 50°C-alkaline wash (1.46% (vol/vol) Ultrasil 110 and 0.11% (vol/vol) Ultrasil 01, Ecolab Inc., St. Paul, MN). Alkaline solution was flushed out with water to neutral pH, and a sanitizer solution (0.42% (vol/vol) Oxonia Active, Ecolab Inc., St. Paul, MN) was circulated through the membranes for 10 min at 75°C. Sanitizer was flushed out with water to neutral pH, and the system was ready to begin milk processing. The process used a feed temperature of 18 to 20°C, baseline pressure of 35 kPa, differential pressure of 103 kPa, volume reduction of 4.0 and diafiltration level of 100% (based on volume of skim milk) with 20%, 30%, 30%, and 20% of the diafiltration water added at vessel one, two, three, and four, respectively. During start-up, the feed and circulating boost pumps were sequentially started and the combined MF concentrate obtained from all four vessels was
recycled back to the balance tank until the desired concentration factor was achieved.

After about 10 min, diafiltration was started and after about 30 min, when the required concentration was reached, the MF concentrate flow was diverted to continuous forward mode and the concentrate was collected in a 2,000-L double-jacketed tank. Two separate batches of 5,500 L of skim milk were processed to obtain 1,324 L of MCC. The total processing time for each batch was 17 h and the average overall flux was 9.3 L/m²h.

Membranes were subjected to a long clean after skim milk processing. The long clean consisted of a water flush until retentate exited clear from the system followed by a 30-min 50°C-alkaline wash (1.46% (vol/vol) Ultrasil 110, 0.11% (vol/vol) Ultrasil 01, and 0.11% (vol/vol) XY-12, Ecolab Inc.), a 45-min 46°C-enzyme wash (0.26% (vol/vol) Ultrasil 110, and 0.84% (vol/vol) Ultrasil 63, Ecolab, Inc.), a 30-min 43°C-acid wash

Figure 3.1 Schematic diagram of four-vessel, polymeric spiral-wound microfiltration unit.
(0.53% (vol/vol) Ultrasil 76, Ecolab Inc.), a second 30-min 50°C-alkaline wash, and a 15-min 32°C-soak wash (0.53% (vol/vol) Ultrasil MP, Ecolab Inc.). Water flushes to neutral pH were performed between each wash step, except after the final soak wash, which was allowed to stay on the membrane during storage for preservation. Pressures for all cleaning cycles were maintained at 69, 83, and 152 kPa, for baseline, stage boost, and membrane inlet pressure, respectively.

Based on the mass of serum protein collected in the permeate relative to the mass of serum protein in the pasteurized skim milk, 70-75% of the serum protein present in the skim milk was removed during the MF process. The composition of the skim milk and MF retentate for the two batches are shown in Table 3.1.

**Vacuum Evaporation.** The 1,324 L of MCC obtained from MF of each batch of skim milk were further processed to produce HC-MCC in a multi-pass falling film vacuum evaporator (Dahmes Stainless, Inc., New London, MN). The two-stage falling film evaporator had a primary stage with three passes and a finisher stage with two passes. The MCC was vacuum-evaporated at a condensing temperature of 63°C and a pressure of -680 mbar (-20 in Hg). The evaporator was started using water as the feed,

<table>
<thead>
<tr>
<th>Component</th>
<th>Skim Milk</th>
<th>MF Retentate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Batch I</td>
<td>Batch II</td>
</tr>
<tr>
<td>TS, %</td>
<td>8.93</td>
<td>8.99</td>
</tr>
<tr>
<td>Fat, %</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>TN, %</td>
<td>2.96</td>
<td>3.24</td>
</tr>
<tr>
<td>NCN, %</td>
<td>0.66</td>
<td>0.72</td>
</tr>
<tr>
<td>NPN, %</td>
<td>0.19</td>
<td>0.21</td>
</tr>
<tr>
<td>CN/TN</td>
<td>0.78</td>
<td>0.78</td>
</tr>
</tbody>
</table>
and after achieving the required operating conditions, MCC was fed to the balance tank. The concentrate from the final pass was recycled back to the feed tank until the desired concentration was achieved. The target concentration was 30% solids for Batch I and 25% solids for Batch II. The solids target was reduced from Batch I to Batch II because excessive fouling of the evaporator was observed with Batch I. After reaching the required concentration, the evaporator was diverted to continuous forward mode, and HC-MCC was collected in a 1,800-L jacketed tank. The HC-MCC was transferred to 1.89-L containers, frozen at -20°C, shipped from South Dakota State University to Utah State University, and stored at -20°C until further analysis. The composition of the HC-MCC from Batch I and II is shown in Table 3.2.

**Composition Analysis.** The pasteurized skim milk, MF retentate, and HC-MCC from each batch were analyzed for total solids, total fat, and ash using standard chemical analysis procedures as described by Hooi et al. (2004). Kjeldahl analysis was used to determine total nitrogen, non-casein nitrogen and NPN (Hooi et al., 2004) with casein

**Table 3.2 Composition of liquid highly concentrated micellar casein concentrate**

<table>
<thead>
<tr>
<th>Component</th>
<th>Batch I</th>
<th>Batch II</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS, %</td>
<td>30.14</td>
<td>24.91</td>
</tr>
<tr>
<td>Fat, %</td>
<td>0.94</td>
<td>0.53</td>
</tr>
<tr>
<td>TN, %</td>
<td>23.02</td>
<td>19.07</td>
</tr>
<tr>
<td>NCN, %</td>
<td>2.3</td>
<td>1.68</td>
</tr>
<tr>
<td>NPN, %</td>
<td>0.32</td>
<td>0.17</td>
</tr>
<tr>
<td>CN/TN</td>
<td>0.90</td>
<td>0.91</td>
</tr>
<tr>
<td>Lactose, %</td>
<td>3.79</td>
<td>3.12</td>
</tr>
<tr>
<td>Organic Acids, %</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>Ash, %</td>
<td>2.33</td>
<td>1.99</td>
</tr>
<tr>
<td>Calcium, %</td>
<td>0.72</td>
<td>0.59</td>
</tr>
</tbody>
</table>
and serum protein content calculated by the difference. Lactose and organic acids were determined using an HPLC-based method as described by Upreti et al. (2006). The HPLC system (Beckman Coulter Inc., Fullerton, CA) included a solvent delivery module (System Gold 125), a 20-μL sample injection loop (Rheodyne, Rohnert Park, CA), a model 631 column heater (Alltech, Deerfield, IL), a multichannel wavelength scanning detector (190–600 nm; System Gold 168 detector), and a refractive index detector (RI-2031, Jasco Corporation, Hachioji, Japan). A 300 x 7.80-mm ion exclusion column (ROAOrganic Acid, Phenomenex Inc., Torrance, CA) maintained at 65°C with sulfuric acid (0.013 N) mobile phase at a flow rate of 0.6 mL/min was used. The mobile phase was prepared by dissolving 360 μL of HPLC-grade sulfuric acid (Sigma-Aldrich, St. Louis, MO) in one liter of HPLC-grade water (Thermo Fisher Scientific, Pittsburgh, PA). Calcium was determined by atomic absorption spectroscopy (AAnalyst 200, PerkinElmer instruments LLC, Waltham, MA) at a wavelength of 423 nm (Metzger et al., 2000).

Dispersibility, Suspendability, and Solubility

Sample Preparation. One-hundred-gram portions of frozen HC-MCC were partially thawed at room temperature (~22°C), and then sufficient HC-MCC (30.02 ± 0.01 g for Batch I and 37.50 ± 0.04 g for Batch II) was added to 200 mL of deionized water to achieve 3% (wt/wt) protein. Water temperatures were adjusted to produce mixtures at 4, 12, 20, and 50°C. After combining HC-MCC and water, ~0.5 mL of antifoam B emulsion (J. T. Baker, Avantor Performace Materials, Inc., Center Valley, PA) were added and pH was adjusted to 6.4, 6.8, 7.0, or 7.2 with 1N HCl or 1N NaOH as needed. Mixing was performed (a) at high shear (HS) using a high-speed mixer (Model GLH; OMNI International, Kennesaw, GA) at 7,500 rpm or (b) at low shear (LS) using a
magnetic stirrer (~800 rpm), or with a combination of LS and HS mixing. High-shear mixing was for 1 min while LS mixing was for 10 or 30 min. In addition, after mixing, some samples were stored overnight at 4°C with LS stirring for 18 h. Each sample was tested once for dispersibility, suspendability, and solubility. Each preparation treatment was performed in at least triplicate.

**Dispersibility.** The dispersibility test was adapted from International Dairy Federation standard method for MPC (Fang et al., 2007). After mixing, the dispersed samples were poured through a sieve (U.S.A. Standard Testing Sieve, VWR International LLC., Radnor, PA) with a mesh size of 250 µm. The beaker was rinsed 3 times using the filtrate. Material retained on the sieve was transferred onto a pre-dried, pre-weighed coffee filter paper (Bunn-o-Matic Cor., Springfield, IL) using ~800 ml water. After draining, the filter paper was placed in a pre-dried and pre-weighted beaker and dried in a vacuum oven at 107°C overnight and weighed. Dispersibility was calculated as the percentage of dry weight of retained particles compared to weight of dry matter in the HC-MCC mixture.

**Suspendability and Solubility.** Once dispersibility reached ~90%, the filtrate through the sieve from the dispersibility test was centrifuged at 80 g or 20,000 g for 5 min to measure suspendability and solubility, respectively. Samples prepared at 4, 12, 20, and 50°C were centrifuged at 4, 12, 20, and 20°C. The rationale being that at 80 g, particles of the HC-MCC gel that were small enough to pass through the 250-µm sieve would be easily sedimented and the remaining material could be considered as being suspended while at 20,000 g, only individual casein micelles would remain in the supernatant and these could be considered as being soluble or fully solvated. The
supernatant liquid was obtained and measured for protein using an infrared milk analyzer (B2000, Bentley Instruments, Inc., Chaska, MN) that had been calibrated with milk standards prior to the measurements. Suspendability and solubility were calculated as the percentage of protein concentration of the supernatant compared to 3% protein in the initial HC-MCC mixture. As a reference for the extent of sedimentation that occurs at 80 and 20,000 g, suspendability and solubility were measured for homogenized pasteurized skim milk.

**Adding Trisodium Citrate.** Dispersibility, suspendability, and solubility were measured on samples of HC-MCC mixed with 60 mM trisodium citrate (TSC) (Thermo Fisher Scientific, Fair Lawn, NJ) instead of water using HS for 1 min at 4 and 20°C with pH adjusted to 7.0 using 1 M HCl. An overnight storage at 4°C was optionally performed after mixing at 4°C. Another mixing was performed with 120 mM TSC at 4°C. Calibration curves were made for correcting protein levels measured by infrared milk analyzer of diluted skim milk of known protein levels with water, and with 60 and 120 mM TSC solution, respectively.

**Rheological Properties**

**Sample Preparation.** Frozen HC-MCC containing 23% protein was partially thawed at room temperature, then ~25g HC-MCC were stirred at LS at 50°C for 10 min to liquefy the gel. To obtain HC-MCC with protein levels of 17% and 20%, sufficient HC-MCC was transferred to another beaker and appropriately diluted with deionized, warm (~50°C) water. Protein content of diluted HC-MCC was tested using a rapid protein analyzer (Sprint, CEM, Matthews, NC).
**Gelation Temperature and Thermal Reversibility.** The rheological properties of HC-MCC were studied using a magnetic bearing rheometer (Model AR-G2; TA Instruments, New Castle, DE). After stirring at 50°C for 5 min, 7.5 mL of HC-MCC were poured into the coaxial cylinder that was already set at 50°C, followed by cooling to 5°C at 1°C/min. Strain was set at 0.5% and frequency at 1.0 Hz. The sample was covered with a solvent trap immediately after sample addition to prevent evaporation. Temperature, storage modulus (G’), and loss modulus (G’’) were recorded at 30-s intervals. The cold-gelling temperature (CGT) was calculated as the temperature at which G’ equals to G’’ as the HC-MCC was cooled. To test thermal reversibility, HC-MCC was cooled from 50 to 5°C, held at 5°C for 30 min, heated to 50°C, held at 50°C for 30 min, followed by another round of cooling, holding, and heating steps at the rate of 1°C/min. Gelation temperature was measured in triplicate and thermal reversibility was measured in duplicate.

**Transmission Electron Microscopy**

Frozen HC-MCC containing 23% protein was partially thawed at room temperature, then ~25 g were heated to 50°C for 10 min to liquefy the gel, poured into a petri dish to about 1-mm thickness, and cold-gelled at room temperature for 20 min. The gel sample was slowly flooded with 2% glutaraldehyde (Electron Microscopy Services, Hatfield, PA) in distilled water and fixed *in situ* for 1 h at room temperature. The fixed gel was cut into small pieces with a razor blade and pieces were carefully transferred into a vial filled with 2% glutaraldehyde. Samples were rinsed in two changes of sodium cacodylate buffer (0.1 M with 16 mM calcium chloride and 4.8% sucrose at pH 7.4) for
10 min each. Samples were post-fixed in 2% osmium tetroxide in cacodylate buffer for 1 h at room temperature followed by a 5-min rinse with nanopure water. Samples were treated with saturated aqueous uranyl acetate for 1 h, then dehydrated through a graded series of ethanol (50% for 10 min, 70% for 10 min, 2x 95% for 10 min, 4x 100% for 10 min) and transitioned into Epon plastic with four changes of 100% acetone, 10 min each. Samples were infiltrated in plastic:acetone 1:1, for 1 h, followed by a change to 3:1 overnight. The next day, samples were infiltrated with pure plastic with three changes, each first for 1 h on a rotator followed by 1 h under vacuum. The specimens were then individually embedded in a flat mold and polymerized overnight at 65°C. Sections (~70 to 100 nm) were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL) using a diamond knife (Diatome, Hatfield, PA). Sections were double-stained for 20 min with saturated aqueous uranyl acetate followed by 10 min with Reynold’s lead citrate. Sections were analyzed using a transmission electron microscope (TEM) (JEM 1400 Plus, JEOL USA, Inc., Peabody, MA) operated at 120 kV, and digital images were captured with a Gatan camera (Gatan Inc., Pleasanton, CA).

**Experimental Design**

Effects of mixing method (HS for 1 min, LS for 30 min, with optional overnight storage) and mixing temperature (4, 12, 20, and 50°C) on dispersibility were analyzed using a 4x4 factorial design. Effects of mixing with overnight storage and mixing temperature on suspendability and solubility were studied using a 2x4 factorial design. Effects of mixing method at 50°C on suspendability and solubility, different mixing pH and addition of citrate on dispersibility, suspendability and solubility were studied using a
completely randomized design. T-test was used to compare dispersibility of HC-MCC in water and 60 mM TSC. Linear regression was performed to investigate the effect of protein levels on CGT. Data were analyzed for statistical significance at 95% confidence level using PROC GLM function in statistical analysis software (SAS version 9.3, SAS Institute Inc., Cary, NC). Significance was declared at \( P < 0.05 \) and trend at \( 0.05 \leq P < 0.1 \). Post-hoc means comparisons were made based on p-values \( (\alpha = 0.05) \) using Tukey-Kramer adjustment to obtain differences of least mean squares.

RESULTS

**Dispersibility, Suspendability, and Solubility**

Two batches of HC-MCC were manufactured and their compositions are listed in Table 3.2. Batch I was used in all experiments whereas Batch II was only used in dispersed when mixed using HS for 1 min or LS for 30 min at 50°C. At lower temperatures there was incomplete dispersion without significant difference between 4, 12, and 20°C \( (P > 0.05, \text{ Table 3.3}) \). Using HS for 1 min increased dispersion at lower temperatures (~65%) compared to LS for 30 min (~38%). Oommen (2004) reported a similar effect of shear rate on the dispersion of powders. When HC-MCC samples which dispersed initially at 4, 12, and 20°C were subsequently stored overnight at 4°C, dispersibility increased to 100% in all cases.

Suspendability (measured as lack of sedimented protein at 80 g) of HC-MCC dispersed at 50°C was 89% with HS and 91% with LS (Table 3.4). Solubility (measured as lack of sedimented protein at 20,000 g) was 85% and 82%, respectively (Table 3.5). Since suspendability and solubility were only determined in samples with dispersibility ≥
Table 3.3 Mean dispersibility\(^1\) of rehydrated HC-MCC, with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50\(^\circ\)C, followed by optional overnight hydration (-O) at 4\(^\circ\)C.

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature ((^\circ)C)</th>
<th>4</th>
<th>12</th>
<th>20</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td></td>
<td>60.2(^b)</td>
<td>67.7(^b)</td>
<td>66.6(^b)</td>
<td>98.8(^c)</td>
</tr>
<tr>
<td>HS-O</td>
<td></td>
<td>98.7(^c)</td>
<td>99.1(^c)</td>
<td>99.8(^c)</td>
<td>100.8(^c)</td>
</tr>
<tr>
<td>LS</td>
<td></td>
<td>37.5(^a)</td>
<td>--(^2)</td>
<td>37.9(^a)</td>
<td>99.4(^c)</td>
</tr>
<tr>
<td>LS-O</td>
<td></td>
<td>99.5(^c)</td>
<td>--(^2)</td>
<td>100.0(^c)</td>
<td>100.7(^c)</td>
</tr>
</tbody>
</table>

\(^{abc}\) Means with the same superscript letter were not significantly different, \(\alpha=0.05\)

\(^1\) %Dispersibility = 100 x (dry matter in MCC – dry weight of particles with size \(\geq 250 \mu m\))/(dry matter in MCC)

\(^2\) Not tested

Table 3.4 Mean suspendability\(^1\) of rehydrated HC-MCC, with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50\(^\circ\)C, followed by optional overnight storage (-O) at 4\(^\circ\)C.

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature ((^\circ)C)</th>
<th>4</th>
<th>12</th>
<th>20</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td></td>
<td>--(^2)</td>
<td>--(^2)</td>
<td>--(^2)</td>
<td>89.1(^A)</td>
</tr>
<tr>
<td>HS-O</td>
<td></td>
<td>58.4(^a)</td>
<td>51.9(^a)</td>
<td>60.9(^a)</td>
<td>87.4(^Ab)</td>
</tr>
<tr>
<td>LS</td>
<td></td>
<td>--(^2)</td>
<td>--(^2)</td>
<td>--(^2)</td>
<td>91.9(^B)</td>
</tr>
<tr>
<td>LS-O</td>
<td></td>
<td>54.9(^a)</td>
<td>--(^*)</td>
<td>60.9(^a)</td>
<td>92.8(^Bb)</td>
</tr>
</tbody>
</table>

\(^{ab}\) Means with the same letter were not significantly different, \(\alpha=0.05\)

\(^{AB}\) Means with the same letter were not significantly different, \(\alpha=0.05\)

\(^1\) %Suspendability = 100 x (protein in HC-MCC − protein in supernatant of 80 g centrifugation)/(protein in HC-MCC)

\(^2\) Not tested as incomplete dispersibility, < 90\% as shown in Table 3.2

\(^*\) Not tested
Table 3.5 Mean solubility\(^1\) of rehydrated HC-MCC, with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50°C, followed by optional overnight storage (-O) at 4°C.

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>HS</td>
<td>--</td>
</tr>
<tr>
<td>HS-O</td>
<td>29.3(^{ab})</td>
</tr>
<tr>
<td>LS</td>
<td>--</td>
</tr>
<tr>
<td>LS-O</td>
<td>26.9(^a)</td>
</tr>
</tbody>
</table>

\(^{abcd}\) Means with the same letter were not significantly different, \(\alpha=0.05\)

\(^{AB}\) Means with the same letter were not significantly different, \(\alpha=0.05\)

\(^1\) % Solubility = 100 x (protein in HC-MCC − protein in supernatant of 20,000 g centrifugation)/(protein in HC-MCC).

\(^2\) Not tested as incomplete dispersibility, < 90% as shown in Table 3.2

90%, these values were not determined for HC-MCC mixed at 4, 12, and 20°C without any overnight storage. When the additional overnight storage at 4°C was included, mean suspendability and solubility were 57% and 29% for HC-MCC mixed at ≤20°C (Tables 3.4 and 3.5). However, solvation at 50°C still provided significantly higher (\(P < 0.001\)) solubility than solvation at 4, 12, or 20°C whether or not overnight storage was included (Tables 3.4 and 3.5). There was no difference in suspendability upon overnight storage at 4°C after mixing at 50°C, and a slight difference in solubility but solubility was still in the range of 80 to 85%. Using overnight storage as a way to achieve improved solvation has commonly been used in reconstituting nonfat dry milk (Berridge, 1952). In our experiment, however, overnight storage did not produce complete solubilization of HC-MCC without prior mixing at 50°C.

While investigating the impact of time on solvation of HC-MCC, we observed that providing 10 min of LS mixing in water at 20°C followed by 1 min of HS mixing
increased dispersibility. Using this mixing method, dispersibility of 94 to 98% was achieved (Table 3.6) compared to only 67% when using HS alone at 20°C. However, both suspendability and solubility remained low with values of ~32% and ~15%, respectively. Modifying pH within the range from 6.4 to 7.2 had only a slight effect with a trend ($P < 0.05$) for increased solubility with increasing pH from 13% at pH 6.4 to 16% solubility at pH 7.2.

Addition of 60 mM TSC significantly ($P < 0.001$) increased dispersibility of HC-MCC at 4 and 20°C to 97% and 99%, respectively (Table 3.7). Suspendability also increased to 74% and 81%, respectively, with solubility values being similar (Table 3.7). Including overnight storage at 4°C after mixing at 4°C had a tendency ($P < 0.10$) to increase suspendability and solubility to 86%. Increasing the citrate concentration to 120 mM citrate was less effective in improving solvation of HC-MCC at 4°C than adding overnight storage.

**Rheological Properties**

Cold-gelling temperature was linearly correlated ($P < 0.001$) with protein concentration (Figure 3.2). Non-diluted HC-MCC (~23% protein) and diluted HC-MCC

### Table 3.6 Mean dispersibility, suspendability, and solubility of rehydrated HC-MCC, with pH adjusted to 7.2, 6.8 or 6.4, mixing using low shearing for 10 min, followed by high shearing for 1 min at 20°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Dispersibility %</th>
<th>Suspendability %</th>
<th>Solubility %</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>98.3$^a$</td>
<td>32.8$^a$</td>
<td>16.0$^b$</td>
</tr>
<tr>
<td>6.8</td>
<td>97.1$^a$</td>
<td>30.8$^a$</td>
<td>14.1$^{ab}$</td>
</tr>
<tr>
<td>6.4</td>
<td>94.0$^a$</td>
<td>32.8$^a$</td>
<td>13.4$^a$</td>
</tr>
</tbody>
</table>

$^{ab}$ Means within a column with the same letter were not significantly different, $\alpha=0.05$
Table 3.7 Mean dispersibility, suspendability, and solubility of rehydrated HC-MCC in 60 or 120 mM trisodium citrate, with pH adjusted to 7.0, mixing using high shearing (HS) for 1 min at 4 or 20°C, followed by optional overnight storage (-O) at 4°C.

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature (°C)</th>
<th>Citrate (mM)</th>
<th>Dispersibility (%)</th>
<th>Suspendability (%)</th>
<th>Solubility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>4</td>
<td>60</td>
<td>96.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>73.9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>HS-O</td>
<td>4</td>
<td>60</td>
<td>99.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HS</td>
<td>20</td>
<td>60</td>
<td>98.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HS</td>
<td>4</td>
<td>120</td>
<td>99.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Means within a column with the same letter were not significantly different, α=0.05

(20% and 17% protein) gelled at a mean temperature of 38, 28, and 7°C, respectively.

For every percent unit decrease of protein, mean CGT decreased about 5°C. When HC-MCC protein concentration had been reduced to 16%, gelation did not occur until the diluted HC-MCC was cooled to 5°C.

At 50°C, non-diluted HC-MCC was fluid and had G’ of 10 Pa (Figure 3.3). Upon gelation and cooling to 5°C, G’ was 3.3 kPa and further increased to 5.1 kPa after holding at 5°C for 30 min. Upon reheating to 50°C, the HC-MCC re-liquefied with G’ dropping to <10 Pa. When HC-MCC was held at 50°C for 30 min, and then re-cooled, the same pattern in G’ was observed. There is a trend (0.05 < P < 0.1) of slightly lower G’ obtained after the second cooling cycle, i.e., 2.2 kPa upon reaching 5°C and 3.6 kPa after holding for 30 min. When heated again, G’ again dropped to <10 Pa. Storage modulus followed a similar pattern with a decrease of ~1.3 kPa in G” of the cold HC-MCC occurring after the second cooling cycle.
Figure 3.2 Cold-gelling temperature of micellar casein concentrate (23% protein and lower protein levels upon dilution with water) determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1.0 Hz. $R^2$, correlation coefficient. Dash line shows extrapolated trendline.

Figure 3.3 Storage modulus ($G'$) (open square, left axis) of highly concentrated micellar casein concentrate during temperature cycling (solid line, right axis) at 1°C/min from 50°C to 5°C measured using 0.5% strain applied at 1.0 Hz frequency.
**Transmission Electron Microscopy**

In transmission electron micrographs of cold-gelled HC-MCC, casein micelles were observed as being close together and evenly distributed with approximately 20 to 50-nm spaces separating them (Figure 3.4). Most of casein micelles were intact, more or less spherical, and fit the typical size range of 20 to 600 nm (McMahon and Oommen, 2013). Some casein micelles were collapsed and others were aggregated (Figure 3.4, asterisks). Several casein micelles were non-spherical in shape, which was also observed in skim milk treated with high pressure (~150 MPa) (Knudsen and Skibsted, 2010). In contrast, casein micelles with more spherical structure have been detected in skim milk or ultrafiltrated skim milk at 20°C (Karlsson et al., 2007, McMahon et al., 2009). Some low electron-dense protuberances of 20 to 30 nm were observed extending from the body of the casein micelles (Figure 3.4, black arrows), which is in accordance with previous observations made by other researchers (Dalgleish et al., 2004, McMahon and Oommen, 2008). Occasionally, small fat droplets in size ranging from 50 to 100 nm were observed between the casein micelles (Figure 3.4, white arrows).

**DISCUSSION**

**Dispersibility, Suspendability, and Solubility**

Similar to studies of milk powders (Westergaard, 2004), we used the term “dispersibility” to refer to the ability of the HC-MCC gel to be sufficiently reduced in size to pass through a 250-µm mesh sieve. Commonly, the assessment of solubility of milk powders is based on lack of sedimentation upon centrifuging at 170 to 750 g for
Figure 3.4 Transmission electron micrographs of thin sections of cold-gelled highly concentrated micellar casein concentrate in low (A) or high (B) magnifications showing casein micelles (grey-black) of various sizes, including chains of protein extending out from casein micelles (black arrows), some casein micelles that appear aggregated (asterisks), as well as some very small milkfat globules (white arrows).
10 to 15 min at 20°C (Moughal et al., 2000, Havea, 2006, Schokker et al., 2011, Crowley et al., 2015). However, this does not mean that the powder particles have been hydrated completely into individual casein micelles, only that any remaining particles are small enough that they do not sediment under the test conditions used. In our view, complete solvation is achieved when only individual, non-aggregated colloidal casein micelle particles are dispersed in the medium. This should apply to powders and was the requirement we set for the HC-MCC gel to be considered to have 100% solubility.

Milk powders need to go through a hydration process to release individual casein micelles contained within the dry powder particles. In contrast, the cold HC-MCC already contains hydrated casein micelles but the casein micelles are locked together as a gel. Release, or dispersion, of all the individual casein micelles from HC-MCC gel particles is necessary for 100% solubility to be obtained. To better understand the reduction in particle size that occurs during shearing and dispersion of the HC-MCC gel into water, we designated the term “suspendability” to refer to particles that were small enough to pass through the 250-µm mesh sieve but were large enough to sediment at a low centrifugal force, i.e., 80 g for 5 min. This was the conditions under which no sediment was observed from pasteurized skim milk.

The three tests used in this study provide an indication of particle size reduction of the HC-MCC gel as it was dispersed, suspended and solubilized in water. The dispersibility test effectively quantified visually-observable macrogel particles (≥ 250 µm). The suspendability and solubility centrifugation tests at 80 g or 20,000 g for 5 min separated microgel particles (~1 to 250 µm) and casein micelle aggregates (0.5 to 1 µm),
respectively, from the fraction of HC-MCC that was solubilized into individual casein micelles (Marchin et al., 2007).

To find optimum conditions of solubilizing HC-MCC, we chose five parameters including shearing, overnight storage, temperature, pH, and the addition of TSC. Combinations of 10-min LS and 1-min HS reached full dispersibility (~98%) of HC-MCC, but only 33% of suspendability and 16% of solubility at 20°C and pH 7.2 (Table 3.6). These results indicate that by solely increasing shearing rate, 65% of the HC-MCC gel was in particles smaller than 250 µm. Still, complete solubilization was not achieved since only 16% particles were found separated into individual casein micelles (Figure 3.5). Increasing shearing rate has been associated with reduced rehydration time or smaller particle sizes of dairy powder dispersions, presumably by physically breaking particles apart, increasing turbulence, accelerating mass transfer and releasing of casein micelles into solution (Hixson and Crowell, 1931b, Oommen, 2004, Bock et al., 2008, Jeantet et al., 2010, Richard et al., 2013, Chandrapala et al., 2014). High shear is usually more effective in reducing particle size and rehydration time during solubilization of dairy powder than LS (Hixson and Crowell, 1931b, Oommen, 2004, Bock et al., 2008, Jeantet et al., 2010, Richard et al., 2013, Chandrapala et al., 2014). More aggregates with larger sizes were observed in sodium caseinate dispersions hydrated using LS for 10 h than using HS for 10 min followed by hydration for 1 h (Oommen, 2004). In our study, increasing mixing temperature seemed to be more effective in solubilizing HC-MCC than overnight storage. The next most effective treatment included mixing under increased shear rate. When followed by overnight storage, all HC-MCC gel particles were reduced in size to < 250 µm with ~30% of the gel dissolved into individual casein micelles.
Figure 3.5 Approximate size distribution of protein particles in HC-MCC and water mixture containing 3% protein. Black bars: mixing with low shear for 10 min and subsequent high shear for 1 min at 20°C. Gray bars: mixing with high shear for 1 min and subsequent overnight storage at 4°C. Bars with diagonal strips: mixing with high shear for 1 min at 4°C with addition of 60 mM of trisodium citrate. White bars: mixing with high shear for 1 min at 50°C. Size distribution was calculated as: >250 µm = dispersibility; 1-250 µm = suspendability – dispersibility; 0.5-1 µm = solubility – suspendability; < 0.5 µm = solubility. Bars=SE, n=3.
Both heating to 50°C and adding 60 mM TSC melted the HC-MCC gel with ~90% and 80% of casein micelles reaching sizes of individual casein micelles, respectively.

Increasing hydration temperature, time, and shear rate are commonly used parameters to increase solubility of dairy proteins in industry. Increasing mixing temperature and time has been associated with a decrease in the amount of sediment or rehydration time of dairy powders (Hixson and Crowell, 1931a, Mimouni et al., 2009, Jeantet et al., 2010, Schokker et al., 2011, Richard et al., 2013, Crowley et al., 2015). Increased solvation after overnight storage at 4°C may partially be attributed to the dissociation of β-casein from casein micelles due to decreased hydrophobic interactions at low temperature. However, other interactions are likely to play more important roles since hydrophobic interaction could not explain higher solubility at 50°C when hydrophobic interactions would be stronger.

Increasing mixing temperature accelerates the release of material from powder particles into solution, which appears to be the rate-limiting step in solubilization of MPC (Mimouni et al., 2009). Moreover, the rehydration process is more sensitive to mixing temperature than to shearing speed (Jeantet et al., 2010, Richard et al., 2013). For example, increasing mixing temperature by 4°C was as effective as doubling stirring rate (from 400 to 800 rpm) in rehydration of MCC powder (Jeantet et al., 2010).

At pH of milk (~6.6), phosphate salts exist mostly in the form of HPO_4^{2-}, which has a buffering capacity from pH of 5.8 to 7.8 (Lucey and Horne, 2009). Therefore, pH change from 6.4 to 7.2 has minimum impact on HC-MCC solvation.
Calcium-Mediated Protein Linkage

Adding 60 mM TSC increased dispersibility of the HC-MCC gel at ≤ 20°C to 100% and solubility to 80% with the remaining 20% being microgel pieces (Figure 3.5). This disruption of HC-MCC into smaller particles with use of TSC suggests that calcium is possibly involved as part of the linkages between caseins in cold-gelled HC-MCC. However, it could also be argued that disruption of the casein micelles by chelating calcium leads to a breakup of the HC-MCC gel particles. In addition to the interlocking of caseins within the casein micelles by nanoclusters of calcium phosphate (McMahon and Oommen, 2008), calcium can bind directly to the caseins as has been shown for metal cations in general (Reddy and Mahoney, 1992). Dispersion of HC-MCC (3% protein) contains about 23 mM of total calcium. And 60 mM of citrate is sufficient to complex all available calcium. It was shown that by adding a calcium-chelating agent (i.e., TSC), free calcium ions favorably attached to TSC and formed a soluble complex (Lucey and Horne, 2009). We hypothesize this to be the reason for the disruption of calcium-mediated protein-protein linkages with the result of increased solvation of the HC-MCC gel.

Existence of non-covalent bonding was suggested in MPC powder (Anema et al., 2006, Havea, 2006). Swelling of casein micelles has been observed upon addition of citrate, urea, or EDTA to casein micelle dispersions, as evidenced by the increased volume of casein micelles after addition of these substances (Sood et al., 1979, Huppertz et al., 2007, de Kort et al., 2011). Similarly, increased particle sizes and decreased turbidity were observed in casein micelles after addition of citrate or urea (Huppertz et al., 2007). These results are in agreement with our observations that addition of calcium
chelators increased the solvation of casein micelles. However, since chelating calcium by adding TSC did not fully solubilize HC-MCC (~23% of the protein remained as microgel pieces) additional factors must be involved in linking casein micelles together in the HC-MCC gels.

It is noted that addition of TSC also causes reduction of casein-bound calcium, which may lead to dissociation of casein micelle in MPC and reduction of casein-bound phosphate (Kaliappan and Lucey, 2011). Dissociated casein molecules can be present as small aggregates, which are smaller and lighter, and therefore, harder to sediment according to Stoke’s Law. Small clusters of particles measuring less than 50 nm were observed in calcium-depleted casein micelles using TEM (Oommen, 2004, McMahon and Oommen, 2013). Thus, the role of calcium chelation in increasing solubilization of HC-MCC gels may involve decreasing calcium-induced interactions between casein micelles and dissociating casein micelles into smaller particles.

**Microstructure of HC-MCC**

The observed close packing of casein micelles in transmission electron micrographs of the HC-MCC gel (separation distances of 20 to 50 nm) can be related to a casein concentration factor of ~4.5 in HC-MCC. In comparison, the average distance between casein micelles in skim milk is ~120 nm (Walstra et al., 1984). The observation of casein micelles in the HC-MCC gel with non-spherical shape may indicate deformation of the periphery of the casein micelles as a consequence of shear during manufacture.

It has been calculated that casein micelles contain from 1 to 8 g water per gram protein depending on how the hygroscopicity is measured (Kumosinski et al., 1988).
Theoretically, fat globules in cream are closely packed (i.e., touching each other without compression) when fat level reaches 72% (Bylund and Pak, 2003). Similarly, if casein micelles contain 4 g water per gram of casein, then MCC would become closely packed when a casein concentration of 14% is approached. Therefore, in HC-MCC draining of water from the outer portion of the casein micelles needs to occur in order to reach ~20% casein during the manufacture. This results in close packing of the casein micelles in which there is overlap of their hydration spheres (Figure 3.6).

Given the closeness of neighboring casein micelles, the gap between casein micelles in HC-MCC is likely to be filled with surface protuberances radiating from the same casein micelles (Figure 3.7). Protuberances of tubular shape with a diameter of 10 to 20 nm extending from the bulk of casein micelles have been reported (Dalgleish et al., 2004, McMahon and Oommen, 2008). Being able to view fine-stranded protein protuberances or tendrils at high magnifications is problematic for at least two reasons. Firstly, the protuberance structure can easily be changed during sample preparation such as coating with heavy metal. Secondly, finely detailed structure with low electron density may be lost if contrast is set too high during image capture (McMahon and McManus, 1998).

If the observed gaps between casein micelles were devoid of protein mass associated with the casein micelles, it would be expected that the casein micelles would be randomly distributed throughout the sample volume. In contrast, these gaps are uniformly, and consistently, of approximately the same size, suggesting that the casein micelles have been pushed together as closely as possible and are held separate
Figure 3.6 Schematic representation of the spatial arrangement of individual casein micelles in relation to casein concentration. (A) Single casein micelle with corresponding hydration sphere. (B) Casein micelles in milk (~2.6% casein) with ample of free space around the micelles. (C) Casein micelles in MCC (~9% casein) have less space between each other but hydration spheres are still separated. (D) Casein micelles in HC-MCC are packed closely with overlapping spheres. Black circles represent colloidal calcium phosphates, gray circle represent individual caseins in casein micelle, and dash line represents surface of hydration sphere of an individual casein micelle.
Figure 3.7 Schematic representation of two adjacent casein micelles with overlapping surface casein protuberances which are connected by free calcium-ion bridges. Black circles represent caseins with associated hydrodynamic spheres (gray), and dash line represents surface of casein micelles.

through steric hindrance of their outer peripheral protein protuberances and tendrils that in the past have been referred to as a hairy layer. In conclusion, based on moisture content, results from TEM and the impact of calcium chelation on solubilization of HC-MCC, it would appear that the outer portions of the casein micelles sterically overlap with each other and some linking between casein micelles occurs via calcium bridging between adjacent peripheral proteins in the cold HC-MCC gel.

Small fat droplets were found interspersed between casein micelles. The presence of some fat droplets in HC-MCC was expected as HC-MCC contains a small amount of fat (0.5 to 1%, Table 3.2). Those fat droplets remaining would be the smallest fat droplets that have too slow a sedimentation rate to be present in the cream factor leaving the cream separator. The shearing and temperature changes (i.e., freezing and thawing) during preparation and handling of HC-MCC may have caused some collapse and
aggregation of casein micelles (Gebhardt, 2014), leading to these unusually large aggregates.

**Cold-Gelation of HC-MCC**

The observation that HC-MCC with 23% protein content forms a gel at temperatures of \( \leq 38^\circ C \) corresponds well with its low dispersibility, suspendability and solubility at temperatures of \( \leq 20^\circ C \) and its high solubility at 50°C when the gel has melted. Such gelling and melting behaviors are mainly temperature-dependent, and much less affected by storage time or mixing speed. Similar \( G' \) patterns upon reheating/recooling cycles indicate that the cold gelation of HC-MCC is thermally reversible (Figure 3.3). The slight decrease (~1.3 kPa) in \( G' \) suggests slightly weaker gel strength after recooling. Although, gel strength may be restored if held for longer than 30 min during recooling.

We propose that cold-gelation of HC-MCC is caused by steric interference\(^3\) between the closely packed casein micelles through overlapping protuberances on their periphery (Figure 3.7), and that these interactions are strengthened through calcium bridging. That is, the casein micelles in HC-MCC are packed so closely together that their outer tendrils overlap and interpenetrate into the hydration sphere of each other. The spatial closeness of casein micelles and their tendrils permits calcium bridging to form between negatively charged locations of the proteins. Cold-gelling of HC-MCC thus occurs when the kinetic energy of the casein micelles is sufficiently reduced to inhibit mobility and ability to move in relation to adjacent casein micelles. At high temperature, 

\( ^3 \) Steric interference refers to the intercalation of protein chains on adjacent casein micelles that limits their mobility
kinetic energy of casein micelles is sufficient to enhance mobility of surface protuberances and the casein micelles themselves, resulting in less drag in moving past each other and the gel melts. When the temperature is again decreased, the reduced kinetic energy leads to restricted movement and gelation of HC-MCC gel. The higher the casein concentration of HC-MCC, the shorter the distance between casein micelles, the sooner steric interference slows down casein micelle mobility and CGT occurs at higher temperature. This casein concentration is reached during the last stages of HC-MCC concentration by MF with further dewatering of the casein micelles occurring during evaporation.

**CONCLUSIONS**

Understanding solubilization of rehydrated frozen HC-MCC and its rheological properties can help in designing process systems for using HC-MCC as an ingredient in liquid food systems. Either mixing at high temperature (~50°C) or addition of TSC can achieve complete dispersion and more than 80% solubility of HC-MCC in water (3% protein). Overnight storage helps to fully disperse HC-MCC, but only reaches ~30% of solubility at ≤20°C. High shearing is more effective than low shearing in increasing dispersibility although it provides no advantage in increasing suspendability or solubility. Cold-gelation of HC-MCC is thermally reversible and reducing protein levels in HC-MCC can decrease its CGT. The HC-MCC with less than 16% of protein does not gel at 5°C. We propose that cold-gelation of HC-MCC occurs when the kinetic energy of the casein micelles is sufficiently reduced to inhibit their mobility in relation to adjacent
casein micelles. Based on these results, using high shear rates followed by moderate heating is suggested to maximize solubility of HC-MCC in liquid foods.

ACKNOWLEDGMENTS

Authors give thanks to Dr. Nabil N. Youssef (Utah State University) for suggestions on TEM sample preparation and comments on manuscript, Dr. Silvana Martini (Utah State University) for her assistance and advice in rheological measurements, the Aggie Creamery (Utah State University, Logan) for donating of cream and its staff for help with protein measurements, and the Electron Microscopy Core Research Facility at University of Utah (Salt Lake City) for use of the transmission electron microscope. Funding for this research was provided by the National Dairy Council (Rosemont, IL) and the Utah Agricultural Experiment Station, Utah State University, and approved as journal paper number 8767. The use of trade names in this publication does not imply endorsement by Utah State University of the products named nor criticism of similar ones not mentioned.

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CHAPTER 5

INVESTIGATING COLD GELATION PROPERTIES OF RECOMBINED HIGHLY CONCENTRATED MICELLAR CASEIN CONCENTRATE AND CREAM FOR USE IN CHEESE MAKING

ABSTRACT

Highly concentrated micellar casein concentrate (HC-MCC), a potential ingredient for cheese making, contains ~20% casein with ~70% of serum proteins removed by microfiltration, and diafiltration of skim milk, followed by vacuum evaporation. Our objective was to investigate cold gelation properties of recombined concentrated milk (RCM) by mixing thawed frozen HC-MCC and cream under different casein levels, pH, protein to fat ratios, and with addition of sodium citrate or calcium. The HC-MCC was recombined with cream using low shear at 50°C for 30 min, and rheological measurements were conducted. Cold gelling temperature (the temperature at which storage modulus (G’) = loss modulus (G’’)) was linearly correlated ($P < 0.001$) with casein levels from 8.6% to 11.5% ($R^2 = 0.71$), pH from 6.6 to 7.0 ($R^2 = 0.96$), and addition of sodium citrate from 0 to 0.36 mmol/g casein ($R^2 = 0.80$). At pH 7.0, gelation occurred at 12, 26 and 38°C with 9%, 10% and 11% of casein, respectively. At pH 6.6, 6.8, and 7.0, RCM with 12% casein gelled at a mean temperature of 12, 26, and 37°C, respectively. Adding calcium chloride at the level of 0.17 mmol/g casein significantly increased CGT from 18 to ≥ 50°C ($P < 0.05$), whereas no significant change was

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observed at levels up to 0.12 mmol/g casein ($P > 0.05$). Different protein to fat ratios ranging from 0.8 to 1.2 did not significantly influence gelling temperature ($P > 0.05$). In transmission electron micrographs of RCM with 12% casein, casein micelles were nonspherical and partially dissociated into small protein strands. Upon addition of calcium chloride at 0.21 mmol/g casein, casein micelles were more spherical and retained colloidal structure with presence of aggregated casein micelles. These gelation processes of RCM with or without addition of trisodium citrate were both reversible. We propose that cold gelation of RCM occurs when protein strands that have been partially released from the casein micelles entangle, restrict their mobility, and form a fine-stranded gel network. Upon addition of high levels of calcium, cold gelation was promoted presumably through direct aggregation of casein micelles. Understanding cold gelation properties can help potentially use RCM for cheese making.

**Keywords:** micellar casein, microfiltration, gelation, microstructure

**INTRODUCTION**

Ultrafiltration (UF) technology has been used in cheese making to increase cheese yield and daily milk processing capacity since the 1970s (Ernstrom et al., 1980, Kosikowski et al., 1985, Govindasamy-Lucey et al., 2004). There are three approaches to using UF for making natural cheese based on low, medium, and high levels of concentration (Fox et al., 2000).

A low level of concentration of 1.2 to 1.8× (by volume reduction) is the most widely used. It increases cheese yield per vat of milk and has the convenience of neither requiring specialized equipment nor large changes in cheese making procedures.
(Govindasamy-Lucey et al., 2004). The practical limit for concentration by UF is about 5× (van Leeuwen et al., 1990). Hard and semi-hard cheeses can be made using UF concentration factors of ~5× provided sufficient whey expulsion after rennet coagulation can be obtained to achieve the final moisture content (Sutherland and Jameson, 1981). This includes diafiltration and pre-acidification of the UF retentate to reduce lactose and calcium phosphate content. Concentrating milk to this level requires use of specialized equipment to handle the higher viscosity compared to milk, and because it is more difficult to expel whey from curd made from a 4 to 6× retentate. An additional challenge is that having more serum proteins retained in the cheese slows down changes in texture and flavor development during aging (Creamer et al., 1987, Lelievre et al., 1990, Bastian et al., 1991).

Ultrafiltering milk to similar concentration can be used directly (without any need for whey expulsion) for making soft and semi-soft cheeses (Tamime and Kirkegaard, 1996, Fox et al., 2000). Such cheeses have sufficiently high moisture content that milk can be concentrated so that it contains the amount of solids needed. Then, acidification and coagulation of the UF retentate yield the finished product with little if any whey drainage.

In the 1990s, microfiltration (MF) was used to concentrate skim milk and produce phosphate caseinate (Pierre et al., 1992), which is commonly called micellar casein concentrate (MCC) in the United States (Saboyainsta and Maubois, 2000, Nelson and Barbano, 2005, Hurt et al., 2010). Unlike UF, MF only concentrates micellar casein but does not concentrate serum proteins, up to 95% of which can be removed with extensive diafiltration (Pierre et al., 1992, Hurt et al., 2010, Marella et al., 2013). Therefore,
micellar casein concentrate obtained through MF process is potentially more suitable for cheese making than UF retentate.

In a similar way to using UF retentate, MF retentate with low concentration levels of 1.2 to 1.8× are suitable for increases cheese yield (Neocleous et al., 2002a, b, Govindasamy-Lucey et al., 2004). Typically, MCC manufactured using MF is only concentrated to 3 to 4× (Nelson and Barbano, 2005, Amelia et al., 2013, Hurt et al., 2015), while higher concentrations of 7 to 8× can be achieved by using milk acidification, or further concentrating using UF or evaporation (Brandsma and Rizvi, 1999, Amelia and Barbano, 2013, Lu et al., 2015). Cheese making using milk retentate with high concentration factors such as 8× MF retentate is difficult and requires specialized equipment in manufacture, and therefore, is not widely used in industry (Brandsma and Rizvi, 1999, Fox et al., 2000, Brandsma and Rizvi, 2001). Medium concentration factor (4 to 5×) of UF retentate is used commercially to produce high-moisture cheese such as Quark, Cream, and Feta cheese, but is not used widely for semi-hard or hard rennet-curd cheese due to changes in cheese texture, flavor and functionality (Green et al., 1981, Fox et al., 2000). Without the interference of serum protein, MF retentate with medium concentration has the potential to make cheese using conventional cheese making equipment while retaining cheese quality.

Because of the fouling issues caused by the fat when microfiltering whole milk, it is more feasible to use an MF skim milk concentrate and recombine it with cream for use in cheese making (Brandsma and Rizvi, 2001, Neocleous et al., 2002b, Govindasamy-Lucey et al., 2007). There has been some research using recombined MF retentate and cream to make Cheddar (St-Gelais et al., 1995, Neocleous et al., 2002a, b), Mozzarella
(Garem et al., 2000, Brandsma and Rizvi, 2001), and pizza cheese (Govindasamy-Lucey et al., 2007). A highly concentrated micellar casein concentrate (HC-MCC) containing ~23% (wt/wt) protein has been manufactured through MF, diafiltration, and vacuum evaporation of skim milk (Lu et al., 2015). The recombined concentrated milk (RCM) obtained by mixing HC-MCC with cream is very suitable for cheese making because of its high casein level (~20% wt/wt) and low serum protein level (< 2% wt/wt). However, HC-MCC tends to gel at temperatures below 50°C which can make it problematic for use in cheese making (Lu et al., 2015). Our objective was to investigate the effect of casein levels, pH, protein to fat ratios, addition of citrate or calcium on cold gelation properties of RCM. To better understand the factors that affect cold gelation, thermal reversibility and microstructure of RCM gel were also studied.

**MATERIALS AND METHODS**

**HC-MCC**

The HC-MCC was manufactured at the Institute for Dairy Ingredient Processing at South Dakota State University (Brookings, SD) as described by Lu et al. (2015). Pasteurized skim milk was concentrated to ~12.5% solids using MF with diafiltration and then further condensed through vacuum evaporation to form the HC-MCC. Frozen HC-MCC was shipped to Utah State University (Logan, UT) and stored frozen until needed. The composition of HC-MCC is shown in Table 4.1.
Table 4.1 Composition of highly concentrated micellar casein concentrate (HC-MCC) made using microfiltration and vacuum evaporation

<table>
<thead>
<tr>
<th>Component</th>
<th>HC-MCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids %</td>
<td>30.14</td>
</tr>
<tr>
<td>Fat, %</td>
<td>0.94</td>
</tr>
<tr>
<td>Total N, %</td>
<td>23.02</td>
</tr>
<tr>
<td>Non-casein N, %</td>
<td>2.30</td>
</tr>
<tr>
<td>NPN, %</td>
<td>0.32</td>
</tr>
<tr>
<td>Casein N/Total N</td>
<td>0.90</td>
</tr>
<tr>
<td>Lactose, %</td>
<td>3.79</td>
</tr>
<tr>
<td>Organic Acids, %</td>
<td>0.39</td>
</tr>
<tr>
<td>Ash, %</td>
<td>2.33</td>
</tr>
<tr>
<td>Calcium, %</td>
<td>0.72</td>
</tr>
</tbody>
</table>

**Rheological Properties**

One-hundred-gram portions of frozen HC-MCC (23% protein) were partially thawed at room temperature (~22°C). The RCM was made by mixing HC-MCC (~18.5 to 27.5 g), warm cream (32% to 44% fat, 50°C) (Aggie Creamery, Utah State University, Logan, UT), and skim milk (purchased from a local supermarket) to reach casein levels from 8.6% to 12.1% with constant protein to fat ratios of 0.8, 1.0, and 1.2, and optional addition of trisodium citrate (0.08 mmol/g casein) (Thermo Fisher Scientific, Fair Lawn, NJ). Another batch of RCM was made by pre-mixing cream (20.4 g) and skim milk (2.1 g) with 0, 0.13, 0.26, or 0.36 mmol/g casein of pH-adjusted citrate at levels of 0.08, or 0.12, or 0.17 mmol/g casein of dihydrate calcium chloride (J. T. Baker, Phillipsburg, NJ), followed by addition of HC-MCC (27.5 g). The RCM was heated to 50°C at low shear using a magnetic stirrer (~800 rpm), and pH was adjusted to 7.0, 6.8, and 6.6 with 1N NaOH as needed. Protein content of diluted RCM was tested using a rapid protein analyzer (Sprint, CEM, Matthews, NC).
Gelation Temperature and Thermal Reversibility

Cold-gelling temperature (CGT) and thermal reversibility were measured using a magnetic bearing rheometer (Model AR-G2; TA Instruments, New Castle, DE) as described by Lu et al. (2015). After stirring at 50°C for 30 min, 7.5 mL of RCM were poured into the coaxial cylinder that was already set at 50°C, followed by cooling to 5°C at 1°C/min. Strain was set at 0.5% and frequency at 1 Hz. The sample was covered with a solvent trap immediately after sample addition to prevent evaporation. The CGT was determined as the temperature at which storage modulus (G') equaled loss modulus (G'”). Thermal reversibility was determined by cooling the mixture from 50 to 5°C, holding at 5°C for 30 min, heating to 50°C, holding at 50°C for 30 min, followed by another round of cooling, holding, and heating steps at the rate of 1°C/min. The CGT was measured in triplicate and thermal reversibility in duplicate.

Transmission Electron Microscopy

Frozen HC-MCC was partially thawed at room temperature, then 55.0 g of HC-MCC was mixed with 39.5 g of warm (50°C) cream (38% fat), and 5.5 g of skim milk, with optional addition of 0.37 g of calcium chloride. The mixture was stirred at 50°C for 30 min, poured into a petri dish to about 1-mm thickness, and allowed to gel at room temperature (~22°C) for 20 min. The gel sample was chemically fixed, processed, and analyzed by transmission electron microscopy (TEM), and digital images were captured as described by Lu et al. (2015).
**Statistical Analysis**

Linear regression was performed to investigate the effects of different casein levels, pH, and citrate addition on CGT and G’. Effects of protein to fat ratios and addition of calcium chloride on CGT and G’ were studied using a completely randomized design. Data were analyzed for statistical significance at 95% confidence level using PROC GLM function in statistical analysis software (SAS version 9.3, SAS Institute Inc., Cary, NC). Significance was declared at $P < 0.05$. Post-hoc means comparisons were made based on p-values ($\alpha = 0.05$) using Tukey-Kramer adjustment to obtain differences of least mean squares.

**RESULTS AND DISCUSSION**

As previously observed when HC-MCC was solubilized in water (Lu et al., 2015), RCM formed by mixing HC-MCC with cream at 50°C also gelled when cooled. The temperature at which cold gelation of RCM occurred depended on the concentration of casein levels, pH, and calcium status.

**Microstructure of Cold Gelled RCM**

Fat droplets ranging from 1 to 12 µm in size were observed in the low-magnification electron micrographs of cold-gelled RCM (Figure 4.1A and B). In the high-magnification micrographs, which were used to study casein micelle appearance, the regions of the sample examined contained only casein micelles.

When cold-gelled RCM was examined using transmission electron microscopy (TEM, Figure 4.1A, C and E), it was observed that the casein micelles were of non-
Figure 4.1 Transmission electron micrographs of cold-gelled (at ~21°C) recombined concentrated milk (12% casein, casein to fat ratio of 0.8, pH of 7.0) with (B, D, and F) or without (A, C, and E) addition of 0.21 mmol/g casein of calcium chloride (f = fat globules, e = loosely entangled protein, white arrows = chains of protein located between protein stains, asterisks = aggregated casein micelles).
spherical shape and had a more ragged and open structure compared to a gel formed by cooling HC-MCC (Figure 4.2). In the gelled HC-MCC (containing 20.7% casein) most of proteins were contained within the casein micelles with a small amount located between the close-packed casein micelles. We had expected that when mixing the HC-MCC with cream, the colloidal nature of the casein micelles would be retained because the serum phase of cream would provide the same calcium phosphate equilibrium as occurs in milk. Instead, the caseins in the RCM had the appearance of being partially dissociated with a greater proportion of smaller, less electron-dense protein material (i.e., the proteins were lighter gray in appearance). This is indicative of less heavy metal staining of the proteins, and suggests a more open structure with reduced crosslinking of protein strands via calcium phosphate. When 0.21 mmol/g casein of calcium chloride was added to RCM (Figure 4.1B, D and F) there was a restoration of the casein into colloidal particles that were more typical of what has previously been observed for skim milk and ultrafiltered milk (Olson, 1992, Karlsson et al., 2007, McMahon et al., 2009, Lu et al., 2015).

Since the RCM contained only 12.6% casein (which is about half of the casein level in HC-MCC), there was a larger relative distance between casein micelles in RCM gels (Figure 4.1) compared to HC-MCC gels (Figure 4.2). Lu et al. (2015) had concluded that HC-MCC underwent cold gelation because the casein micelles are closely packed together causing steric interference between their peripheral protein tendrils. The reason for gelation of RCM when the casein micelles are spaced further apart is not as apparent.

In comparison to the observed microstructure of other types of milk gels, the RCM gels contain many more small protein strands. In most cases, there was a lack of direct aggregation between neighboring casein micelles. This is opposite to what has
Figure 4.2 Transmission electron micrographs of cold-gelled micellar casein concentrate showing casein micelles (grey-black) of various sizes, including chains of protein extending out from casein micelles (black arrows), some casein micelles that appear aggregated (asterisks), as well as some very small milkfat globules (white arrows). (Reprinted with permission from Lu et al. (2015)).
been observed for milk gels formed by rennet or acid coagulation of milk. For example, in rennet-coagulated milk, the casein micelles are more spherical in appearance and occupy a smaller volume fraction than those in RCM, but they are linked together into chains formed by direct aggregation of the casein micelles (Figure 4.3) (unpublished data, A. H. Vollmer). Such renneted casein micelles have had κ-casein macropeptides cleaved from their periphery and form into clusters and chains interlinking to each other.

Similarly, in milk gels formed by acidification at 20 or 30°C, casein micelles also appear circular and directly form into chains (Figure 4.4) (McMahon et al., 2009). Although in this case, it is not known what rearrangements have occurred in casein micelle structure as the pH of the milk is lowered to 4.8. There is an initial dissociation of protein from the casein micelles as milk is acidified to ~ pH 5.2, then as pH 4.8 is approached the proteins are re-associated into spherical colloidal particles just prior to formation of the gel network.

Casein micelles in milk have been observed using TEM to have a somewhat spherical structure, and depending on resolution, appear to have a relatively smooth periphery in their native form (McMahon et al., 2009, McMahon and Oommen, 2013). Non-spherical casein micelles or small strands of proteins are considered as non-native or dissociated structures and have been observed in skim milk treated with high pressure (~150 MPa) (Oommen, 2004, Knudsen and Skibsted, 2010). Thin strands of protein are also observed in sodium caseinate solutions (Oommen, 2004, Knudsen and Skibsted, 2010).

Dissociated casein micelles in RCM can be attributed to the calcium reduction (~22%) that has occurred during microfiltration with diafiltration during manufacture of
Figure 4.3 Transmission electron micrographs of thin sections of milk gel coagulating for 30 min after rennet addition at 31°C.
Figure 4.4 Transmission electron micrographs of acid milk gels at pH 4.8 formed after acidification of skim milk by glucono-δ-lactone at (A) 40°C, (B) 30°C, (C) 20°C, and (D) 10°C (bar = 1 μm) (Reprinted with permission from McMahon et al. (2009)).
the HC-MCC. Extensive diafiltration or washing is commonly used to reduce the lactose and soluble calcium level. There is generally 1.3 millimole of calcium per gram of casein in milk, of which about two thirds (i.e., ~0.9 mmol/g casein) is bound to casein within the casein micelles and is known as colloidal calcium phosphate (CCP) (Singh et al., 1996). As calcium is removed, CCP is solubilized from the casein micelles to maintain ionic calcium and phosphate at their solubility limit, eventually resulting in dissociation of casein micelles (Lucey and Horne, 2009). Loss of CCP is known to cause dissociation of casein micelles in MPC and in casein micelles dispersions dialyzed against water (McMahon and Oommen, 2008, Kaliappan and Lucey, 2011).

In transmission electron micrographs of calcium-depleted casein micelles, small protein clusters less than 50 nm in size were observed (Oommen, 2004, McMahon and Oommen, 2013). Therefore, dissociated casein micelles caused by calcium reduction during HC-MCC processing can be present as non-spherical clusters or small protein strands. However, it is interesting that the casein micelles in HC-MCC would dissociate so extensively in cream given that the ionic strength of milk was maintained. In HC-MCC gels with 20.7% casein, most casein micelles still appeared intact without any apparent dissociation (Figure 4.2). Also in comparison, concentrated casein micelles are widely used in casein micelle studies in which they are suspended in milk permeate, without any dissociation of casein micelles being reported (Pierre and Brule, 1981, Farrell Jr et al., 1988).

Upon addition of 0.21 mmol/g casein of calcium chloride to RCM, casein micelles appeared to be more spherical in shape and more colloidal in structure (Figure 4.1B, D and F). Compared to RCM without calcium addition, there was a smaller
proportion of less electron-dense protein strands, but a higher proportion of aggregated colloidal particles (Figure 4.1D, asterisks) and small protein strands. Some of these protein strands located between and appeared to connect adjacent casein micelles (Figure 4.1F, white arrows). Casein micelles appeared to be less compact to each other, and their peripheral edges were less spherical with a more open structure than that in HC-MCC gel (Figure 4.2). Addition of calcium may increase calcium-mediated bridges between negatively charged protein side chains such as carboxyl or phosphoserine groups, resulting in aggregation of dissociated casein micelles.

**Factors Influencing Cold Gelation**

*Different Casein Levels.* The CGT was linearly correlated ($P < 0.001$) with casein concentration in RCM (Figure 4.5). The mixture with mean casein levels of 9, 10, and 11% gelled at mean temperatures of 12, 26, and 38°C, respectively. For every percent unit decrease of casein level, mean CGT decreased about 5°C. Although G’ at CGT was

![Graph showing the relationship between casein percentage and cold gelation temperature (CGT)](image)

**Figure 4.5** Cold-gelling temperature (CGT, °C) of recombined concentrated milk (pH 7.0 and protein to fat ratio of 0.8) at different casein levels, determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz.
also linearly associated ($P < 0.001$) with casein levels in RCM, the change of $G'$ is relatively small ($\sim 2$ Pa) for every percent change of casein level.

The CGT was reported to be linearly correlated with protein concentration in HC-MCC gel, resulting from steric interference between overlapping protuberances on the periphery of closely packed casein micelles (Lu et al., 2015). Although casein micelles in RCM were not as closely packed as in HC-MCC, the strands of dissociated casein micelles possibly also cause steric interference by limiting movement of other close-by strands or particles. The higher casein level in the mixture, the shorter distance between surrounding caseins, the sooner steric interference reduces casein mobility, and the higher temperature at which CGT occurs.

**Different protein to fat ratios.** The CGT and $G'$ at CGT were both not significantly ($P > 0.05$) influenced by different protein to fat ratios ranging from 0.8 to 1.2 (Table 4.2), with mean CGT of 31°C and $G'$ of 11 Pa. This indicates that within the testing range of casein levels in nonfat portion from 12.0-12.6% and fat levels from 10.5 to 15%, there is no significant influence on steric interference between casein micelles. The lack of significant effect of protein to fat ratio is probably due to the small testing range used in this study, which considered levels relevant to cheese manufacture.

**Different pH.** The CGT was in a linear relationship ($P < 0.001$) with pH (Figure 4.6). At pH 7.0, 6.8, and 6.6, RCM gelled at a mean temperature of 37, 26, and 12°C, respectively. For every 0.1 unit decrease of pH, mean CGT decreased about 7°C. When pH of RCM had been reduced to 6.5, gelation did not occur until the mixture was cooled to 5°C. Although $G'$ at CGT was also linearly associated ($P < 0.05$) with pH in RCM, the
Table 4.2 Different protein to fat ratios (P:F), casein and fat levels, and casein levels in nonfat portion (casein% in NF) in recombined concentrated milk at pH 7.0, determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz.

<table>
<thead>
<tr>
<th>P:F</th>
<th>Casein %</th>
<th>Fat %</th>
<th>Casein % in NF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>10.7</td>
<td>15.0</td>
<td>12.6</td>
</tr>
<tr>
<td>1.0</td>
<td>10.7</td>
<td>12.3</td>
<td>12.1</td>
</tr>
<tr>
<td>1.2</td>
<td>10.8</td>
<td>10.5</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Figure 4.6 Cold-gelling temperature (CGT, °C) of recombined concentrated milk (12% casein, protein to fat ratio of 1.2) at pH 6.6, 6.8, and 7.0, determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz. Dashed line shows extrapolated trend line.

\[ y = 66.56x - 427.92 \]
\[ R^2 = 0.96 \]
change of $G'$ is relatively small (1 Pa) for every 0.1 unit change of pH.

Increasing pH from 6.7 to 7.1 was associated with increasing levels of caseins (i.e., $\alpha_s$-casein and $\beta$-casein) dissociating from casein micelles, resulting in decreasing diameter of casein micelle in recombined skim milk and micellar casein solutions (Anema and Klostermeyer, 1997, Post et al., 2012, Anema et al., 2014). Dissociation of casein micelles could increase the number of protein strands, which could possibly enhance steric interference by increasing entanglement between different particles. This entanglement could further limit mobility of protein strands and form a three-dimensional gel structure, resulting in cold gelation of RCM at a higher temperature.

**Effect of calcium and citrate.** The CGT was linearly associated ($P < 0.001$, $R^2 = 0.81$) with citrate addition (i.e., 0, 0.13, 0.26, and 0.36 mmol/g casein, Figure 4.7). For every 0.1 mmol/g casein increase of added citrate, mean CGT increased about 6°C. The effect of calcium addition to CGT varies at different addition levels. Adding 0.17 mmol/g

![Graph](image)

**Figure 4.7** Cold-gelling temperature (CGT, °C) of recombined concentrated milk (12% casein, casein to fat ratio of 0.8, and pH 6.6) with addition of trisodium citrate (TSC, mmol/g casein) determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz.
casein of calcium chloride significantly ($P < 0.05$) increased CGT from 18 to $\geq 50^\circ$C (Figure 4.8). However, no significant change ($P > 0.05$) occurred in CGT when up to 0.12 mmol/g casein of calcium chloride was added.

Addition of citrate has been reported to increase solubilization of casein micelles in HC-MCC gel, supposedly resulting from calcium-mediate protein linkage or dissociation of casein micelles (Lu et al., 2015). Previously, addition of trisodium citrate has been associated with reducing levels of CCP through calcium chelation, resulting in dissociation of casein micelle in MPC (Kaliappan and Lucey, 2011). Dissociated casein micelle could possibly increase entanglements of protein strands, resulting in an increased steric interference and higher CGT.

The discontinuous change of CGT by addition of calcium indicates that cold gelation at low (i.e., $\leq 0.12$ mmol/g casein) and high calcium levels (i.e., $\geq 0.17$ mmol/g casein) are probably caused by a different mechanism. Addition of calcium at $\geq 0.17$

![Figure 4.8 Cold-gelling temperature (CGT, °C) of recombined concentrated milk (12% casein, casein to fat ratio of 0.8, and pH 6.6) with addition of calcium chloride (mmol/g casein) determined upon cooling from 50°C to 5°C at 1°C/min using a strain of 0.5% and a frequency of 1 Hz.](image-url)
mmol/g casein could possibly strengthen the gel by calcium-mediated protein linkage. Non-covalent bonding has been suspected to occur in HC-MCC gel and MPC powder (Anema et al., 2006, Havea, 2006, Lu et al., 2015). Increasing voluminosity and swelling of casein micelle has been observed upon addition of citrate, urea or EDTA to casein micelle dispersions (Sood et al., 1979, Huppertz et al., 2007, de Kort et al., 2011). The calcium-mediated protein linkage could be weakened by addition of calcium chelators or be strengthened by addition of calcium. Strengthened interaction between casein micelles could cause aggregations of casein micelle, which agrees with the observation in transmission electron micrographs of RCM with calcium addition (Figure 4.1D, asterisks). The formation of aggregates expedites formation of a three-dimensional gel structure at higher temperatures (i.e. resulting in higher CGT).

**Thermal Reversibility**

At 50°C, RCM is fluid and when containing 10% protein had G’ of < 0.1 Pa (Figure 4.9). Upon gelation and cooling to 5°C, G’ was about 200 Pa, and further increased to 360 Pa after holding at 5°C for 30 min. Upon reheating to 50°C, the mixture re-liquefied with G’ decreasing to 0.1 Pa. When held at 50°C for 30 min, and re-cooled, the same pattern in G’ was observed.

Addition of 0.08 mmol/g casein trisodium citrate to RCM increased G’ at 50°C over ten-fold to 2 Pa. When cooled, G’ reached 480 Pa upon gelation and cooling to 5°C, and further increased to 640 Pa after holding at 5°C for 30 min. Upon reheating to 50°C, G’ of the citrate-treated RCM decreased to 3 Pa. The same pattern in G’ was observed after holding at 50°C for 30 min, and re-cooling to 5°C. At any specified temperature, G’ of RCM with citrate addition was higher than that without addition (Figure 4.9). These
Figure 4.9 Storage modulus (G’, kPa) of recombined concentrated milk (10% protein, protein to fat ratio of 0.8, pH of 7.0) during temperature cycling (solid line, right axis, °C) with (black open square, left axis) or without (grey open triangle, left axis) addition of 0.08 mmol/g casein of trisodium citrate during temperature cycling (solid line, right axis) at 1°C/min from 50°C to 5°C measured using 0.5% strain applied at 1 Hz frequency.

similar G’ patterns upon reheating or re-cooling cycles indicate that the cold gelation of RCM is thermally reversible as was previously observed for HC-MCC (Lu et al., 2015).

**Casein Micelle Dissociation**

In our previous work (Lu et al., 2015), we concluded that cold gelation of HC-MCC gel was attributed to steric interference of overlapping protuberances on the periphery of closely packed casein micelles. However, transmission electron micrographs of RCM indicate that casein micelles are no longer intact or closely packed to each other. Instead, casein micelles extensively dissociated into loosely non-spherical aggregates and small protein strands, leaving empty channels in-between (Figure 4.1C and E). In such a system, possibly an increased volume is occupied by proteins that dissociated from casein micelles, resulting in entanglements between small protein strands and dissociated casein
micelles. These entanglements restrict mobility of particles and cause gelation, especially at cold temperatures. Loose protein aggregates and non-spherical casein micelles have been observed in acidified skim milk at 10°C, indicating dissociation of casein micelles occurs at low temperatures (McMahon et al., 2009). This agrees with our observation that when increasing pH or adding citrate, dissociation of casein micelles is promoted, causing a higher degree of entanglement between proteins, resulting in cold gelation at a higher temperature.

The RCM of 4.6x concentration factor (i.e. 11.6% casein) under casein to fat ratio of 0.8 and pH of 6.6 gelled at 18°C. Similarly, Orme (1998) observed a viscoelastic gel property at low temperatures in a 5x whole milk retentate (pH 6.6) produced by UF and diafiltration. The diafiltration process reduced calcium by about 50% in HC-MCC, which would decrease casein-bound calcium, resulting in dissociation of casein micelles. Interesting, although some non-spherical casein micelles are present in HC-MCC gel, extensive dissociation of casein micelle has not been observed (Lu et al., 2015). A possible reason is that draining of water from the outer portion of casein micelles in HC-MCC probably occurs during the concentrating process, causing hydration spheres of casein micelles overlapping and leaving no room for loosening casein micelle structure.

**CONCLUSIONS**

Understanding cold gelation of RCM and its rheological properties can help in designing process systems for using RCM as an ingredient in food applications. At pH 6.6, an RCM with 12% casein does not gel until cooled below 12°C and so would not interfere with cheese making. If the pH is higher than 6.6, then gelation occurs at higher
temperatures unless the casein concentration is lowered. For example, at pH 7.0 RCM with 12% casein gels at 37°C. Based upon the relationship between pH and cold gelation temperature it was predicted that RCM with 12% casein would not gel even when cooled to 5°C. Addition of either sodium citrate or high levels of calcium (i.e., ≥ 0.17 mmol/g casein) increased CGT, although low levels of calcium (i.e., ≤ 0.12 mmol/g casein) did not impact CGT.

Cold gelation of RCM was thermally reversible, even when citrate was added to partially chelate calcium. Compared to cold-gelled HC-MCC, the cold-gelled RCM, the casein micelles were less closely packed together and appeared as being partially dissociated. We propose that cold gelation of RCM occurs when protein strands that have been partially released from the casein micelles entangle, restrict their mobility and form a fine stranded gel network. Such a network consists of these fine strands of proteins as well as the modified casein micelles.

In general, lowering temperature, increasing pH above 6.6, or adding chelating calcium accelerates dissociation of casein micelles. Formation of a gel network that is dependent upon entanglement of protein strands that are only loosely associated with the casein micelles would be favored when there is increased dissociation of casein micelles. When high levels (21 mmol/g casein) of calcium are added to RCM, the casein micelles were less dissociated but gelation was promoted, presumably through direct aggregation of casein micelles. Provided pH of RCM is not above the normal pH of milk, RCM at a casein level of 12% (wt/wt) has potential for use in cheese making.
ACKNOWLEDGMENTS

Authors give thanks to Dr. Lloyd E. Metzger (South Dakota State University) and Mr. Anil Kommineni (South Dakota State University) for supplying the HC-MCC. We also thank Dr. Nabil N. Youssef (Utah State University) for suggestions on TEM sample preparation, Dr. Silvana Martini (Utah State University) for her assistance and advice in rheological measurements, the Aggie Creamery (Utah State University, Logan) for donating of cream and its staff for help with protein measurements, and the Electron Microscopy Core Research Facility at the University of Utah for use of the transmission electron microscope. Author Lu was supported in her Ph.D. studies by the Western Dairy Center, +MD (Logan, UT). Funding support was also provided by the Utah Agricultural Experiment Station, Utah State University, and approved as journal paper number 8767. The use of trade names in this publication does not imply endorsement by Utah State University of the products named nor criticism of similar ones not mentioned.

REFERENCES


CHAPTER 6

INVESTIGATING RENNET COAGULATION PROPERTIES OF RECOMBINED HIGHLY CONCENTRATED MICELLAR CASEIN CONCENTRATE AND CREAM FOR USE IN CHEESE MAKING

ABSTRACT

Highly concentrated micellar casein concentrate (HC-MCC), a potential ingredient for cheese making, containing ~20% casein with ~70% of serum proteins removed by microfiltration, and diafiltration of skim milk, and then further concentrated by vacuum evaporation. Our objective was to investigate rennet coagulation properties of recombined concentrated milk (RCM) by mixing thawed frozen HC-MCC and cream under different casein levels and pH, with addition of various levels of rennet, and coagulate under different temperatures. The HC-MCC was recombined with cream using low shearing at 50°C for 10 min, followed by cooling to 31, 28 or 25°C, adding rennet, and measuring rheological properties. Rennet coagulation time (RCT, the time at which storage modulus (G*) = loss modulus (G'')), was not significantly different (P > 0.05) at casein levels ≥ 5.7%, although slightly decreased from 8.7 to 7.4 min (P < 0.05) when increasing casein level from 3.2 to 5.7%. The mean initial G'' (G''₀) increased about 10 times (P < 0.05) when increasing casein level from 3.2 to 10.9%, whereas no significant change (P > 0.05) in initial G' (G'₀). Logarithm of G' at RCT (G'₁, R² = 0.99), G' at 1.5 times of RCT (G'₁₅, R² = 0.94), and G' at 2 times of RCT (G'₂, R² = 0.95) were linearly associated with casein level (P < 0.001). Reducing the coagulation temperature of RCM

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5
from 31 to 25°C significantly increased mean RCT from 7.4 to 9.5 min \((P < 0.001)\), mean \(G''_0\) from 0.2 to 1.2 Pa \((P < 0.05)\), mean \(G'_1\) from 2 to 3.6 Pa \((P < 0.05)\), whereas decreased mean \(G'_2\) from 1107 to 827 Pa \((P < 0.05)\). No significant change was found in \(G'_0\) and \(G'_{1.5}\) \((P > 0.05)\). Pre-acidify RCM from pH 6.6 to 6.2 significantly reduced mean RCT from 11.9 to 6.5 min \((P < 0.001)\), whereas significantly increased \((P < 0.05)\) mean \(G'_{1.5}\) and \(G'_2\) from 110 to 290 Pa, and 340 to 880 Pa, respectively. No significant change was found in \(G'\), \(G''_0\) and \(G'_1\) \((P > 0.05)\). Reducing rennet level from 20 to 5 µL/100 g mixture significantly increased RCT from 8.8 to 28.9 min \((P < 0.001)\), without significant changes in \(G'\) or \(G''\) \((P > 0.05)\). The RCT was linearly associated with inverse of rennet level \((P < 0.001, R^2 = 0.99)\). For every 0.05 unit increase in inverse of rennet level, mean RCT increase about 30.7 min. In transmission electron micrographs of RCM coagulum (≈11% casein), protein strands are long and highly inter-linked extensively.

Understanding rennet coagulation properties can help potentially use RCM for cheese making.

**Keywords:** micellar casein, rheology, microstructure, coagulation

**INTRODUCTION**

Cheese manufacturing using concentrated milk through ultrafiltration (UF) has benefits of increasing cheese yield and daily milk processing capacity (Ernstrom et al., 1980, Kosikowski et al., 1985, Govindasamy-Lucey et al., 2004). The milk concentrated by microfiltration (MF), however, is potentially more suitable for cheese making compared to UF milk since it contains much less serum proteins, which negatively affect cheese texture and flavor development during aging (Creamer et al., 1987, Lelievre et al.,
1990, Bastian et al., 1991). Limited research has been done using RCM to make cheese, such as Cheddar (St-Gelais et al., 1995, Neocleous et al., 2002a, b), Mozzarella (Garem et al., 2000, Brandsma and Rizvi, 2001), or cheese without standard of identity (i.e., pizza cheese) (Govindasamy-Lucey et al., 2007).

A highly concentrated micellar casein concentrate (HC-MCC) containing ~20% (wt/wt) protein has been manufactured through MF, diafiltration, and vacuum evaporation of skim milk (Lu et al., 2015). The recombined concentrated milk (RCM) by mixing HC-MCC with cream is very suitable for cheese making because of its high casein level (17 to 20% wt/wt) and low serum protein level (< 2% wt/wt). However, because of the high protein level, both HC-MCC and RCM exhibit a cold gelation property (i.e., forming a gel at low temperatures), which could interfere with cheese making process. In our previous work, we found that RCM of medium concentration (11-12% casein) is potential for cheese making since it does not cold-gel at cheese making temperature (Lu et al., 2016).

There are three approaches of using milk retentate in cheese making based on the concentration factor (Fox et al., 2000). Retentate with low concentration factors up to 1.8× is most widely applied because it neither needs specialized equipment nor large changes in cheese making procedures (Neocleous et al., 2002a, b, Govindasamy-Lucey et al., 2004). Cheese making using milk retentate with high concentration factors up to 8× is not widely used in industry due to inconvenience of requiring specialized processing equipment (Brandsma and Rizvi, 1999, Fox et al., 2000, Brandsma and Rizvi, 2001). The UF retentate of medium concentration factor (4 to 5×) is used in industry for high-moisture cheese production, but not widely used for semi-hard or hard rennet-curd cheese.
because of changes in cheese texture, flavor and functionality (Green et al., 1981, Fox et al., 2000). Without the interference of serum protein or cold gelation, MF retentate with medium concentration has the potential to make cheese using conventional cheese making equipment while retaining cheese quality.

A challenge of cheese making using concentrated milk is that as milk concentration increasing, rennet coagulation time is reduced, whereas both curd firming rate and curd hardness are increasing (Sharma et al., 1993, Orme, 1998). Therefore, understanding of rennet coagulation properties of RCM is important for its use in cheese manufacture. Our objective was to investigate the effect of casein levels, coagulation temperature, pH, and rennet level on rennet coagulation properties of RCM. To better understand the factors affect rennet coagulation, microstructure of RCM was also studied.

MATERIALS AND METHODS

HC-MCC

The HC-MCC was manufactured at the Institute for Dairy Ingredient Processing at South Dakota State University (Brookings, SD) as described by Lu et al. (2015). Pasteurized skim milk was concentrated to ~12.5% solids using MF with diafiltration then further condensed through vacuum evaporation to form the HC-MCC. Frozen HC-MCC was shipped to Utah State University (Logan, UT) and stored frozen until needed. Compositions of 2 batches of HC-MCC are shown in Table 5.1.
Table 5.1 Composition of highly concentrated micellar casein concentrate (HC-MCC) made using microfiltration and vacuum evaporation

<table>
<thead>
<tr>
<th>Component</th>
<th>HC-MCC Batch I</th>
<th>HC-MCC Batch II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids %</td>
<td>30.14</td>
<td>27.04</td>
</tr>
<tr>
<td>Fat, %</td>
<td>0.94</td>
<td>0.67</td>
</tr>
<tr>
<td>Total N, %</td>
<td>23.02</td>
<td>18.63</td>
</tr>
<tr>
<td>Non-casein N, %</td>
<td>2.30</td>
<td>1.62</td>
</tr>
<tr>
<td>NPN, %</td>
<td>0.32</td>
<td>0.21</td>
</tr>
<tr>
<td>Casein N/Total N</td>
<td>0.90</td>
<td>0.91</td>
</tr>
<tr>
<td>Ash, %</td>
<td>2.33</td>
<td>1.93</td>
</tr>
<tr>
<td>Calcium, %</td>
<td>0.72</td>
<td>0.54</td>
</tr>
</tbody>
</table>

**Rheological Properties**

The RCM was made by mixing HC-MCC, warm cream (32% to 44% fat, 50°C) (Aggie Creamery, Utah State University, Logan, UT, or Dean foods, Dallas, Texas), and skim milk (Dean foods, Dallas, Texas) to reach protein to fat ratio of 0.8, and casein levels of 3.2%, 5.7%, 8.4%, or 10.9% as described by Lu et al. (2016). After mixing at low shear using a magnetic stirrer (~800 rpm) at 50°C for 10 min, RCM was cooled to 31°C and adjusted to pH 6.6, 6.4, or 6.2 using 1N HCl or NaOH (J. T. Baker, Phillipsburg, NJ), followed by optional further cooling to 28 or 25°C using a water bath at room temperature. Protein content of diluted RCM will be tested using a rapid protein analyzer (Sprint, CEM, Matthews, NC).

**Rennet Coagulation Time**

Rennet coagulation time (RCT) was measured using a magnetic bearing rheometer (Model AR-G2; TA Instruments, New Castle, DE). After reaching temperature
of 31, 28, or 25°C, 20, 10, 6.7, or 5 µl of rennet (Maxiren Double Strength, DSM Food Specialties, Eaglesville, PA) diluted in 2 ml of distilled water were added to RCM and mixed for 30 s. Then 7.5 mL of RCM was immediately poured into the coaxial cylinder that was already set at same temperature, followed by a time sweep at strain of 0.01, and frequency of 1.0 Hz. The sample was covered with a solvent trap immediately after sample addition to prevent evaporation. The RCT was determined as the time when storage modulus ($G'$) equals loss modulus ($G''$). Gel development was monitored for 2 or 3 times of RCT. Each RCM was tested once and each treatment was performed in triplicate.

**Transmission Electron Microscopy**

Frozen HC-MCC was partially thawed at room temperature, then RCM was made by mixing 58.0g of HC-MCC with 32.5g of warm cream (44% fat), and 9.5g of skim milk. Then RCM was stirred at 50°C for 10 min and cooled down to 31°C. An aliquot of 5 g of RCM was transferred to a 50-mL test tube, and mixed with 0.2 mL of 50% (wt/wt) glutaraldehyde (Electron Microscopy Services, Hatfield, PA) by gently hand shake. After sitting for 5 min, 5.2 mL of 3% (wt/wt) warm low-melting agarose (~50°C) was added and mixed by vortexing. The mixture was poured into a petri dish to about 1-mm thickness, and solidity at 22°C for 20 min. Another group of RCM was made, stirred at 50°C for 10 min and cooled down to 31°C. Rennet of 20 µL was added to 100 g of RCM and gently stirred for 30 sec. The mixture was immediately poured into 2 petri dishes to about 1-mm thickness, with the dishes being covered, sealed, and placed in a water bath at 31°C. At 8 and 24 min after rennet addition, dishes were taken out from the water bath,
slowly flooded with 2% glutaraldehyde and fixed in situ for 1 h at room temperature. All samples were cut, stored, processed, viewed by transmission electron microscopy (TEM), and digital images were captured as described by Lu et al. (2015).

**Statistical Analysis**

Linear regression was performed to investigate the effects of casein level on logarithm of $G'$, and inverse of rennet level on inverse of RCT. Effects of coagulation temperature, pH, and rennet level on $G'$ were studied using a completely randomized design. Data were analyzed for statistical significance at 95% confidence level using PROC GLM function in statistical analysis software (SAS version 9.3, SAS Institute Inc., Cary, NC). Significance was declared at $P < 0.05$. Post-hoc means comparisons were made based on $p$-values ($\alpha = 0.05$) using Tukey-Kramer adjustment to obtain differences of least mean squares.

**RESULTS AND DISCUSSION**

Two batches of HC-MCC were manufactured. Batch I was used in all experiments whereas Batch II was only used in studying RCM microstructure and rennet coagulation at different rennet levels.

**Factors Influencing Rennet Coagulation**

*Different Casein Levels.* The mean RCT was not significantly different ($P > 0.05$) at casein level $\geq 5.7\%$, although slightly decreased from 8.7 to 7.4 min ($P < 0.05$) when increasing casein level from 3.2 to 5.7%. The mean of initial $G''$ ($G''_0$) increased about 10 times ($P < 0.05$) when increasing casein level from 3.2 to 10.9%, whereas no
significant change ($P > 0.05$) in initial $G'$ ($G'_0$) with a mean value < 0.1 Pa. The $G'$ in RCM with higher casein level increased faster, as indicated by the steeper slopes of $G'$ over time and less time needed to reach a specified $G'$ value (Figure 5.1). Logarithm of $G'$ at RCT ($G'_{1}$, $R^2 = 0.99$), $G'$ at 1.5 times of RCT ($G'_{1.5}$, $R^2 = 0.94$), and $G'$ at 2 times of RCT ($G'_{2}$, $R^2 = 0.95$) were linearly associated with casein level ($P < 0.001$, Figure 5.2). For every percent unit decrease of casein level, mean $G'$ increased about 2 Pa.

The RCT reduction from 3.2 to 5.7% casein agrees with Orme (1998) and Sharma et al. (1993), who found that at milk native pH, RCT decreases as UF milk concentration increasing. No significant impact of UF milk concentration on RCT was also reported (Sandra et al., 2011). Increased $G''_0$ in RCM of high casein levels indicates an increased viscosity in those samples. The increase in viscosity is as expected and caused by increased solids level in high-casein RCM. A similar relationship has also been found in UF retentate (Orme, 1998, Sandra et al., 2011). In our study, the value of $G'_{1.5}$ indicates

![Figure 5.1 Storage modulus ($G'$, Pa) of rennet gel of recombined concentrated milk (pH 6.4 and protein to fat ratio of 0.8) at different casein levels: 3.2% (□), 5.7% (○), 8.4% (△), and 10.9% (◇), as well as gel of whole milk (+), coagulated at 31°C, under strain of 0.01, and frequency of 1.0 Hz. Curves represent means of three replications.](image)
curd firmness at cutting time, which is about 1.5 times of RCT. The value of $G''_2$ is used to compare curd firming rate regardless of rennet level used (McMahon and Brown, 1984b). The linear relationships between $G'$ and casein level indicate that curd firmness at coagulation time and cutting time, as well as curd firming rate, all increased with high casein level. Increased curd firming rate and maximum curd firmness have been observed in concentrated and ultrafiltrated milk (Dalgleish, 1980, 1981, Guinee et al., 1996, Sandra et al., 2011). The linear relationship observed between logarithm of $G'$ and casein level in RCM agrees with Guinee et al. (1996), who have found a power law relationship between protein concentration in skim milk UF retentate and $G'$ of rennet curd – $G' \propto P^n$ ($n > 1$). They have also found a similar relationship between protein concentration and maximum curd firming rate.

Figure 5.2 Linear relationship between logarithm of storage modulus of recombined concentrated milk (pH 6.4 and protein to fat ratio of 0.8) at rennet coagulation time (●), at 1.5 times of rennet coagulation time (■), and at 2 times of rennet coagulation time (▲), under different casein levels, strain of 0.01, and frequency of 1.0 Hz.
milk (e.g. UF or MF milk) is primarily formed by aggregation of small clusters of individual casein micelles under high concentration (Orme, 1998). Instead of the thin strands and highly branched matrix in rennet milk curd, the small-sized clusters in rennet concentrated milk curd are able to interpenetrate and form thick strands with a low degree of branching and small whey pockets, resulting in increased curd firmness (Orme, 1998). The fast coagulation in concentrated milk is mainly attributed to increased frequency of successful inter-micellar collisions, resulting from decreased mean distance between casein micelles in highly concentration milk (Garnot et al., 1982, Orme, 1998, Sandra et al., 2011).

**Different Coagulation Temperature.** Reducing the coagulation temperature of RCM from 31 to 25°C significantly increased mean RCT from 7.4 to 9.5 min (P < 0.001, Table 5.2 and Figure 5.3), mean $G''_0$ from 0.2 to 1.2 Pa (P < 0.05), mean $G'1$ from 2 to 3.6 Pa (P < 0.05), whereas decreased mean $G'2$ from 1107 to 827 Pa (P < 0.05). No significant change was found in $G'_0$ and $G'_1.5$ (P > 0.05, Table 5.2).

Previously, it has been reported that decreasing coagulation temperature (32 to 28°C) increases RCT of UF milk, mainly resulting from low hydrophobic interaction of hydrolyzed casein micelles at low temperature (Sharma et al., 1993) The RCT is less dependent on temperature as UF milk is more concentrated, indicating that the higher concentration causes a higher collision rate of casein micelle, resulting in a less impact on RCT (Orme, 1998).

The increase in $G''_0$ at 28°C indicates the viscosity of RCM increases as temperature is decreasing, which is as expected in fluids. As coagulation temperature decreasing, curd firmness rate significantly decreased, as evidenced by both decreased
Table 5.2 Mean rennet coagulation temperature (RCT, min), initial storage modulus \((G'_0, \text{ Pa})\) and loss modulus \((G''_0, \text{ Pa})\) within 4 min after rennet addition, storage modulus at 1, 1.5, and 2 times of rennet coagulation time \((G'_1, G'_1.5, \text{ and } G'_2)\) of recombined concentrated milk (11% casein and protein to fat ratio of 0.8) coagulated at different temperatures, under strain of 0.01, and frequency of 1.0 Hz.

<table>
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<tr>
<th>Temperature (°C)</th>
<th>RCT (min)</th>
<th>(G'_0)</th>
<th>(G''_0)</th>
<th>(G'_1)</th>
<th>(G'_1.5)</th>
<th>(G'_2)</th>
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</thead>
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<td>31</td>
<td>7.4\textsuperscript{a}</td>
<td>0.1\textsuperscript{a}</td>
<td>0.2\textsuperscript{a}</td>
<td>2.0\textsuperscript{a}</td>
<td>335.8\textsuperscript{a}</td>
<td>1107.2\textsuperscript{b}</td>
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<td>28</td>
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<td>1.0\textsuperscript{ab}</td>
<td>2.8\textsuperscript{ab}</td>
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<td>908.5\textsuperscript{ab}</td>
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<tr>
<td>25</td>
<td>9.5\textsuperscript{b}</td>
<td>0.9\textsuperscript{a}</td>
<td>1.2\textsuperscript{b}</td>
<td>3.6\textsuperscript{b}</td>
<td>258.3\textsuperscript{a}</td>
<td>827.0\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Means with the same superscript letter within the same column were not significantly different, \(\alpha=0.05\)

Figure 5.3 Storage modulus \((G', \text{ Pa})\) of rennet gel of recombined concentrated milk (11% casein and protein to fat ratio of 0.8) coagulated at different temperatures: 31(Δ), 28 (○), and 25°C (□), as well as gel of whole milk (+) coagulated at 31°C, under strain of 0.01, and frequency of 1.0 Hz. Curves represent means of three replications.
G’₂ and slope of G’ curve (Figure 5.3). The impact on curd firmness is minimum with a slight increase at RCT and no impact at cutting time. Decreased curd firming rate has been observed in rennet coagulated UF milk as coagulation temperature decreasing from 32 to 28°C (Sharma et al., 1993). Although rennet activity also decreased, decreased curd firming rate is primarily resulting from decreased hydrophobic interaction of casein micelles at lower temperature (Sharma et al., 1993, Orme, 1998). Considering the high casein level in RCM (i.e., 11%), mean distance between casein micelles is rather short, probably resulting in formation of small-sized clusters, thick strands, and low degree of branching, and eventually firm body of rennet RCM curd. This process is not or only weakly influenced by change of coagulation temperature ranging from 31 to 28°C.

**Different pH.** Pre-acidify RCM from pH 6.6 to 6.2 significantly reduced mean RCT from 11.9 to 6.5 min ($P < 0.001$, Table 5.3 and Figure 5.4), whereas significantly increased ($P < 0.05$) mean $G'_1$, $G'_2$ from 110 to 290 Pa, and 340 to 880 Pa, respectively. No significant change was found in $G'_0$, $G''_0$ and $G'_1$ ($P > 0.05$, Table 5.3).

The changes in $G'$ and $G''$ indicate that as decreasing pH from 6.6 to 6.2, firmness of RCM curd at cutting time, as well as firmness rate both increased, whereas no significant impact on initial RCM viscosity or its firmness at RCT. Previously, it has been reported that reducing pH from 6.8 to 5.8 decreases RCT, increased curd firmness and firming rate of UF concentrated milk (Sharma et al., 1993, Orme, 1998). A similar effect of pH on curd firming rate has been also reported in regular milk (Kowalchyk and Olson, 1977). Under low pH, charges of casein micelles in concentrated milk are decreased, resulting in increased collision frequency, and therefore, a faster coagulation, as evidenced by decreased RCT (Orme, 1998). The increased collision frequency also
Table 5.3 Mean rennet coagulation temperature (RCT, min), initial storage modulus ($G'_0$, Pa) and loss modulus ($G''_0$, Pa) within 4 min after rennet addition, storage modulus at 1, 1.5, and 2 times of rennet coagulation time ($G'_1$, $G'_{1.5}$, and $G'_2$) of recombined concentrated milk (11% casein and protein to fat ratio of 0.8) coagulated at different pH at 25°C, under strain of 0.01, and frequency of 1.0 Hz.

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<th>$G'_{1.5}$</th>
<th>$G'_2$</th>
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<td>342.5a</td>
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<tr>
<td>6.4</td>
<td>9.5b</td>
<td>0.9a</td>
<td>1.2a</td>
<td>3.6a</td>
<td>258.3b</td>
<td>827.0b</td>
</tr>
<tr>
<td>6.2</td>
<td>6.5a</td>
<td>0.4a</td>
<td>0.6a</td>
<td>2.7a</td>
<td>289.4b</td>
<td>877.9b</td>
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</table>

Means with the same superscript letter within the same column were not significantly different, $\alpha=0.05$

Figure 5.4 Storage modulus ($G'$, Pa) of rennet gel of recombined concentrated milk (11% protein and protein to fat ratio of 0.8) at different pH: 6.6 (□), 6.4 (○), and 6.2 (Δ), coagulated at 25°C, as well as gel of whole milk (+) coagulated at 31°C, under strain of 0.01, and frequency of 1.0 Hz. Curves represent means of three replications.
expedites the formation of small-sized clusters in rennet concentrated milk curd, which interpenetrate and form thick strands with a low degree of branching and small whey pockets, resulting in increased curd firmness and curd firming rate (Orme, 1998).

**Different Rennet Level.** Reducing rennet level from 20 to 5 µL/100 g mixture significantly increased RCT from 8.8 to 28.9 min ($P < 0.001$), without significant changes in $G'$ or $G''$ ($P > 0.05$). The RCT was linearly associated with inverse of rennet level ($P < 0.001$, $R^2 = 0.99$, Figure 5.5). For every 0.05 unit increase in inverse of rennet level, mean RCT increase about 30.7 min.

As expected, decreasing rennet level increases time for hydrolysis of κ-casein, resulting in increased RCT. No significant impact was found in viscosity, curd firmness, or curd firming rate in rennet RCM curd. The inverse relationship between rennet level and RCT has been reported in rennet milk coagulation previously (McMahon and Brown, 1983).

![Figure 5.5](image)

**Figure 5.5** Linear relationship between inverse of rennet level (1/R, 1/µL) and rennet coagulation temperature (RCT, min) in recombined concentrated milk (11% casein and protein to fat ratio of 0.8) coagulated at 31°C, under strain of 0.01, and frequency of 1.0 Hz.
**Microstructure of RCM**

In RCM fixed at 31°C (~11% casein), some casein micelles were spherical, whereas some were aggregated into clumps (asterisks in Figure 5.6B and 5.6C). Small strands of protein located between and appeared to link adjacent casein micelles (white arrows, Figure 5.6C). The casein micelles observed in RCM were close to ones previously observed in HC-MCC (Lu et al., 2015), whereas less spherical compared to casein micelles typically observed in skim milk or UF milk (Olson, 1992, Karlsson et al., 2007, McMahon et al., 2009) In our previous work, we found that in cold gelled RCM (~12% casein, at 21°C), casein micelles were less spherical and had a more ragged and open structure, and upon addition of calcium chloride, casein micelles became more spherical and aggregated (Figure 4.1). It is possible that increased temperature (i.e., from 21 to 31°C) decreases solubilization of colloidal calcium phosphate, resulting in less dissociation and more spherical shape of casein micelles in RCM. The aggregated casein micelles and protein stains located between casein micelles were also observed in cold gelled RCM with addition of calcium chloride at 21°C. The relative distance between casein micelles in RCM fixed at 31°C was larger than cold gelled RCM, which is as expected due to dilution of RCM after addition of agarose. Fat molecules and agarose fibers were also observed (level f and black arrows, Figure 5.6A).

**Rennet Coagulum of RCM.** When rennet coagulum of RCM was examined using TEM, it was observed that the casein micelles were more spherical than RCM before adding rennet and they aggregated together (Figure 5.7). Compared to strands in rennet milk gel (Figure 4.3), strands formed in RCM coagulum were longer and more interlinked to each other. Coagulum of RCM formed at 3 times of RCT (Figures 5.7B, 5.7D,
Figure 5.6 Transmission electron micrographs of recombined concentrated milk, glutaraldehyde-fixed at 31°C and agarose-solidified at 22°C (~11% casein, casein to fat ratio of 0.8) (f = fat globules, asterisks = aggregated casein micelles, white arrows = chains of protein located between protein stains, and black arrows = agarose fibers).
Figure 5.7 Transmission electron micrographs of rennet coagulum of recombined concentrated milk at ~31°C (~11% casein, casein to fat ratio of 0.8) at rennet coagulation time (A, C, and E) or 3 times of rennet coagulation time (B, D, and F) (f = fat globules).
and 5.7F) has thicker and longer chains of aggregated casein micelles than that formed at RCT (Figures 5.7A, 5.7C, and 5.7E). Fat molecules with various sizes approximately ranging from 2 to 30 µm were observed (level f, Figures 5.7A and 5.7B).

Previously, it has been suggested that high frequency of casein micelles in UF concentrated milk leads to the formation of smaller casein micelle clusters with a low degree of κ-casein hydrolysis (Orme, 1998). These small clusters could easily attach to the interior gel structure and form thicker protein strands with large whey pockets, resulting in a more rigid coagulum matrix (Orme, 1998). We found that RCM coagulum has a more inter-linked and rigid gel structure. And both large and small empty spaces between protein strands were observed. Protein strands in coagulum of RCM are much longer and inter-connected than rennet milk gel, probably resulting in a stronger network. This agrees with the increased curd firmness (G’) we observed in RCM curd with high protein levels.

CONCLUSIONS

Understanding rennet coagulation of RCM can help in designing process systems for using RCM in cheese making. Reducing rennet level can increase coagulation time of RCM (11% casein) without impact on curd firmness or firming rate. Decreased coagulation temperature helps to increase coagulation time and decrease curd firmness rate, but also increases the initial viscosity of RCM. Pre-acidify RCM has no advantage in increasing coagulation time, decrease curd firmness or firming rate. Decreasing rennet level can effectively reduce RCT without influence on curd firmness. Microstructure of RCM and its coagulum indicate that the increased curd firmness probably results from
the highly inter-linked and longer protein strands in concentrated milk. Reducing rennet level can be applied to slow down rennet coagulation of RCM (11% casein) in cheese making.

ACKNOWLEDGMENTS

Authors give thanks to Dr. Lloyd E. Metzger (South Dakota State University) and Mr. Anil Kommineni (South Dakota State University) for supplying the HC-MCC. We also thank the Aggie Creamery (Utah State University, Logan) for donating of cream and its staff for help with protein measurements, and the Electron Microscopy Core Research Facility at the University of Utah for use of the transmission electron microscope. Author Lu was supported in her Ph.D. studies by the Western Dairy Center, +MD (Logan, UT). Funding support was also provided by the Utah Agricultural Experiment Station, Utah State University, and approved as journal paper number 8767. The use of trade names in this publication does not imply endorsement by Utah State University of the products named nor criticism of similar ones not mentioned.

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Standardization of milk using cold ultrafiltration retentates for the manufacture of

Use of cold microfiltration retentates produced with polymeric membranes for

Effect of use of milk concentrated by ultrafiltration on the manufacture and

properties of retentates obtained by ultrafiltration of skim milks heated to

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Solubilization of rehydrated frozen highly concentrated micellar casein for use in


CHAPTER 7

GENERAL SUMMARY

Understanding solubilization, cold gelation, and rennet coagulation properties of HC-MCC can help in designing process systems for its use in cheese making. Using high shear rates followed by moderate heating (∼50°C) is suggested to maximize solubility of HC-MCC in liquid foods. Addition of either sodium citrate or high levels of calcium (i.e., ≥ 0.17 mmol/g casein) increased CGT, although low levels of calcium (i.e., ≤ 0.12 mmol/g casein) did not impact CGT and sodium citrate helps to solubilize HC-MCC at low casein level of ∼3%.

Cold gelations of HC-MCC mixing with water or cream are both thermal reversible and even when citrate was added to partially chelate calcium. Reducing casein levels in HC-MCC or RCM can both decrease its CGT. The HC-MCC with less than 16% of protein does not gel at 5°C. At pH 6.6, an RCM with 12% casein does not gel until cooled below 12°C and so would not interfere with cheese making.

We propose that cold-gelation of HC-MCC occurs when the kinetic energy of the casein micelles is sufficiently reduced to inhibit their mobility in relation to adjacent casein micelles. Compared to cold-gelled HC-MCC, in the cold-gelled RCM, the casein micelles were less closely packed together and appeared as being partially dissociated. We propose that cold-gelation of RCM occurs when protein strands that have been partially released from the casein micelles entangle, restrict their mobility and form a fine stranded gel network. Such a network consists of these fine strands of proteins as well as the modified casein micelles.
To solve the problem of fast coagulation, increased curd firmness, and firming rate, rennet coagulation of RCM was studied. Reducing rennet level can increase coagulation time of RCM (11% casein) without impact on curd firmness or firming rate. Decreased coagulation temperature helps to increase coagulation time and decrease curd firmness rate, but also increases initial viscosity of RCM. Pre-acidify RCM has no advantage in increasing coagulation time, decrease curd firmness or firming rate. Microstructure of RCM and its coagulum indicate that the increased curd firmness probably results from the highly inter-linked and longer protein strands in RCM curd.

Overall, provided pH of RCM is not above the normal pH of milk, RCM at a casein level of 11-12% (wt/wt) has potential for use in cheese making. Reducing rennet level can be applied to increase its rennet coagulation time.

My suggestions for future research involving HC-MCC and RCM include: 1. Investigate rennet coagulation of RCM at decreased coagulation temperature as well as lower pH. Since reducing pH can decrease viscosity of RCM and accelerate coagulation and curd firming, whereas decreasing coagulation acts oppositely, it would be interesting to find out whether applying both under careful monitor could slow down coagulation and curd firming, and also decrease the initial viscosity of RCM simultaneously. 2. In this study, rennet coagulation properties of RCM were investigated without acidification or further cheese making process. Reducing coagulation temperature could slow down the coagulation and curd firming, but may also decrease activity of starter culture, which could impede the acidification process. It would be interesting to manufacture cheeses at pilot plant and see how these treatments could affect cheese quality. 3. We only investigated the impact of directly acidifying RCM using acid, it would be interesting to
see the impact of acidification using starter culture, which decreases pH more slowly and may lessen the effect of accelerating coagulation and curd firming.
## APPENDIX A. STATISTICS FOR CHAPTER 3

Table A.1 ANOVA of dependent variables for dispersibility of rehydrated highly concentrated micellar casein

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Table A.2 ANOVA of dependent variables for suspendability of rehydrated highly concentrated micellar casein after overnight storage

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Table A.4 ANOVA of dependent variables for solubility of rehydrated highly concentrated micellar casein after overnight storage

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Table A.5 ANOVA of dependent variable for solubility of rehydrated highly concentrated micellar casein at 50°C

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Table A.8 ANOVA of solubility of rehydrated highly concentrated micellar casein at different pH

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Table A.9 ANOVA of dispersibility of rehydrated highly concentrated micellar casein with addition of trisodium citrate

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Table A.10 ANOVA of suspendability of rehydrated highly concentrated micellar casein with addition of trisodium citrate

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Table A.11 ANOVA of solubility of rehydrated highly concentrated micellar casein with addition of trisodium citrate

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</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>3</td>
<td>667.1569729</td>
<td>222.3856576</td>
<td>8.30</td>
<td>0.0058</td>
</tr>
</tbody>
</table>
APPENDIX B. STATISTICS FOR CHAPTER 5

Table B.1 ANOVA of rennet coagulation time of recombined concentrated milk at different casein levels

<table>
<thead>
<tr>
<th>Source</th>
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</tr>
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<tbody>
<tr>
<td>Casein</td>
<td>3</td>
<td>4.29769231</td>
<td>1.43256410</td>
<td>7.37</td>
<td>0.0085</td>
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</tbody>
</table>

Table B.2 ANOVA of rennet coagulation time of recombined concentrated milk at different coagulation temperatures

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>2</td>
<td>7.78566667</td>
<td>3.89283333</td>
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<td>0.0006</td>
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</table>

Table B.3 ANOVA of rennet coagulation time of recombined concentrated milk at different pH levels

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>2</td>
<td>43.84888889</td>
<td>21.92444444</td>
<td>40.11</td>
<td>0.0003</td>
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</table>

Table B.4 ANOVA of initial $G'$ ($G'_o$) of recombined concentrated milk at different coagulation temperatures

<table>
<thead>
<tr>
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<th>F Value</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>2</td>
<td>1.59624232</td>
<td>0.79812116</td>
<td>4.02</td>
<td>0.0689</td>
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</tbody>
</table>
Table B.5 ANOVA of initial $G''$ ($G''_0$) of recombined concentrated milk at different coagulation temperatures

<table>
<thead>
<tr>
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<th>F Value</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>2</td>
<td>1.94021952</td>
<td>0.97010976</td>
<td>7.62</td>
<td>0.0175</td>
</tr>
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</table>

Table B.6 ANOVA of $G'$ at rennet coagulation time ($G'_1$) of recombined concentrated milk under different coagulation temperatures

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
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<td>3.99025365</td>
<td>1.99512683</td>
<td>8.04</td>
<td>0.0153</td>
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Table B.7 ANOVA of $G'$ at 1.5 times of rennet coagulation time ($G'_{1.5}$) of recombined concentrated milk under different coagulation temperatures

<table>
<thead>
<tr>
<th>Source</th>
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>2</td>
<td>13340.31383</td>
<td>6670.15692</td>
<td>4.27</td>
<td>0.0613</td>
</tr>
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</table>

Table B.8 ANOVA of $G'$ at 2 times of rennet coagulation time ($G'_2$) of recombined concentrated milk under different coagulation temperatures

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</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>2</td>
<td>147620.1682</td>
<td>73810.0841</td>
<td>5.94</td>
<td>0.0311</td>
</tr>
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</table>
Table B.9 ANOVA of initial $G'$ ($G'_{0}$) of recombined concentrated milk at different pH

<table>
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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2</td>
<td>0.57477622</td>
<td>0.28738811</td>
<td>2.02</td>
<td>0.2134</td>
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</table>

Table B.10 ANOVA of initial $G''$ ($G''_{0}$) of recombined concentrated milk at different pH

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>2</td>
<td>0.63195267</td>
<td>0.31597633</td>
<td>2.97</td>
<td>0.1267</td>
</tr>
</tbody>
</table>

Table B.11 ANOVA of $G'$ at rennet coagulation time ($G'_{1}$) of recombined concentrated milk under different pH

<table>
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<tr>
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2</td>
<td>1.03424067</td>
<td>0.51712033</td>
<td>2.23</td>
<td>0.1885</td>
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</table>

Table B.12 ANOVA of $G'$ at 1.5 times of rennet coagulation time ($G'_{1.5}$) of recombined concentrated milk under different pH

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</thead>
<tbody>
<tr>
<td>pH</td>
<td>2</td>
<td>54832.26083</td>
<td>27416.13041</td>
<td>13.15</td>
<td>0.0064</td>
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</table>
Table B.13 ANOVA of $G'$ at 2 times of rennet coagulation time ($G'_2$) of recombined concentrated milk under different pH

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>pH</td>
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<td>393850.4023</td>
<td>196925.2011</td>
<td>20.69</td>
<td>0.0038</td>
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</table>

Table B.14 ANOVA of initial $G'$ ($G'_0$) of recombined concentrated milk with addition of different levels of rennet

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Rennet</td>
<td>3</td>
<td>0.17018692</td>
<td>0.05672897</td>
<td>0.49</td>
<td>0.7006</td>
</tr>
</tbody>
</table>

Table B.15 ANOVA of initial $G''$ ($G''_0$) of recombined concentrated milk with addition of different levels of rennet

<table>
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<tr>
<th>Source</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rennet</td>
<td>3</td>
<td>0.08364744</td>
<td>0.02788248</td>
<td>0.56</td>
<td>0.6535</td>
</tr>
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</table>

Table B.16 ANOVA of $G'$ at rennet coagulation time ($G'_1$) of recombined concentrated milk with addition of different levels of rennet

<table>
<thead>
<tr>
<th>Source</th>
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rennet</td>
<td>3</td>
<td>3.00428633</td>
<td>1.00142878</td>
<td>2.16</td>
<td>0.1632</td>
</tr>
</tbody>
</table>
Table B.17 ANOVA of $G'$ at 1.5 times of rennet coagulation time ($G'_{1.5}$) of recombined concentrated milk with addition of different levels of rennet

<table>
<thead>
<tr>
<th>Source</th>
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<th>F Value</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rennet</td>
<td>3</td>
<td>17157.77308</td>
<td>5719.25769</td>
<td>0.47</td>
<td>0.7114</td>
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</table>

Table B.18 ANOVA of $G'$ at 2 times of rennet coagulation time ($G'_{2}$) of recombined concentrated milk with addition of different levels of rennet

<table>
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<tr>
<th>Source</th>
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<th>F Value</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rennet</td>
<td>3</td>
<td>160317.2935</td>
<td>53439.0978</td>
<td>1.11</td>
<td>0.3957</td>
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</table>

Table B.19 ANOVA of $G'$ at 3 times of rennet coagulation time ($G'_{3}$) of recombined concentrated milk with addition of different levels of rennet

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>Rennet</td>
<td>3</td>
<td>343169.5151</td>
<td>114389.8384</td>
<td>1.90</td>
<td>0.1994</td>
</tr>
</tbody>
</table>
APPENDIX C

03/03/2016
Almut Vollmer
8700 Old Main Hill,
Utah State University,
Logan, UT, 84322

Dear Dr. Almut Vollmer

I am in the process of preparing my dissertation in the Nutrition, Dietetics, and Food Sciences department at Utah State University. I hope to complete my degree program in Nutrition and Food Science.

I am requesting your permission to include the attached material as shown. I will include acknowledgments and/or appropriate citations to your work as shown and copyright and reprint rights information in a special appendix. The bibliographic citation will appear at the end of the manuscript as shown. Please advise me of any changes you require.

Please indicate your approval of this request by signing in the space provided, attaching any other form or instruction necessary to confirm permission. If you charge a reprint fee for use of your material, please indicate that as well. If you have any questions, please call me at the number below.

I hope you will be able to reply immediately. If you are not the copyright holder, please forward my request to the appropriate person or institution.

Thank you for your cooperation,

Ying Lu
4355126674

I hereby give permission to Ying Lu to reprint the following material in his/her dissertation.

CHAPTER 6: INVESTIGATING RENNET COAGULATION PROPERTIES OF RECOMBINED HIGHLY CONCENTRATED MICELLAR CASEIN CONCENTRATE AND CREAM FOR USE IN CHEESE MAKING (Pages 118-140)

Fee: __________________

Signed: __________________ 3-3-16
CURRICULUM VITA

Ying Lu
Dept. of Nutrition, Dietetics, and Food Sciences
Utah State University
Logan, UT, 84322-8700
Phone: (435) 512-6674
E-mail: Y.L@aggiemail.usu.edu

Education

Ph. D. Nutrition and Food Science Advisor: Dr. Donald J. McMahon 04/2016
Utah State University
Thesis: “Investigation of Solubilization, Cold Gelation, and Rennet Coagulation Properties of Highly Concentrated Micellar Casein Concentrate for Use in Cheese Making”

M. S. Nutrition and Food Science Advisor: Dr. Donald J. McMahon 07/2012
Utah State University
Thesis: “Effects of Sodium Chloride Salting and Substitution with Potassium Chloride on Whey Expulsion of Cheese”

B. E. Biological Engineering 06/2007
Nanchang University, Nanchang, Jiangxi, China

Research Interests

Dairy Protein Chemistry Cheese Science Food Product Development
Dairy Processing and Technology Food Chemistry Data Analysis

Professional Practice

Research Assistant Utah State University Logan, UT Sep. 2010-present

♦ Five years of dairy product research and development experience including reduced sodium cheese, potential food usage of a concentrated casein gel, cream cheese filled rice flour dumpling, cottage cheese dip, Cheddar cheese, and ice cream
♦ Developed 2 formulas for diabetic people in China based on latest scientific publications and recommendations of American Diabetes Association
♦ Identified new dairy technologies, conducted trials both on bench top and in pilot plant
♦ Developed skills of analytical test, documentation, statistical experiment design, data analysis (SAS), and formulation (Genesis R&D)
♦ Published articles as the first author in peer-reviewed journals
♦ Presented at national meetings for both academic and industrial audiences

◆ New product development based on knowledge of food ingredients, functional food additives and the physic-chemistry of food ingredient interactions.
◆ Developed 1 novel product—a Chinese BBQ filled baking bun from concept, and improved 2 existing formulas—a fried bean tot and grilled cheese sandwich for national school lunch program
◆ Evaluated acceptance of products by conducting a sensory test of 60 panelists
◆ Prepare and present prototype products to management team and customers
◆ Developed and conducted a sensory training program for 10 trainees from management and quality assurance team
◆ Ensured all products following school lunch program requirements and code of federal regulations
◆ Worked in a team of 8 to solve problems of packaging line in Gooding Cheese Plant
◆ Wrote thorough reports including formulas, processing, labeling, and recommendations to customers
◆ Worked closely with culinary experts, vendors, purchasing, business development and customers to meet targets

◆ Taught lectures, graded homework and exams for a class of 30 students

◆ Led a 20-member resident assistant team and conducted residential programs for thousands of residents

Presentations

◆ Lu, Y., D. J. McMahon, and L. Metzger. 2014. Dispersibility, suspension ability, solubility, and gelation properties of rehydrated frozen highly concentrated micellar casein. Poster presentation at American Dairy Science Association Annual Meeting,
Kansas City, MO, Jul 20-24th
♦ Lu, Y., and D. J. McMahon. 2012. Whey syneresis upon salting of Cheddar cheese curd. Western Dairy Center Annual Meeting, Utah State University, Logan, UT, May 8th
♦ Lu, Y., and D. J. McMahon. 2012. Influence of salt and potassium chloride on whey syneresis from Cheddar cheese curd. Poster presentation at IFT Bonneville Section, Sandy, UT, April 10th

Publications

Activities and Skills
♦ Organizer and holder, BUILD Dairy Program, Utah State University, Logan, UT, 2014
♦ Finalist, Dairy Research Institute New Product Competition, 2014
♦ Team leader, First Prize for developing ’Dipeese’ – a cottage cheese dip at Idaho Milk Processors Association Dairy Product Development Contest, Sun Valley, ID, 2013
♦ First prize Poster Presentation Award in Institute of Food Technologists Bonneville Section, Sandy, UT, 2012
♦ Participant, saltiness descriptive sensory panel, Utah State University, Logan, UT, 2012
♦ Completed workshop for International Teaching Assistants, The Intensive English Language Institute, Utah State University, Logan, UT, 2012
♦ Proficient in speaking and writing Chinese and English
**Workshops**

- Preventive Controls for Human Food 2016
- Grant Writing Workshop 2012
- Quality Control Workshop (GMP) 2012
- HACCP Workshop 2012
- Employee "Train-the-Trainer" Food Safety Workshop 2012
- Statistical Process Control Workshop 2012
- Safe Quality Foods Workshop 2012
- International Teaching Assistants 2012

**Professional Affiliations**

- American Dairy Science Association
- Institute of Food Technologists
- USU Food Science club