ENVIRONMENTALLY SUSTAINABLE AND BIOCOMPATIBLE CELLULOSE-CASEIN-MINERAL COMPOSITES

A Thesis in
Agricultural and Biological Engineering
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

May 2011
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ABSTRACT

The binding affinity of the bovine casein protein system for nano- and micron- sized microbial and plant cellulosic fibers and three calcium-containing minerals was studied. A simplified reversed-phase high performance liquid chromatography method was designed for separation of the four casein subunits: alphas1-, alphas2-, beta, and kappa. Binding assays depicted the affinity of whole casein and an alphaS mixture to nano-sized microbial cellulose, micron-sized plant-derived cellulose, calcium carbonate, calcium phosphate, and hydroxyapatite as a function of protein concentration.

The binding affinity data of the whole and alphaS casein systems to cellulose and calcium carbonate were used to create a paper composite material that is both biocompatible and biodegradable. Dynamic mechanical analysis was used to determine Young's Modulus, the metric to assess paper strength. The Young’s Modulus of the cellulose-calcium carbonate control was an average of 2.86 GPa, the whole casein loaded sample, 3.12 GPa, and the alphaS loaded sample, 3.32 GPa. A second composite matrix was created where cellulose fibers were first coated with the cationic chitosan, to determine if strength was altered by enhancing the electrostatic attraction of the materials involved. In this matrix the calcium carbonate-cellulose/chitosan control had an average Young’s Modulus of 2.74 GPa, the whole casein loaded composite, 2.65 GPa and the alphaS loaded composite, 2.98 GPa. An analysis of variance was employed at the 95% confidence level to determine if the average Young's Moduli of controls and protein composites within and between both matrices were significantly different.

Note: alphaS casein is constituted of >70% pure alpha,1 and alpha,2 casein (Sigma C6780), confirmed by RP-HPLC (results not shown).
# TABLE OF CONTENTS

LIST OF FIGURES.....................................................................................................................v

LIST OF TABLES......................................................................................................................vii

ACKNOWLEDGEMENTS..........................................................................................................viii

CHAPTER I INTRODUCTION.....................................................................................................1

CHAPTER II LITERATURE REVIEW.........................................................................................6
  2.1 Historical Background of Cellulose and Casein Use....................................................7
  2.2 Issues Pertaining to the Paper Industry .......................................................................8
  2.3 Cellulose, Casein, and Minerals as Composite Materials ...........................................9
  2.4 State of the Art: Cellulose-Casein-Mineral Composite Production .............................19

CHAPTER III GOALS, OBJECTIVES AND HYPOTHESES....................................................21
  3.1 Overview of Goals and Hypotheses .............................................................................21
  3.2 Specific Objectives .....................................................................................................24

CHAPTER IV MATERIALS AND METHODS....................................................................28
  4.1 Materials Used .........................................................................................................28
  4.2 Experimental Phases and Design ..............................................................................29
  4.3 Laboratory Use and Equipment Description ................................................................41

CHAPTER V RESULTS AND DISCUSSION......................................................................42
  5.1 Phase I Results: Casein Subunit Fractionation and Confirmation ............................42
  5.2 Phase II Results: Whole Casein and AlphaS Binding Affinity .................................46
  5.3 Phase III results: Cellulose(+-chitosan):Casein:Mineral Composites .......................58

CHAPTER VI CONCLUSIONS AND FUTURE WORK..........................................................71

References.............................................................................................................................76
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Sources of pollutants in the pulp and paper industry</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>The cellulose molecule and its geometric chair conformation</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Horne’s “dual-bonding” model of casein micelle formation</td>
<td>15</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Protein-carbohydrate binding due to carbohydrate-π interactions</td>
<td>22</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Research schematic: casein binding affinity tests and composite creation</td>
<td>30</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Binding affinity assay schematic</td>
<td>33</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>1% Blotting paper method and schematic for handsheet creation</td>
<td>36</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Determination of whole casein and alphaS for complete functionalization of calcium carbonate</td>
<td>37</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Tension test method used to calculate Young’s Modulus</td>
<td>39</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Paper composite procedures</td>
<td>41</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>RP-HPLC chromatogram of whole bovine casein</td>
<td>42</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>Fraction 2 mass spectrometry profile and molecular weight</td>
<td>43</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Fraction 3 mass spectrometry profile and molecular weight</td>
<td>44</td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>Fraction 4 mass spectrometry profile and molecular weight</td>
<td>45</td>
</tr>
<tr>
<td>Figure 5.5</td>
<td>Binding affinity of whole casein to cellulose substrates</td>
<td>49</td>
</tr>
<tr>
<td>Figure 5.6</td>
<td>Binding affinity of whole casein for mineral substrates</td>
<td>50</td>
</tr>
<tr>
<td>Figure 5.7</td>
<td>Binding affinity of alphaS casein to cellulose substrates</td>
<td>53</td>
</tr>
<tr>
<td>Figure 5.8</td>
<td>Binding affinity of alphaS casein to calcium carbonate and calcium chloride</td>
<td>53</td>
</tr>
<tr>
<td>Figure 5.9</td>
<td>Binding affinity of alphaS casein to calcium phosphate and hydroxyapatite</td>
<td>54</td>
</tr>
</tbody>
</table>
Figure 5.10 Comparison of the binding affinity for the microcrystalline substrates between whole casein and alphaS systems………………………………………………………………..54

Figure 5.11 Representative control samples from matrix I and their stress-strain curves to illustrate Young’s Modulus derivation……………………………………………………61

Figure 5.12 Representative casein samples from matrix I and their stress-strain curves to illustrate Young’s Modulus derivation…………………………………………………….62

Figure 5.13 Young’s Modulus of matrix I………………………………………………………………..64

Figure 5.14 Representative control samples from matrix II and their stress-strain curves to illustrate Young’s Modulus derivation………………………………………………………….65

Figure 5.15 Representative casein samples from matrix II and their stress-strain curves to illustrate Young’s Modulus derivation………………………………………………………..66

Figure 5.16 Young’s Modulus of matrix II………………………………………………………………..68

Figure 5.17 Comparing strain at break (%) between matrices I and II……………………………..69
LIST OF TABLES

**Table 4.1** Optimized parameters for RP-HPLC separation of whole bovine casein ................32

**Table 4.2** Composite sample matrices.................................................................38

**Table 5.1** Amount of whole casein bound to cellulose and mineral substrates.................50

**Table 5.2** Amount of alphaS casein bound to cellulose and mineral substrates...............55

**Table 5.3** Matrix I tension test results.................................................................61

**Table 5.4** Matrix II tension test results.................................................................63

**Table 6.1** Average Young’s Modulus for matrix I and II........................................73
ACKNOWLEDGEMENTS

This work was funded by the USDA National Needs Graduate Fellowship Competitive Grant No. 2007-38420-17782 from the National Institute for Food and Agriculture. A special thank you to my committee members: Dr. Catchmark, Dr. Brown, and Dr. Puri, and to my labmates: Erin, Jing, Jin, Yang, Fanglin, Niharika, and Gulten.

To my brothers and sisters, who are sources of pride (and comedy) with each day: Greg, Will, Ari, Alicia and Aubree- you have much to contribute and I am fortunate to be your big sister. Mom- thank you for all of the support and your continual efforts to help me figure out my path; Dad- it has been wonderful spending time with you these last few years. I am very lucky to have such loving parents.

To Vivek Verma, thank you for all of your help and support in my first few months as a research scientist. Thank you for all of the calculus tutoring sessions; and congratulations on all of your continuing successes. Thank you to Dr. Brown who I have sought out not only as a committee member but as a friend. Your support has been unwavering and very much appreciated.

Finally, thank you to my advisor Dr. Catchmark. You are an inspiration and a significant motivational factor for me in graduate school and life beyond. Thank you for being a continual source of knowledge and enthusiasm.
CHAPTER I
INTRODUCTION

The digital age ushered in an explosion of new technology and the transformation of numerous products and manufacturing processes in many branches of society. The speed of knowledge dissemination and the acquisition of new ideas and resources vastly altered the market landscape. The projection that the computer would render conventional paper use obsolete, however, has not been realized. On the contrary, with a projected increase of paper consumption from 300 million tons in 2007 to over 400 million tons by the year 2010, the world’s dependence on paper products is growing (The Technical Association of the Pulp and Paper Industry (TAPPI), 2009). In 2002 the national economic census bureau recorded over five thousand paper manufacturing facilities in the United States with a total product value of over 150 billion dollars (United States Economic Census, 2002). Rising public pressure to cultivate renewable energy and improve recycling habits has catalyzed many paper mills to implement physicochemical and biological clean-up practices (Pokhrel and Viaraghavan, 2004). To aid with this industry transformation, the opportunity for new biocompatible paper and packaging products is another market opportunity to help “green” the pulp and paper industry both in manufacturing (using “green” chemistry and processing) and recycling (biocompatibility coupled with biodegradability).

Paper-making is an integral part of the American economy and is still working to achieve the environmentally friendly make-over of many of its industrial counterparts. Environmental guidelines vary from country to country and an extensive regulatory system of pollutant management and remediation is not fully in practice both here in the United States and abroad
The disposal of solid waste, the exhaustion of water supplies, and the use of chemicals contribute to the carbon footprint of the paper industry. Currently, about 60% of solid waste found in streams comes from paper materials (Nassar et al., 2009). Figure 1 outlines the sources of various pollutants during the processing of pulp and paper.

**Figure 1.1 Sources of pollutants in the pulp and paper industry (Pokhrel and Viaraghavan, 2004).** *(BOD = biochemical oxygen demand, COD = chemical oxygen demand, AOX = adsorbable organic halides, VOCs = volatile organic compounds).*
The fundamental component of paper and paper products is wood-derived cellulose. Cellulose abundance and biodegradability, in tandem with its rigidity and subsequent enhancement of mechanical properties make it the key ingredient in paper, past and present. However, the interest in using cellulose as a fuel source for ethanol production and its extraction from virgin forests underpin the importance in optimizing its content in paper products. Despite its biocompatibility, the separation of cellulose from lignin and other impurities requires harsh chemicals and/or expensive enzymes (Sun and Cheng, 2002). The extensive de-lignification process and the heightened desire to reduce the environmental damage from cutting down trees for paper materials reinforces the need for paper composite materials with reduced fiber content without compromising strength. Cellulose fiber dispersion and alignment are critical in dictating paper integrity and management of the amount of cellulose needed for the product. Proper fiber manipulation can reduce the amount of cellulose necessary for a high-performance paper material.

The application of nanotechnology in cellulose research, particularly the attention paid to optimizing a biocompatible method for liberation of elementary nanofibrils (the building blocks of wood), will hopefully make vast advances in the near future. Such advances will enable the creation of environmentally friendly cellulose composite materials with enhanced mechanical properties and functionality. The examination of the intricate crystalline cellulose structure, the mechanism of cellulose synthesis and crystal formation, and the functionalization of cellulose end groups are areas currently being explored in cellulose research. In addition, the molecular interactions between cellulose and other compounds that can lead to novel high-performance products are just a few of the areas for breakthroughs in bio-composite creation (Sun and Cheng, 2002).
A current focus of the biological materials laboratory in the Agricultural Engineering Department is on bio-molecules that are environmentally compatible and candidates for composite incorporation, namely proteins. Proteins are abundant, highly functional, and biodegradable; a perfect counterpart to cellulose in the quest to create a sustainable and enhanced composite. If proteins can be harnessed to act in concert with cellulose and exhibit desirable properties, the possibilities for new composite materials are endless! The proposed research intends to use a particular protein system, casein, which historically has been shown to interact with cellulose, to link a cellulose substrate with a calcium-containing mineral. Casein has a biological function to solubilize and transport calcium-containing minerals to the mammalian neonate for nutrition and bone deposition (Fox and McSweeney, 2006). By capitalizing on casein’s natural ability to solve dispersion and interfacial compatibility issues of materials with differing hydrophobicity and hydrophilicity, it is believed that cellulose fiber percolation can be engineered using the biochemical interactions that happen within the casein protein system. Casein will simultaneously function as a biodegradable binding intermediate for cellulose and a calcium-containing mineral, calcium carbonate, which is currently used by the paper industry as a coating and filler material. Casein’s unique protein functionality creates a variety of exciting new applications and events; which, if harnessed properly on the nanoscale, can become the macro-composite products with potential use in a wide variety of applications.

Aside from the quest for environmentally sustainable materials, it is important to explore areas in economic need and identify a value-added product that can bridge the needs of such an industry. Casein protein can be extracted from a variety of mammals, but the proposed research will focus on bovine casein from cow’s milk. If composite functionality is attained, another valuable product will be added to the dairy industry which is struggling, particularly in
Pennsylvania. The creation of a cellulose-casein-mineral composite will combine sustainability with economic aid to an integral part of Pennsylvania revenue, the fourth largest dairy state in the United States (Pennsylvania Dairy Task Force Economic Development Committee, 2008). Ultimately the goal of this research is to materialize dreams of a completely “green” industry by way of sustainable materials engineering geared toward paper manufacturing and recycling. There is great motivation to maintain the integrity of the paper and pulp industry by progression into a new era of clean production technology, less energy and water waste, and the production of environmentally compatible materials.
CHAPTER II

LITERATURE REVIEW

The exploration in studying cellulose fiber nanotechnology is a response to the need and for sustainable, environmentally compatible manufacturing processes, products, and recyclability of used goods. Extensive nanofiber research is occurring presently, but there is still much to be elucidated in regard to cellulose nano-composite applications. Lab-scale successes have not yet fully translated to economically viable production methods to address the macro-scale needs of the paper industry and others.

The interest in cellulose together with casein, for use in a novel biodegradable composite comes from the historic use of cellulose and casein blends, two completely renewable and natural materials. Novel binding studies of whole casein and the casein subunits will elucidate the trend behind the molecular interactions between the two materials. To this researcher’s knowledge, there has not been any quantitative assessment of cellulose-casein affinity. The incorporation of the optimal combinations of the casein subunits to bind to cellulose will be based on the results from this binding affinity assay and used for incorporation into a paper composite material. Casein’s function of mineral sequestration and binding makes this polysaccharide-protein combination the ideal duo for the enhancement of current paper materials, where calcium carbonate and other minerals are used as filling and coating material. Cellulose and casein, both abundant and inexpensive, with varying functional properties based on environmental and solvent conditions, offer a variety of engineering applications based on experimental manipulations.
The integral component of the proposed research pertains to creation of a cellulose-casein composite, where the casein material converts a calcium-containing mineral from a mere “filler” to a binder, allowing for more efficient dispersion of mineral throughout the cellulose matrix and thus a reduction of fiber material in the paper. The casein’s function as an intermediate between the mineral and the fiber will function as a biodegradable material without a reduction in mechanical properties often seen with the incorporation of conventional proteins into composite matrices. Thus it is important to understand the historic role of cellulose and casein in composite materials so that this novel biocompatible paper composite can aid in sustaining the economic importance of the pulp and paper industry given the current climate of “green” energy and materials.

2.1 Historical Background of Cellulose and Casein Use

2.1.1 Cellulose

Cellulose, the main component of plant cell walls, has been extensively studied since it was first isolated from wood in 1837 by Anselme Payen and has been applied in a variety of products throughout the years (Hon, 1994). Cellulose is an un-branched, linear polymer of \( \beta(1,4)-D \)-glucose used to make textiles, plastics, and paper, among other items.
Figure 2.1 The cellulose molecule (a) and its geometric chair conformation (b) (Hon, 1994) showing the exposed hydroxyl groups which are able to react with various chemicals to create new cellulose derivatives for material applications.

The role of cellulose as the rigid structural reinforcement of plant materials, leads to improved load-bearing and mechanical performance of composites, which are of particular interest to engineers. The complexity of cellulose molecular interactions and behavior in various solvent systems leads to present day research in which the foundation of synthesis and assembly is still being elucidated. The focus on cellulose synthesis and assembly translate to materials engineering through manipulation of cellulose functionality and external conditions as researchers are attempting to discover and exploit novel properties for composite materials. Much of the research in cellulosic materials is devoted to discovering a way to synthesize and
degrade cellulose efficiently, cost-effectively, and without the need for harsh chemicals (Brown et al., 1996).

2.1.2 Casein

Casein, comprising 80% of the proteins found in milk, is the most studied food protein dating back to the early part of the 1800s (Fox and McSweeney, 2003). Historically, the strong binding capacity of casein was exploited for blends and agents to make desirable products with compatible materials, such as wood glues, paints, and packaging materials (Clemons, 2008). This strong binding capacity and the unique structure make casein an attractive candidate for various applications with other proteins, polysaccharides, and minerals.

2.2 Issues Pertaining to the Paper Industry

Approximately 900 years ago the Chinese discovered a method to make paper sheets using bast fibers, old clothes, and agricultural residual waste (Hon, 1994). Thus, paper-making was born, crowning cellulose as the king of paper materials from that point in history to the present day. The environmental effects of the “modern” paper industry are vast and well-documented. Although some disagreement exists concerning the extent of the impact on terrestrial ecosystems, it is undisputed that there are two major challenges faced by the industry today: solid and chemical waste disposal and the sizable amount of water used for manufacturing. As of 2009 about 60% of the solid waste in municipal streams consisted of agricultural waste with about 30% as paper and paper products (Nassar et al., 2009; EPA, 2009). The potential leaching of pollutants used in paper manufacturing outlined previously consist of organic compounds and suspended solids, and chemical wastes such as strong acids, bases, and
organic halides (Pokhrel and Viraraghavan, 2004). The potential to eliminate chemical output from papermaking includes advances in separating cellulose from lignin and hemicelluloses (not discussed in the scope of this research) and in generating paper materials that use natural, aqueous-based solvents and eliminate the need for harsh chemicals for production. This latter technique applies to the proposed research as nanoscale manipulation of binding affinity will preclude the need for chemical cross-linkers, resins, and other materials typically used to make novelty paper products.

As far as the other critical issue, the recyclability of paper materials, biodegradable paper materials come into play here. The Environmental Protection Agency (EPA) cites that approximately 1/3 of municipal waste is comprised of paper products (EPA, 2009). Land destruction in order to create new landfills is slated to increase with the exponential increase in paper consumption, unless biodegradability of paper materials is further explored. Using recycled paper is one way to combat the issue of waste generation from the paper industry, however, there is still an extensive production process in re-making paper out of already used paper products, requiring the removal of inks, coatings, and adhesives (Nasser et al., 2009). A biodegradable composite material coupled with environmentally friendly substitutes for potentially harmful additives and processing chemicals would be a valuable product for manufacture by the paper industry.

2.3 Cellulose, Casein, and Minerals as Composite Materials

2.3.1 Synthetic versus bio-composites
A composite is made up of two or more distinct materials that together, provide new and unique material properties. Synthetic polymer composites consist of plastics (the matrix) and a reinforcing material (fibers or particles). Paper is a composite material with the wood-derived cellulose matrix and the addition of calcium carbonate particles, clays, and synthetic filler materials. Completely natural fibers, like cellulose, usually lack the high performance of their synthetic counterparts, but through understanding binding interactions on the nanoscale, it may be possible to replace synthetic materials by capitalizing on binding interactions thus preventing the need to compromise on mechanical properties (Berger, 2008).

2.3.2 Cellulose as a composite material

Chemical modification of cellulose molecules has been performed by scientists since the late 1800s. These modifications include: acetylation, acid hydrolysis, and methylation among others (Hon, 1994). The widespread exposure of hydroxyl groups on the cellulose chains enable reactivity with various chemicals to yield new cellulose derivatives with varying properties, functionalities, and subsequent composite applications. Figure 2.1 depicts the linear structure of cellulose and its associated geometric chair conformation (Hon, 1994).

Acid hydrolysis, one method to remove the lignin naturally found alongside cellulose in wood, uses harsh chemicals rendering cellulose fibers that must go through extensive pre- and post treatments to be marketed for consumer products. Enzymatic hydrolysis, another avenue to break cellulose chains, is expensive and not yet feasible for scale-up from the laboratory to processing in industry (Sun and Cheng, 2002).

2.3.3 The importance of nanocellulose
On a macro level, cellulose is the most abundant bio-available polymer and comprises approximately 90% of cotton and 50% of wood (Patel, 2008). Cellulose has been embraced as the paper-making feedstock of choice for decades and should continue to be so with new insights into its molecular make-up and manipulation of its assembly properties on the nanoscale. With the ability to understand a polysaccharide’s functionality through its individual monosaccharide subunits, its geometrical and spatial properties, and the chemical bonds linking the units together, creation of new materials with adjustable, functional properties is now possible (Fishman, 2004). The increased surface area of nanosized fibers opens up a slew of new sites for interaction with other materials. Research has already shown that nanofibers exhibit a higher modulus of strength than their micro- and macro counterparts; thus, breakthroughs in improvement of strength and optical properties of paper materials using less energy and materials is on the horizon (Berger, 2008).

Cellulose nanofibers from bacteria are also an exploratory and important aspect of nanocomposite research as their high degree of crystallinity, high aspect ratio, and high bio-compatibility are desirable qualities for various applications (Eichorn et al., 2001). Again, there is still much research to be done to combat the issues with the material uniformity manifest in the deformation properties of cellulose fibers. Given the material anisotropy and challenge in measuring local deformations, cellulose fibers are difficult to mechanically characterize and understand, including at nano dimensions (Eichorn et al., 2001).

2.3.4 Casein as a composite material

Casein, comprised of four individual subunits (alpha_1-, alpha_2-, kappa-, beta-), is an abundant amphiphilic mammalian protein secreted in the mammary glands to solubilize and
transport calcium-containing minerals to the neonate for nutrition (Damodaran et al., 1997). The particular interest in using the bovine casein protein system as an integral part of a cellulose composite is two-fold; to provide another product for the dairy industry and to explore new structure-function relationships between casein as a function of its affinity for cellulose.

The dairy industry provides over 40,000 jobs making Pennsylvania the fourth largest dairy state in the United States; an industry that needs additional stable products to boost vitality (Pennsylvania Dairy Task Force Economic Development Committee, 2008). Presently, most dairy farmers are running on borrowed credit as a surplus of milk supply in the market has farmers earning less than production costs (Malawsky, 2009). If a casein-composite material can be scaled-up for industrial use, the demand for milk generated by the dairy industry could allow for a boost in revenue, repayment of credit lines, and subsequent prevention of farm foreclosure; an unfortunate incident that has occurred steadily over the past decade (Malawsky, 2009).

The second interest in using casein as the protein of choice for a composite material stems from the paradigm shift in protein structure-function relationship that is spanning the biological sciences. For many years casein was thought to lack higher order structure because of its inability to be crystallized, its high thermal stability, and its high proline content. Through infrared spectroscopy methods, proteins thought to be “unstructured” or disordered are now viewed with a new degree of order; defined as “molten globule” and “pre-molten globule” intermediate states. This is important because instead of the catch-all “native” or “denatured” states, protein conformation is understood as a dynamic event with a range of energy states and conformations possible depending on external conditions (Fishman et al., 2006). This paradigm shift in the view of protein functionality ushers in new thoughts about how to engineer proteins with polymers and other macromolecules for many different applications (Fishman et al., 2006).
Viewing casein as a protein with a range of stable intermediate states, each state able to be manipulated for subsequent interactions, will open up the ability to harness varying functionalities and interactions both with its fellow protein subunits and other molecules.

Despite the fact that the primary structure of each casein subunit is well-established, the interactions of the subunits i.e. the casein micelle, is still being debated (Fox and McSweeney, 2003; Horne, 1998; Damodaran et al., 1997). The minimal requirements for a casein micelle are alpha casein, kappa casein, and a divalent cation (Ho and Waugh, 1964). Infrared spectra support the hypothesis that the sites for calcium binding are organic phosphate groups with binding occurring primarily through the varying quantities of seryl residues located along the subunits (Cross et al., 2005). Various models have been published concerning bulk casein interactions among the subunits in a variety of experimental conditions. One popular model is shown below in Figure 2.2 (Horne, 1998). This “dual-bonding model” shows bonding between the casein’s hydrophobic regions (black rectangles) and the colloidal calcium phosphate (CCP), and between the casein’s hydrophilic regions (curly lines) and the CCP.
Horne’s model shows kappa casein as the limiting factor in micelle formation because of its inability to bond more mineral, containing at most only one phosphoseryl residue. In addition, kappa casein’s clearly defined hydrophilic region preferentially interacts with the aqueous solvent thereby limiting further growth. This model is included to visualize the complexity of the binding interactions that exist among casein itself and to promote possible insight into how calcium-containing minerals could be incorporated into the array of bonding. Alphas,2 and alpha,1 are the most calcium-sensitive caseins with the highest amount of phosphoseryl residues (10-13 and 8-9, respectively) (Fox and McSweeney, 2003).

Akiyoshi and Sunamoto have shown that amphiphilic molecules spontaneously aggregate in solution and can form a varying degree of self-aggregates, micelles, membranes and vesicles (Akiyoshi and Sunamoto, 1996). This tendency for aggregation has not yet been studied using various forms of cellulose as a substrate material simultaneously incorporating minerals that bind through the phosphorylated serine residues found on the majority of casein subunits, to the knowledge of this author. The interaction of cellulose with whole casein and each of the four individual subunits will be studied varying experimental conditions to elucidate the binding capabilities and optimal parameters for production of a composite material(s). Mineral incorporation will include not only calcium carbonate, but hydroxyapatite for other applications incorporating osteoinductive calcium-containing materials that can be further developed for

Figure 2.2 Horne’s “dual-bonding” model of casein micelle formation; with bonding between casein subunits hydrophobic and hydrophilic moieties and CCP (Horne, 1998).
biocompatible polymer bone scaffolds and other tissue engineering applications as will be discussed in later sections.

2.3.5 Nano-composites and the bigger picture

To enhance the environmental compatibility of paper-based materials currently on the market it is necessary to understand the binding interactions that occur on the nanoscale in an effort to manipulate parameters to scale-up from the laboratory to industry efficiently without adversely affecting the environment. As the interest in cellulose as an alternative fuel continues to increase, the more efficient use of the natural feedstocks of this biopolymer that provides a substantial portion of paper products, clothing, building, and biomedical materials will be necessary.

In a 2007 conference discussing new perspectives in paper chemistry, Professor Tom Lindstrom stated that novel composite formulas for packaging materials is the sector of the paper industry most likely to be developed in the immediate future. He emphasized the importance of wood-derived cellulose nanofibers for the creation of “nanopaper,” which would be much stronger than any previous man-made item (Lindstrom, 2007). Not long after Lindstrom’s statement, “nanopaper” created by isolation of the nanometer thick individual cellulose strands from plant cell walls was successfully completed using fibers 10-40 nm in width. The resultant product can withstand about 2/3 more force than cast iron before yielding (Patel, 2008).

Recent study in nanocellulosic films for application to paper coatings and packaging materials has had success in enhancing certain mechanical properties. A Japanese research team has developed a process to successfully create a transparent and flexible nanocellulosic film with a high tensile strength, high oxygen barrier properties and a low thermal expansion (Fukuzumi et
Research done by Yang et al testing various solvents to create casein-cellulose blends recognizes the opportunity for new products due to the extensive hydroxyl groups on cellulose to bond with other materials, specifically proteins. The results from this study indicate that cellulose/casein blend membranes can be formed with varying degrees of miscibility and tensile strength based on the casein concentration and solvent system used. More research is needed to optimize the blend process for commercial interest (Yang et al., 2000). While the goal of this proposed research involves testing various concentrations of casein with cellulose for composite creation, the novelty arises with its incorporation of a mineral binder typically used in paper materials.

More recent study in protein-cellulose binding for composites involved purification of cellulose-binding domains (CBDs) from cellulases, forming two into a cellulose cross-linking protein (CCP) (Levy et al., 2002). The CCP was immersed onto cellulose paper and its mechanical properties tested. It was found that the tensile strength increased, brittleness decreased, and the energy to break increased. It was also found that the cellulose cross-linking protein treatment enhanced the water-repellent capability of the cellulose filter paper; a necessary attribute for products to be used by consumers.

As can be seen there are a variety of nanocellulosic composite studies presently, especially geared toward nanoscale paper applications through creation of coatings/laminations and packaging material with enhanced protection against water and oxygen penetration. The creation of a high-performance, biodegradable product that is able to scale-up efficiently has still
not been realized but potentially can approach fruition with development of the proposed cellulose-casein-mineral composite.

2.3.6 Biomedical industry

Cellulose is being studied as a desirable matrix for bone and tissue engineering as it is biocompatible and has mechanical properties that closely resemble hard and soft tissue. Its porosity and ability to form into a variety of shapes makes cellulose an attractive candidate for implants and drug delivery devices (Wan et al., 2006). Hydroxyapatite, the main constituent of bone and a form of calcium-phosphate, is the mineral of choice to promote osteoinduction in decaying or damaged bone tissue. Current opinion is that cellulose does not have enough reactivity to grow cellular hydroxyapatite on its own and that the cellulose fibers cannot make a tight enough bond with the bone (Wan et al., 2006; Fricain et al., 2001).

Cellulose is also being studied as a cartilage and tissue scaffold after activation by calcium hydroxide solution (Muller et al., 2006). Typically, microbial cellulose, with a higher Young’s Modulus and crystallinity than plant-derived cellulose and almost 200 times more surface area than softwood cellulose, makes it an appropriate candidate for biomedical research (Wan et al., 2006). Microbial cellulose is also very moldable and has already experienced considerable success in implants and with wound-care and regeneration (Czaja et al., 2006).

2.4 State of the Art: Cellulose-Casein-Mineral Composite Production

The challenge of maintaining high-performance in the presence of water, scaling-up composite production from laboratory to industry, and the optimization of binding through
understanding molecular interactions, are areas that need further exploration in the realm of cellulose and casein composite materials. Composite creation requires a delicate interplay between materials, binding interactions, and external solvent, temperature, and processing conditions. There are a variety of issues with scaling-up from the laboratory to the industry and implementing sustainable processing techniques throughout the product’s lifecycle. Once a bio-composite is successfully created and functionalized, it has potential for useful applications spanning many fields.

The goal of this research is to bridge two ubiquitous, biodegradable materials (cellulose and casein) with inorganic calcium-containing minerals used in the paper industry. While the immediate goal is to improve the strength to weight ratio of paper, there are limitless applications for a composite material of this type in the biomedical field and elsewhere. For instance, in the biomedical field, the proposed research could enhance osteoinduction through a cellulose-casein-hydroxyapatite composite resulting in adequate bone cell adhesion and proliferation. The cellulose-casein-mineral composite may also act as a tissue scaffold material, which over time can be engineered to degrade in the body leaving only the hydroxyapatite to stimulate cell proliferation and bone growth.

Additionally, the use of casein as an integral component of this research and subsequent product use greatly enhances its value which could aid the struggling dairy industry in Pennsylvania and across the nation. Cellulose has been extensively studied for a variety of materials and bio-composite applications but has not been assessed for its binding ability to the biocompatible, flexible, and mineral-carrying bovine casein protein system. Ultimately, it is believed that development of a labscale composite production method could be scaled-up to an industrial system which would use entirely “green” processing techniques and materials. This
creates a sustainable product lifecycle which is environmentally compatible from creation through degradation.

CHAPTER III

GOALS, OBJECTIVES AND HYPOTHESES

The overarching research goal is to develop an environmentally compatible laboratory-scale method to create a novel casein-cellulose-mineral composite using the casein protein system as a binding intermediate between cellulose fibers and calcium-containing minerals. Immediate applications will be able to address some of the issues in the paper industry, namely, the weakening of paper materials by filler materials such as calcium carbonate, and the reduction of fiber material in products due to the enhanced binding realized by understanding nanoscale interactions between casein, cellulose, and calcium-containing mineral.

3.1 Overview of Goals and Hypotheses

It is hypothesized that casein-cellulose binding is a function of non-covalent interactions, specifically: electrostatic, hydrophobic, and carbohydrate-π (stacking) interactions. Binding events between the components will depend heavily on casein’s primary structure and external solvent environment. Electrostatic interactions occur between casein’s polar amino acid residues and the negatively-charged hydroxyl groups on the cellulose chains. Because casein is a highly negatively charged protein an additional matrix to composite creation will involve the coating of cellulosic fibers with a positively-charged bio-polymer (chitosan) with the hopes of initiating a
stronger binding event predominately through electrostatic interactions. Hydrophobic interactions will involve casein’s hydrophobic amino acid regions and cellulose C3 groups. The carbohydrate-π interaction is a relatively recent molecularly-modeled phenomenon that accounts for a mechanism of recognition between proteins’ aromatic amino acids and the sugar groups on polysaccharides (Kiehna et al., 2007). Figure 3.1 models the recognition and subsequent binding of a protein-carbohydrate system due to this type of reaction.

Figure 3.1 Recognition between a protein and carbohydrate due to carbohydrate-π interactions (Kiehna et al., 2007).

It is hypothesized that carbohydrate-π interactions will encompass another means of casein-cellulose binding through the protein’s aromatic amino acids with the numerous carbon rings throughout the cellulose chains. Since tyrosine is implicated in the binding of cellulose in cellulose-binding domain proteins (Tormo et al., 1996), it is hypothesized that casein’s regions of tyrosine clusters will participate in this binding reaction. With the various non-covalent binding interactions in mind, it is believed that casein-cellulose binding will be heavily dependent on the
protein primary structure and availability of amino acid side chains due to protein subunit association.

The literature details many studies that show casein strongly binds divalent cations by its phosphoseryl residues (Ho and Waugh, 1964; Damodaran and Paraf, 1997; Fox and McSweeney, 2003; Cross et al., 2005). This ability will manifest as a mechanism to incorporate minerals into the cellulose fiber network. It is believed that the casein subunits with the highest amount of phosphoserine residues and aromatic amino acids, will optimally link cellulose and mineral. It is also hypothesized that the strength of binding between casein, cellulose, and minerals can be adjusted by the amount and existence of the various subunits in the material and external conditions such as pH which dictates amino acid residue charge. These properties will allow casein to be a novel linker between cellulosic fibers and minerals (i.e. calcium carbonate for paper products and hydroxyapatite for biomedical materials).

The main objectives of the research are to efficiently purify/separate whole bovine casein into its four subunits. These subunits, in addition to whole casein, will be bound to cellulose to derive the binding affinity of the casein to cellulose fibers microcrystalline cellulose derived from plants and microbial cellulose nanofibers. The binding between the caseins and calcium carbonate and hydroxyapatite will also be determined for paper product applications and biomedical device applications, respectively. Determination of the binding affinity will allow for creation of cellulose-casein-mineral composites or coatings imparting high mechanical performance in paper products. Other composite types consist of biocompatible composites with controllable attachment for biomedical materials applications.
Whole casein, being the most economically viable (low cost and ease of preparation) will be the intended focus of the composite studies. However, it is feared that complex data with difficulty in interpretation due to whole casein’s intricate micelle structure and subunit interactions may preclude elucidation of the exact molecular events behind binding to cellulose and mineral. Therefore, a mixture of alpha\(_2\) and alpha\(_1\), the proposed optimal binders based on amino acid sequences will be used in the experimental matrix to compare with the whole casein and non-casein control. Alpha\(_1\) is also the most abundant bovine fraction and possesses a specific architecture hypothesized to be the most amenable to cellulose and mineral binding. Isolation of the two alpha\(_S\) units together from the rest of the casein system is considerably more economic than further separation into individual subunits. This could translate into the feasibility of a scale-up process if the proposed binding hypothesis is supported and paper composite properties are enhanced when using the combination of alpha\(_S\) subunits.

Reversed-phase high performance liquid chromatography (RP-HPLC), while effective, is not a viable method to scale-up for production in separation of casein into its four subunits. This is a direct result of the chemical and labor input, the small microgram output of each protein fraction per run, and further downstream preparation purification requirements so that the protein fraction may be used in paper materials. However, it is still advantageous to optimize a RP-HPLC method for future use in laboratory studies with the individual casein subunits, and therefore will be done for future laboratory-scale experimentation.

3.2 Specific Objectives

Objective I
To separate/purify bovine casein into its four individual subunits: alphas1-, alphas2-, beta-, and kappa- casein using a simplified and repeatable RP-HPLC method.

**Objective II**

To depict binding affinity for both whole bovine casein and alphaS for cellulose (microbial and plant-derived) and three different calcium-containing minerals: calcium carbonate, calcium phosphate, and hydroxyapatite.

- **Hypothesis IIA**

  $H_0$: There is no significant difference in binding affinity to cellulose between the two casein systems.
  
  1. $\mu_{wcn-mcc} = \mu_{as-mcc}$
  2. $\mu_{wcn-cnw} = \mu_{as-cnw}$

  $H_a$: There is a statistically significant difference between the two types of casein systems studied, alphaS or whole casein, in binding cellulose. *This affinity will be a function of the differences in amino acid sequences of each subunit and the tendency for self-aggregation.*

  1. $\mu_{wcn-mcc} \neq \mu_{as-mcc}$
  2. $\mu_{wcn-cnw} \neq \mu_{as-cnw}$

- **Hypothesis IIB**

  $H_0$: There is no significant difference between the casein systems’ affinities for microscale cellulose versus nanoscale cellulose.
1. \( \mu_{\text{wcn-mcc}} = \mu_{\text{wcn-cnw}} \)
2. \( \mu_{\text{as-mcc}} = \mu_{\text{as-cnw}} \)

\( H_0: \) There is a significant difference between the casein system’s affinities for microscale versus nanoscale cellulose. \textit{This is due to the increased surface area of the nanoscale substrate to participate in the tyrosine-glucose ring recognition expected to orchestrate casein-cellulose affinity.}

1. \( \mu_{\text{wcn-mcc}} \neq \mu_{\text{wcn-cnw}} \)
2. \( \mu_{\text{as-mcc}} \neq \mu_{\text{as-cnw}} \)

- \textbf{Hypothesis IIC}

\( H_0: \) There is no significant difference in the binding affinity of the two casein systems for calcium-containing minerals (calcium carbonate, calcium phosphate, or hydroxyapatite).

1. \( \mu_{\text{wcn-caco3}} = \mu_{\text{as-caco3}} \)
2. \( \mu_{\text{wcn-cahpo4}} = \mu_{\text{wcn-cahpo4}} \)
3. \( \mu_{\text{wcn-HA}} = \mu_{\text{as-HA}} \)

\( H_0: \) There is a significant difference in the binding affinity of the two casein systems to the calcium-containing minerals.

1. \( \mu_{\text{wcn-caco3}} \neq \mu_{\text{as-caco3}} \)
2. \( \mu_{\text{wcn-cahpo4}} \neq \mu_{\text{as-cahpo4}} \)
3. \( \mu_{\text{wcn-HA}} \neq \mu_{\text{as-HA}} \)

\textbf{Objective III}
To create a high performance cellulose-casein-mineral paper composite using the binding affinity data to identify adequate protein concentration for a 10% (w/w to cellulose) mineral load. To use the Young’s Modulus as a metric for strength of composite materials containing alphaS versus whole casein.

- **Hypothesis IIIA**

H₀: There will be no significant difference in strength as quantified by the Young’s Modulus between the mineral-cellulose control composite and the casein-loaded composite.

1. \( \mu_{BP-\text{caco3}} = \mu_{WCN-BP-\text{caco3}} \)
2. \( \mu_{BP-\text{caco3}} = \mu_{\text{as}-BP-\text{caco3}} \)

Hₐ: There will be a significant difference between the Young’s Modulus of the mineral-cellulose control composite and that of the casein-loaded composite. *It is initially hypothesized that the alphaS system will be the optimal casein counterpart for the composite materials, but binding affinity data may indicate otherwise.*

1. \( \mu_{BP-\text{caco3}} \neq \mu_{WCN-BP-\text{caco3}} \)
2. \( \mu_{BP-\text{caco3}} \neq \mu_{\text{as}-BP-\text{caco3}} \)

- **Hypothesis IIIB**

H₀: There will not be a significant difference in Young’s Modulus between the whole casein composites and the alphaS composites in both matrices.

1. \( \mu_{WCN} = \mu_{\text{as}} \)
2. \( \mu_{WCN-CS} = \mu_{\text{as-CS}} \)
Ha: There will be a significant difference in Young’s Modulus between the whole casein composites and the alphaS composites in both matrices.

1. \( \mu_{\text{wcn}} \neq \mu_{\text{as}} \)
2. \( \mu_{\text{wcn-CS}} \neq \mu_{\text{as-CS}} \)

Objective IV

To create a second composite matrix by first coating the cellulose fibers with a 1% (w/v) chitosan solution. The strength, as quantified by the Young’s Modulus, of the cellulose-chitosan-casein-mineral composites will be compared with the first composite matrix without chitosan present.

- Hypothesis IV:

Ha: There will not be a significant difference in Young’s Modulus between the control composites and the protein-loaded composites in matrix II.

1. \( \mu_{\text{BP-CS-caco3}} = \mu_{\text{wcn-BP-CS-caco3}} \)
2. \( \mu_{\text{BP-CS-caco3}} = \mu_{\text{as-BP-CS-caco3}} \)

Ha: There will be a significant difference in Young’s Modulus between the control composites and the protein-loaded composites in matrix II. Casein’s highly negative charge might preclude binding with cellulose via carbohydrate-\( \pi \) interactions thus necessitating a positively-charged fiber matrix to electrostatically enhance binding. The chitosan composite matrix might show statistically significant different strength properties than the composites not containing the chitosan as a result of better casein distribution among the fibers.

1. \( \mu_{\text{BP-CS-caco3}} \neq \mu_{\text{wcn-BP-CS-caco3}} \)
2. \( \mu_{\text{BP-CS-caco3}} \neq \mu_{\text{as-BP-CS-caco3}} \)
CHAPTER IV

MATERIALS AND METHODS

The fabrication of a high-performance composite or coating for paper materials using casein as a linker between cellulose and a calcium-containing mineral requires knowledge of the portion of casein with the optimal binding affinity for the two materials. This section will outline the materials used and the research phases that begin with protein separation and purification and end with composite assembly.

The research project hinges on casein-cellulose binding and casein-mineral binding as a function of protein concentration with the two protein systems consisting of: whole bovine casein and an alpha_s1 and alpha_s2 (denoted “alphaS”) mixture. The importance of protein concentration in binding affinity is a function of the complex molecular interactions that are believed to promote binding between the protein and polysaccharide and the protein and mineral. It is hypothesized that casein concentration and solvent system pH will affect binding to cellulose and mineral due to the variation of binding type (hydrophobic, electrostatic, et al) and from steric hindrance, which are related to casein concentration and variation in solvent conditions.
4.1 Materials Used

Whole bovine casein (Sigma C7078) and alphaS casein (Sigma C6780) were lyophilized and suspended in filtered water prior to use. Whole casein was raised to a pH of 10 using sodium hydroxide and heated at 40°C for 30 minutes to maximize solubility. The minerals used consisted of: calcium carbonate (EM Science CXO120-2), dibasic calcium phosphate (Baker 1430-01), hydroxyapatite (Sigma 677418), and calcium chloride (Sigma 223506). Low molecular weight chitosan (Sigma 448869) was used for matrix II of the composites. The cellulose substrates consisted of microcrystalline cellulose (Sigma 435236) and nano-sized cellulose which was created from microbial cellulose of the *Acetobacter xylinum* strain 700178. Their size dimensions were reduced by hydrochloric acid hydrolysis and nanoscale dimensions were confirmed by transmission electron microscopy performed by another labmate. All pH adjustments were done with sodium hydroxide (VWR 1310-73-2) or acetic acid (EMD AX0073-9). Cellulose blot paper was ordered from Dick Blick Art Materials, Galesburg, Illinois.

4.2 Experimental Phases and Design

The research project consisted of six phases, which are schematically represented in Figure 4.1.
4.2.1 Phase 1- Objective I: Separation of bovine casein subunits

Phase 1 involved the separation of the bovine casein subunits by reversed-phase high performance liquid chromatography (RP-HPLC). Whole bovine casein was purchased lyophilized, simplifying the sample preparation for chromatography. The method protocol was optimized based on published chromatography work with bovine caseins (Leonil et al., 1994) but optimized factoring in a new chromatography column, RP-HPLC system, and the desired solvent system preparation and conditions.

The motivation behind studying the subunit that optimally binds to cellulose stems from the fact that the amino acid sequences of all of the casein subunits are known; thus determination
of the subunit that interacts optimally with cellulose would support the hypothesis that a particular amino acid sequence either sterically permits or has amino acid motif(s) which through structure, charge, and behavior, allow preferential binding to cellulose.

Whole casein solution was prepared by mixing 5.0 mg/ml whole bovine casein (Sigma C7078) in sodium hydroxide (NaOH)-adjusted reverse osmosis (RO) water (pH 10) with 0.01% beta-mercaptoethanol added as a reducing agent. The protein was pumped through the HPLC system and fraction collected when eluting off of the column in the order of increasing hydrophobicity. Approximately 75 runs were attempted of different temperatures, column size, solvent conditions, and gradients. The most optimal and simplified conditions are listed in Table 4.1. The instrument used was a Shimadzu LC-20 A/B pump system. Un-separated whole bovine casein was also used for testing binding affinity to cellulose and minerals allowing direct advancement to Phase 3.
Table 4.1 Optimized parameters for RP-HPLC separation of whole bovine casein

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>25 °C</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Solvent A</td>
<td>0.05% Trifluoroacetic Acid (TFA): HPLC-grade water</td>
</tr>
<tr>
<td>Solvent B</td>
<td>80% Acetonitrile: 20% of Solvent A</td>
</tr>
<tr>
<td>Column size</td>
<td>Viva C4 250X4.6 mm ID</td>
</tr>
<tr>
<td>Gradient</td>
<td>0-85% B 0-30 min., 85%-100% B 30-33 min., 100% B 33-34 min., 100-0% B, 34-35 min.</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>19,000XG for 15 minutes to pellet any insoluble casein portion so as not to damage column</td>
</tr>
<tr>
<td>Monitored wavelength</td>
<td>210 and 280 nm</td>
</tr>
</tbody>
</table>

4.2.2 Phase 2- Objective I: Confirmation of subunit separation

Phase 2 confirmed casein subunit separation following fractionation by RP-HPLC. This confirmation was achieved by tandem liquid chromatography-mass spectrometry using the known amino acid sequence, phosphorylation, and glycosylation pattern of the individual subunits.
4.2.3 Phase 3- Objective II: Binding analysis- testing hypotheses IIA, IIB, and IIC

Phase 3 involved the determination of the binding trends of whole casein and the alphaS mixture with the five substrates of interest: microcrystalline plant cellulose, nanofiber microbial cellulose, calcium carbonate, calcium phosphate, and hydroxyapatite. The binding affinity of the alphaS casein system for calcium chloride was also tested. The experimental method to determine binding affinity is a binding assay designed in the laboratory using ultraviolet-visible spectroscopy. The adjusted parameters included pH, water system, and protein concentration in both whole casein and alphaS cases. Figure 4.4 outlines the experimental matrix for the assay.

**Figure 4.2 Binding affinity assay schematic.**
Different casein concentrations and a non-protein control, were mixed with one of the five substrates (5.0 mg/ml) in two different solvent systems to study the effects of pH. The casein-cellulose or casein-mineral solution rotated at 40 rpm for 30 minutes to allow for binding, followed by centrifugation at 19,000X G for 20 minutes to ensure that all cellulose or mineral was pelleted with bound caseins. The supernatant (containing unbound protein) was extracted for ultraviolet (UV) spectroscopy. The absorbance of the unbound casein protein was read at 280 nm and concentration was calculated based off of an established casein calibration curve in the case of whole casein, or the published extinction coefficient for alphaS (Creamer et al, 2009; Fox and McSweeney, 1998). In the case of whole casein the absorbance value was deducted from the original absorbance of the stock casein protein solution giving the amount of casein that was bound to the substrate. The binding affinity assay was done for two different sample sets (whole casein and alphaS) and repeated three times using different preparations of casein to ensure randomization.

Through quantification of protein concentration before and after binding, binding curves were generated of the particular casein system’s affinity for cellulose and mineral in the various treatments. By plotting the concentration of casein bound versus the initial concentration of casein and using the Langmuir equation, one mathematical model for molecular adsorption, binding constants can be generated for each casein system to the substrates of interest. The assumptions for use of the Langmuir equation include i) monolayer coverage of substrate, ii) equivalence (uniformity) of substrate surface sites, iii) independence of binding sites on the substrate, that is to say, no interaction between adjacent adsorbed molecules, and iv) the existence of a dynamic equilibrium between adsorbing molecules and the free molecules in solution (Nelson, 2001; Liu, 2006). These are hefty assumptions to make in regard to a casein
solution when there is not uniformity between the subunits themselves. However, if the binding data can be fit with this simplified equation a binding constant could theoretically be generated and used to predict concentration-dependent binding between the different casein systems and the variety of substrates tested. Additionally, an analysis of variance (ANOVA) between the different means of the sample sets will determine if there are statistically significant differences between the various means (as outlined by the hypotheses) using a 95% confidence level (alpha=0.05).

4.2.4 Phase 5- Objectives III and IV: Composite creation using binding affinity data

Phase 5 used whole casein and the alphaS casein to create paper composite films with calcium carbonate as the preferred mineral system. Ideally whole casein and calcium carbonate will create composite materials with heightened mechanical properties due to the difficulty in large-scale casein separation and the low cost of calcium carbonate, however, the alphaS casein system was also incorporated into composites and tested. The handsheet creation method was developed by a laboratory co-worker and modified after substantial collaboration. Figure 4.3 contains the detailed paper-making methods for each composite sheet.
1% Blot Paper Control Method:
1) By hand, tear 80.8 g of cellulose blot paper into ~10X10 mm pieces
2) Put 20.2 g of paper into four, 4000 ml flasks
3) Add 1000 g filtered water (FW) to each flask
4) Soak for 1 hour
5) Using blender on “blend” setting, mix each solution for 5 seconds (3X)
6) Pour a flask’s content of the blot paper solution into the white bucket and add 1020 g more FW water (2020 g total) [do 4X for each flask]
7) Stir with electronic mixer for 2 days on 50 rpm in large bucket, seal when finished until use

0.1% Blot paper control:
1) Weigh out 110 g blot paper solution
2) Bring up to 1100 g with FW
3) Use handsheet apparatus to make paper sheets
4) Empty out bucket, replace FW, rinse screen between each sheet
5) Make sheets and layer between filter paper
6) Press at ~2800 lbs for 5 minutes
7) Heat at 80°C for 1 hour

Figure 4.4 details the method for determining the amount of whole casein and alphas to be mixed with calcium carbonate to ensure complete functionalization of the mineral. The calculations were based on the ultraviolet spectroscopy binding affinity data (results in following chapter).
• How much casein to add to 110 mg CaCO3 f(10% w/w of cellulose) for composites?
  – According to binding affinity study
    • the range of alphaS binding by 2.5 mg CaCO3 was 0.034-0.127 mg, thus, 5.2 mg alphaS casein should be completely bound by 110 mg CaCO3
    • The range of whole casein binding by 2.5 mg CaCO3 was 0.009-0.099 mg, thus, 4 mg of whole casein should be completely bound by 110 mg CaCO3
• Wanted to ensure more than maximal coverage since watching out excess—therefore, 6 mg used of each casein type.
• How to mix casein + CaCO3?
  – Dissolve 6.0 mg whole casein in NaOH-adjusted filtered water (pH 10) by heating at 40°C for 20 min; note, if using alphaS use filtered water as is (pH 7)
  – Add 110 mg CaCO3 to casein solution and rotor for 30 min
  – Centrifuge 19,000 X g for 15 min
  – Dump supernatant (unbound caseins)
  – Wash pellet gently and decant
  – Centrifuge 10,000Xg for 15 min
  – Decant, re-suspend pellet in paper solution

Figure 4.4 Determination of the amount of whole casein and alphaS for complete functionalization of calcium carbonate.

After mixing for approximately two hours, the solution was poured through a screen and formed into a paper film using a press with approximately 2800 pounds (+/- 300 pounds) of force applied for five minutes. The sample matrix is listed in Table 4.2. Each sample was made in triplicate. The weight of blot paper solution per sheet and the applied force for pressing comply with TAPPI 2009 standards for handsheet preparation and testing (TAPPI 220 “Physical Testing of Handsheets”, 2009).
Table 4.2 Composite sample matrices

<table>
<thead>
<tr>
<th>Matrix I-pH 7</th>
<th>Matrix II-pH 5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% BP control</td>
<td>0.1% BP+CS</td>
</tr>
<tr>
<td>10% CaCO3</td>
<td>10% CaCO3+BP/CS</td>
</tr>
<tr>
<td>10% CaCO3+WCN</td>
<td>10%CaCO3+WCN+BP/CS</td>
</tr>
<tr>
<td>10% CaCO3+AS</td>
<td>10% CaCO3+AS+BP/CS</td>
</tr>
</tbody>
</table>

BP= blot paper, CaCO3=calcium carbonate, CS= chitosan, WCN= whole casein, AS= alphas

4.2.6 Phase 6-Composite property tests: testing hypotheses III and IV

Phase 6 included testing the mechanical properties of the composite films using dynamic mechanical analysis (DMA). The strength of a composite material is its capacity to sustain a load without failing or excessive deformation (Hartog, 1977). A few tensile strength methods were developed and tested; the optimal method for force application is shown in Figure 4.5. All tests were carried out at 35 degrees Celsius after 30 minutes of temperature equilibration. Relative humidity, an important parameter dictating paper properties, was not controlled in these experiments and may be a significant source of variation. Certain physical properties that are important in paper products can be tested by the dynamic mechanical analyzer including tensile strength, elongation at break, damping, and creep. This particular study focused on the tensile strength and the static load and stress at break. The DMA data will be used to quantify the hypothesized correlation between binding trends and mechanical properties.

An important test for strength, is quantifying a material’s stress to strain ratio (Young’s Modulus or modulus of elasticity) by increasing the tension force applied to the material over a
period of time and measuring the force required to cause material yielding. The Young’s Modulus hinges on the extent of fiber-bonding in the material, therefore results can be indicative of the degree of binding between the different components of the composite. Elongation at break is the percent increase in the material’s length during the tension test. The damping test quantifies the energy dissipation in a material (tan δ), giving information about a material’s ability to store energy. Tan(δ) is the ratio of the energy loss in internal motion over the elastic response. It is quantifiable for materials that lie between the viscous and elastic limits. The creep test measures the progressive deformation of the material under a constant stress at an elevated temperature. This test is important in determining if the material can withstand performance at higher temperatures (Institute of Paper Science and Technology-Georgia Tech, 2007). Further mechanical tests, such as those listed above, are avenues of future data analysis, time permitting.

1. Equilibrate at 35°C
2. Isothermal for 60 min
3. Force=0.1 N
4. Ramp force 0.1 N to 5 N
5. Force=0.5 N
6. Isothermal for 5 min
7. Force=0.1 N
8. Ramp force 0.1 N to 5N
9. Force=0.5 N
10. Isothermal for 5 min
11. Force=0.1 N
12. Ramp force 0.1 N to 1 N
13. Force= 1 N
14. Isothermal for 5 min
15. Force=0.1 N
16. Ramp force 0.1 N to 1 N
17. Force= 1 N
18. Isothermal for 5 min
19. Force= 0.1N
20. Ramp force 0.1 N to 18 N

Figure 4.5 Tension test method used to calculate Young’s Modulus.
All paper composites created for the mechanical tests conformed to TAPPI standards in paper sample preparation and testing methodology (TAPPI 220 “Physical Testing of Handsheets”, 2009). The implicit null hypothesis is that the composites will exhibit no change during variations in treatments and substrate conditions. To determine if there is a significant different in strength the standard error between samples will be calculated and graphically compared. An analysis of variance (ANOVA) will statistically assess if there are significant differences in the means between various sample sets as dictated by the hypotheses, using a 95% confidence interval (alpha= 0.05). The major hypothesis that the incorporation of casein into a cellulose-calcium-containing mineral composite altered the material’s properties that are beneficial in paper-making.

Comparison of two different composite matrices (Table 4.2) against relevant controls was performed. In the first matrix, pure cellulose was the fundamental unit to which a casein-functionalized mineral pellet was added and mixed. The second matrix involved chitosan-coated cellulose fibers as the fundamental unit to which the functionalized mineral was then added. The casein-cellulose-mineral composite(s) reflecting the optimal outcomes of the mechanical tests, i.e. highest tensile strength, will be attractive candidates for future paper products potentially involving the creation of the highest degree of paper strength with minimal fiber input. Figure 4.6 details the procedure for composite-making of both sample matrices.
1% Chitosan (CS) solution:
1. 98.5 ml RO water
2. Add 500 ul acetic acid
3. Stir in 1 g low molecular weight chitosan
4. Stir for ~30 min or until dissolved

0.1% BP paper (pH 7):
1. 110 g 1% blot paper solution
2. Fill to 1100 g with FW
3. Make sheets

0.1% BP-CS paper (pH 5.5):
1. Add 1.1 ml of CS solution into 1100 ml of 0.1% BP solution (already adjusted to pH 5.5 with AA)
2. Stir ~1 hr
3. Make sheets

10% Mineral paper (pH 7):
1. ~110 g 1% BP solution
2. Fill to 1100 g with FW
3. Add ~100 mg mineral
4. Stir ~1.5 hr at ~120 rpm
5. Make sheets

10% Mineral BP-CS paper (pH 5.5):
1. ~110 g 1% BP solution
2. Fill to 1100 g with FW
3. Adjust pH to 5.5 (180 ul AA) → let stir for 20 min
4. Add 1.1 ml CS solution, stir for ~45 min
5. Add 110 mg mineral
6. Stir ~1.5 hr at ~120 rpm
7. Make sheets

10% Mineral+Casein paper (pH 7):
1. ~110 g 1% BP solution
2. Fill to 1100 g with FW
3. Add mineral+casein pellet (~116 mg)
4. Mix for ~1.5 hours at ~120 rpm
5. Make composite sheets

10% Mineral+Casein BP-CS paper (pH 5.5):
1. ~110 g 1% BP solution
2. Fill to 1100 g with FW
3. Adjust pH to 5.5 (180 ul AA) → let stir for 20 min
4. Add 1.1 ml CS solution, stir for ~45 min
5. Add mineral+casein pellet (~116 mg)
6. Mix for ~1.5 hours at ~120 rpm
7. Make sheets

**Figure 4.6** Paper composite procedures. (CS=chitosan, casein=either whole or alphaS)

### 4.3 Laboratory Use and Equipment Description

All laboratory research was carried out in the nanotechnology biological materials laboratory in the Agricultural Engineering building. The laboratory contains the HPLC and DMA machines. Mass spectrometry was performed in the proteomics laboratory in the Althouse building.
CHAPTER V
RESULTS AND DISCUSSION

5.1 Phase I Results: Casein Subunit Fractionation and Confirmation

The separation of whole casein into its four subunits was performed using reversed-phase high pressure liquid chromatography (RP-HPLC). Figure 5.1 shows the RP-HPLC chromatogram. Before mass spectrometry it was hypothesized that the four peaks beginning to elute at 15 minutes corresponded to the four subunits in the following elution order: alphaS2, kappa, alphaS1, and beta.

![Figure 5.1 RP-HPLC chromatogram of whole bovine casein.](image-url)
The molecular weights of the final three fractions of the chromatographic profile were resolved by mass spectrophotometry analysis. Figures 5.2-5.4 show the mass spectrometry results of those three fractions from the RP- HPLC chromatogram. The mass spectrometry profile of the first peak was insufficiently clear to glean a predominate molecular weight. It was assumed that the first peak corresponded to alphaS2 based on published chromatography work with the caseins by Leonil et al (1999) who carried out RP-HPLC and attained a similar chromatogram. The molecular weights of the last three peaks corresponded to the molecular weights of the remaining three casein subunits (kappa, alphaS1 and beta), thus, the order of elution was determined to be: alphas2, kappa, alphas1, and beta.

**Figure 5.2 Fraction 2 mass spectrometry profile and molecular weight.**
Figure 5.3 Fraction 3 mass spectrometry profile and molecular weight.
Originally the objective involved fraction collecting the casein subunits for study of each subunit in the binding affinity assay and incorporation into composite materials. However, the inability to resolve the peaks from each other despite various attempts and the minimal amount of protein fraction collected per run, resulted in a process that was not economical to obtain the casein fractions for laboratory experimentation. While this simplified RP-HPLC method for casein subunit fractionation involves little sample preparation and yielded good reproducibility, the feasibility of scale-up was not realized in the course of this research.
5.2 Phase II Results: Whole Casein and AlphaS Binding Affinity

A binding affinity assay was designed as described in the previous chapter to quantify the amount of casein bound to five different substrates of interest: microcrystalline plant cellulose, nanoscale microbial cellulose, calcium carbonate, calcium phosphate, hydroxyapatite, and in the case of alphaS, an additional sixth substrate, calcium chloride, was added. Initially the goal was to generate binding curves to derive a binding constant by the Langmuir isotherm equation. The Langmuir isotherm is one mathematical model for molecular adsorption. Figure 5.5 shows the binding affinity of whole casein to the two cellulose substrates while Figure 5.6 shows the binding affinity of whole casein to the three mineral substrates. Table 5.1 details the three different sample sets, averages, and standard deviations of the binding of whole casein to the cellulose substrates.

Based on the assumptions of the Langmuir equation described in the previous chapter, and a detailed derivation of the original Langmuir equation (Nelson, 2001) a graphically represented adsorption isotherm pertaining to this specific case of casein binding to substrate can be adapted as: Y = M (abC)/(1+aC) where:

Y = concentration of casein adsorbed
M = concentration of substrate
C = equilibrium concentration of casein
a = graphically-determined constant, and
b = graphically-determined constant
(adapted from http://water.vccs.edu/math/langmuir.html).
Manipulation of the equation to the linear form creates: $1/Y/M = (1/ab)(1/C)(1/b)$. This equation shows that the slope of the isotherm is $1/ab$ and the y-intercept is $1/b$. Once deriving values “a” and “b” from the graph it is possible to re-plug these values into the original equation and obtain the amount of substrate to bind for any equilibrium concentration of the casein.

When tabulating the values of whole casein before and after binding (matching to values of $Y$ to apply in the equation), it became clear that the concentrations do not produce an adsorption isotherm typical when applying the Langmuir equation (Figures 5.5-5.6). Ultimately it was decided that an alternate binding model was necessary to describe binding trends. Unfortunately taking a step back to look at the data from the three sample stocks overall produced inconsistencies that were too statistically significant to proceed with comparison of binding between sample stocks. However, some basic observations from two of the three sample stocks became apparent which will be discussed.

Table 5.1 depicts the intriguing binding trends of whole casein to the cellulose and mineral substrates. At initial low concentrations, nearly all of the casein is bound up with the cellulose in both micron and nano-sized substrates. As the concentration of casein increases however, markedly lower amounts of casein bind to the cellulose. The lowest concentration point of casein tested (0.0625 mg/ml) is ~95% bound on average by the cellulose substrates, decreasing as concentration increases to ~50%, ~20%, ~1.5% (by averages). This trend shows that at the concentrations tested, the saturation point of binding occurs at a concentration lower than the lowest concentration point tested. This prevented quantification of the binding constant using the standard Langmuir method.
The binding affinity assays involved a whole casein system that had been dissolved at pH 10, which was expected to dissociate any micelle formation and allow each of the four subunits to bind to cellulose in preference to other subunits. However, it is possible that complete dissociation of the casein subunits from each other was not realized, compounding the complexity of the interactions occurring. In addition, the different sample stocks may have contained subtle differences in the fraction of subunits entering the binding assay and resulted in instances where casein had a higher prevalence for itself over the cellulosic substrate as the casein concentration increased. Finally, the inaccessibility of the alphaS subunits may have played a role in the decreased binding to cellulose as concentration increased. It had been hypothesized that these two subunits with their high number of tyrosine residues and the specific motif of alpha_1, with one end of the polypeptide covered in tyrosine residues and the other end with phosphoserines, would be the optimal binders. The binding trends of the alphaS system were later examined to verify if removal of the beta and kappa casein subunits would enhance the binding affinity for the cellulosic substrates.

Inspecting the binding trend of whole casein to the calcium-containing minerals shows a similar trend of a marked high proportion of binding initially, when casein concentration was at the two lowest starting concentration points, followed by a progressive decrease in the amount of casein bound to the substrate. This trend was also unexpected since it was believed that a concentration of calcium-containing mineral an order of magnitude higher than the highest casein concentration tested, would completely bind up all of the casein resulting in an increasing binding curve. Again, the conclusions behind the mineral binding trend falls back on the fact that casein’s high preferential binding to itself may counteract the binding to the substrate in this particular binding affinity assay method at these concentrations of protein and mineral tested.
Figure 5.5 Binding affinity of whole casein to microcrystalline cellulose (MCC) and cellulose nanowhiskers (CNW).

Figure 5.6 Binding affinity of whole casein for mineral substrates; calcium carbonate (CaCO3), calcium phosphate (CaHPO4), and hydroxyapatite (HA).
Table 5.1 Amount of whole casein bound to cellulose and mineral substrates

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The complex binding profile of whole casein to cellulose and calcium-containing mineral substrates was not expected to occur with the alphaS casein samples. Instead of four interacting subunits, there are only two present, which is hypothesized to diminish the affinity of the casein subunits for other casein subunits through hydrophobic interactions and disulfide linkages. Figure 5.7 details the binding affinity trend of alphaS for the two cellulose substrates while Figures 5.8-5.9 show alphaS affinity for the calcium-containing mineral substrates. Table 5.2 shows the data for all three sample sets and the average and standard error of binding.

Immediately it is evident that the standard error for each sample precludes a clear and accurate conclusion of a binding trend. Upon further inspection of the data it can be seen that for the two cellulose substrates a fairly consistent amount of alphaS was bound when viewing the average of the three sample sets. When looking at the sample sets individually however, a discernable pattern of binding is not evident. For the microcrystalline cellulose substrate, alphaS sample set one had a trend of increased binding as the protein concentration increased. This indicates that the saturation point had not yet been reached by the highest concentration point tested. However, sample set two and three showed more sporadic concentration readings with no clear binding trend. For nanosized microbial cellulose the binding trend of sample set one was similar to microcrystalline cellulose, with an increasing amount of casein bound as the protein concentration increased. Sample sets two and three showed sporadic binding data with a mixture of increased and decreased binding as alphaS concentration increased. This could indicate that the alphaS composition, whether it be the proportion of the alpha,1 and alpha,2, or possible impurities in the form of other subunits present may be varied between sample set stock solutions. Extreme care was taken to exactly mimic the preparation conditions between sample sets thus minimizing experimental error, although that certainly may be a factor in the similarity
between sample sets two and three and the extreme difference when juxtaposed with sample set one.

The binding trend of alphaS for the calcium-containing minerals also exhibited a high standard error. When looking at the average alphaS bound per mineral substrate the results are masked by the incredible deviation present. Again, breaking down the sample sets and viewing them individually showed no clear binding trend of alphaS for any of the mineral substrates.

Figure 5.7 Binding affinity of alphaS casein to microcrystalline cellulose (MCC) and cellulose nanowhisker (CNW) substrates.
Figure 5.8 Binding affinity of alphaS casein to calcium carbonate (CaCO3) and calcium chloride (CaCl2).

Figure 5.9 Binding affinity of alphaS casein to calcium phosphate (CaHPO4) and hydroxyapatite (HA).
Figure 5.10 Comparison of the binding affinity for the microcrystalline substrate between whole casein and alphaS systems.

Figure 5.10 shows the comparison between the two casein protein systems and microcrystalline cellulose. The numbers 1-4 correspond to the initial casein amount entering the binding assay (1= 0.0625 mg, 2= 0.125 mg, 3= 0.250 mg, 4= 0.500 mg). The large standard deviation of the alphaS system precludes drawing conclusions about the rest of the binding behavior (corresponding graphs for the other five substrates it not shown). But it is clear that there is less whole casein binding at the highest concentration tested than both previous points of whole casein and in comparison to equal loading of alphaS casein. It also appears that the alphaS system has a higher capacity for binding than whole casein.
Table 5.2 Amount of alphaS casein bound to cellulose and mineral substrates.

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</tr>
<tr>
<td>0.250</td>
<td>0.0960</td>
<td>0.1420</td>
<td>0.1080</td>
<td>0.1153</td>
<td>0.0239</td>
<td></td>
</tr>
<tr>
<td>0.375</td>
<td>0.1160</td>
<td>0.0320</td>
<td>0.0760</td>
<td>0.0747</td>
<td>0.0420</td>
<td></td>
</tr>
<tr>
<td>0.500</td>
<td>0.1350</td>
<td>0.0240</td>
<td>0.1100</td>
<td>0.0897</td>
<td>0.0582</td>
<td></td>
</tr>
</tbody>
</table>

Generation of binding curves to quantify binding constants using the Langmuir isotherm equation for the whole casein and the alphaS systems was not possible due to the fact that for whole casein the saturation point was reached too quickly (not enough points were tested before saturation) and because of the inconsistencies and large standard error calculated from the three sample sets for each protein system, specifically in the alphaS case. In the case of whole casein it appears as if the binding saturation point was already reached by the second concentration point tested. Dilution down between points one and two showed experimental inconsistencies due to the 0.01% beta-mercaptoethanol used to reduce the protein. Even though manufacturers assured that this molarity of solution would not interfere with ultraviolet spectroscopy, this researcher did not find that to be the case when used with low concentrations of whole casein.

It was found that it is impossible to glean, with confidence, particular binding trends from these data sets except for perhaps the inconsistent and/or inhomogeneous nature of solutions of
both whole casein and the alphaS subunits in neutral filtered water and filtered water raised by sodium hydroxide (to pH 10). It is possible that casein preferentially binds to itself based on a continuum of micelle dissolution at pH 10 and the possible presence of various casein micelle architectures that are influenced by composition of the other subunits in the mixture, pH, solvent environment, and substrate. The number of compounding factors that could have played a role was not eliminated sufficiently to quantitatively derive a binding constant, or to determine the precise molecular binding trend between the whole casein and alphaS casein systems and the cellululosic or mineral substrates. The overall binding mechanism is still believed to be a complex array of hydrophobic interactions between the casein system and most likely the carbohydrate-π stacking interactions that occur between polysaccharides and aromatic amino acids such as tyrosine. In the case of the mineral, electrostatic interactions between the positively charged calcium groups and negative phosphate groups on the serines and the carboxylic acid groups on the casein were implicated in binding as the literature states.

A promising observation of this preliminary binding study was that whole casein was bound in every case from the lowest starting point (0.0625 mg) until right before the highest (0.500 mg) in both the cellulose and mineral cases. This showed that an optimal range for testing the protein binding was picked, and more importantly, that there is in fact binding occurring. Similarly, alphaS was bound in all cases to both substrate sets, except for the anomalous point in sample set two/CaCO3/0.09375 mg (see Table 5.2). However, in the case of the alphaS system, higher concentrations should be tested since the binding saturation point is never reached.

While quantitative binding trends were not generated using the Langmuir isotherm, the relative low deviation in the whole casein data indicates that perhaps another binding equation
would be more appropriate to interpret the data. This would have different assumptions than the Langmuir which were already proposed to be particularly limiting for this casein system. Additionally, the pragmatic application of this data was to provide a starting point to determine the optimal loading of whole casein and alphaS for complete calcium carbonate functionalization in the paper composites (refer back to Figure 4.8).

Analysis of variance (ANOVA) tests were performed on the means of the three sample sets for each binding event at the 95% confidence level ($\alpha = 0.05$) to determine if any null hypotheses could be rejected. Hypothesis IIA, that there is a statistically significant difference between the two casein systems’ binding affinities for cellulose was not shown for any of the points in the assay except for the highest loading point (0.5 mg) with a $p$-value of 0.042. The sub-hypotheses under Hypothesis IIB, that there is a significant difference between the two casein system’s affinities for microscale versus nanoscale cellulose, were not accepted using the 95% confidence interval. Finally, the alternate hypotheses under Hypothesis IIC, that there is a significant difference in the binding affinity of the two casein systems for the calcium-containing minerals, were also all rejected at every single concentration point tested for this particular confidence level.

5.2 Phase III Results: Cellulose (+/-Chitosan):Casein:Mineral Composites

The major hypothesis involving the paper composites was that casein would bind calcium carbonate, the most common filler in paper materials, simultaneously binding to cellulose fibers increasing the dispersion of the mineral throughout the composite matrix. This casein “binder”
would lead to an increase in the strength of the composites overall and ultimately leading to a reduction of cellulosic fiber in paper materials.

This hypothesis expanded from using whole casein to include a theory on the optimal binder of the four casein subunits. It was proposed that the casein subunit to exhibit optimal binding of both cellulose and mineral would be alpha,1 due to its distinct region of phosphorylated serines (calcium-binding) and cluster of tyrosines (cellulose-binding). The difficulty in separating alpha,1 from the other fractions required the use of a mixture of both alpha,1 and alpha,2 (denoted ‘alphaS’) for the binding and composite experiments. However, the hypothesis remained that the two-domained structure of alpha,1 and the high number of phosphate centers of alpha,2 would result in optimal binding to mineral (Farrell, 2002; Fox and McSweeney, 1998) and cellulose substrates without the steric limitations and self-aggregation when using all four subunits. This was the premise behind the samples in matrix I for the composites.

Sample matrix II incorporated the observation that at pH 7, the pH where the various composite materials were mixed together, casein has a highly negative charge. This negative charge occurs because its isoelectric point is found at a lower pH (4.6) which is a function of its primary sequence. This primary sequence includes many negatively charged amino acids, i.e. aspartate and glutamate, around a neutral pH. It was hypothesized that casein’s highly negative regions would interfere with cellulose binding, itself being an anionic polysaccharide. This necessitated a biodegradable material that could in effect “coat” the cellulose fibers with a positive charge. The cationic polysaccharide chosen was chitosan; which has been show to form films with cellulosic fibers due to an extensive electrostatic attraction (Hasegawa, 1992). The chitosan was added as 1% w/w of the cellulose fibers from a 1% w/v solution coating. This amount was chosen from a study which found that 1-3% (w/w) of chitosan to cellulose was
sufficient to coat the cellulose fibers when making paper composites (Mucha, 1999). The chitosan-cellulose base was the primary system behind matrix II, to which the calcium carbonate, whole casein, and alphaS casein was added. It was believed that the positively charged polymer duo (cellulose-chitosan) would attract the highly negatively charged casein protein thus improving casein dispersion over the fibers ultimately resulting in a better dispersed mineral and ultimately better mechanical properties.

The two matrices, the first without chitosan at pH 7 and the second with chitosan at pH 5.5 (the lowered pH preserved chitosan solubility), were subjected to the tension test using dynamic mechanical analysis (DMA) in order to calculate the Young’s Modulus, an indicator of material strength. This mechanical parameter was used to quantify and compare the strength of the composites. It was hoped that both matrices exhibited a marked increase in strength versus the control cellulose-mineral system; the first matrix involving less material and steps for scale-up production feasibility, the second involving a more intricate binding optimization scheme.

The DMA output of the change in strength and strain over the course of the test was used to derive the Young’s Modulus. The linear portion of the final force ramp, of which there are five (see tension test method in preceding section), was used to find the Young’s Modulus from the slope of the graph when plotting stress versus strain. The equation for the linear portion was forced to $R^2 = 0.998$ for each sample. Representative plots of the samples both before and after fitting are given in Figures 5.11-5.12. The slope multiplied by 100 is the Young’s Modulus value in megapascals (MPa).
Figure 5.11 Representative control samples from matrix I and their stress-strain curves to illustrate Young’s Modulus derivation.
Figure 5.12 Representative casein samples from matrix I and their stress-strain curves to illustrate Young’s Modulus derivation.

Table 5.3 shows the quantitative outputs of the DMA test for sample matrix I. It can be seen that the addition of calcium carbonate to the basic blot paper material actually improved strength as measured by the Young’s Modulus. This was counterintuitive based on the literature associating the weakening of conventional paper materials by fillers such as calcium carbonate and other minerals (Roberts, 1996; Yan, 2004). It is possible that the laboratory-scale composite production method, which differs from industrial paper-making, imparts a weaker cellulosic control. It is also possible that fiber alignment may vary between these processes as cellulosic fibers were allowed to drain on a mesh wire screen resulting in a decrease in overall strength of
the blot paper alone. In some cases the orientation of the fibers may be more amenable to a higher force load. It is inconclusive however, that this reason would be responsible for the repeatable trend of higher strength when ten percent filler material was loaded into the composite.

Figure 5.13 shows the Young’s Modulus of the samples in matrix I in graphical form. The trends of increasing mechanical strength from the control to whole casein system to the alphaS system are in agreement with the original hypothesis. However, the standard deviation of the different samples potentially eliminates a statistically significant difference in Modulus at the 95% confidence level. ANOVA tests found that at this particular confidence level none of the average Young’s Modulus values were statistically significant from each other in matrix I. This indicates that a significant increase in strength by employing the casein system as a binder between mineral and cellulose may in fact be masked by the electrostatic repulsion between casein and the cellulose fibers; testing this hypothesis was the motivation behind composite matrix II creation.
Table 5.3 Matrix I tension test results

| Sample Set- | Weight (g) | Young’s Modulus (MPa) | Static force at break (N) | Strain at break (%) | Stress at break (MPa) | Dimensions (width, thickness) (mm) |
| pH 7        |            |                      |                           |                    |                      |                                    |
| 0.1% Blot paper |         |                      |                           |                    |                      |                                    |
| 1           | 0.875     | 1112                | 3.236                    | 1.89               | 11.74               | 3.4567, 0.0797                     |
| 2           | 0.971     | 1011                | 3.784                    | 2.183              | 12.6                | 3.5633, 0.0843                     |
| 3           | 0.914     | 928.2               | 3.308                    | 1.96               | 10.41               | 3.7700, 0.0843                     |
| 10% CaCO3  |            |                      |                           |                    |                      |                                    |
| 1           | 0.882     | 2868                | 6.949                    | 2.122              | 26.69               | 3.9033, 0.0667                     |
| 2           | 1.060     | 3008                | 7.167                    | 2.153              | 25.57               | 3.4600, 0.0810                     |
| 3           | 1.005     | 2718                | 6.269                    | 2.042              | 21.98               | 3.4900, 0.0817                     |
| 10% CaCO3-Whole Casein | |                      |                           |                    |                      |                                    |
| 1           | 0.913     | 2569                | 5.753                    | 2.063              | 21.73               | 3.2967, 0.0803                     |
| 2           | 1.01      | 3284                | 7.279                    | 3.583              | 29.37               | 3.5400, 0.0700                     |
| 3           | 0.883     | 3511                | 4.878                    | 1.181              | 23.06               | 3.2200, 0.0657                     |
| 10% CaCO3-alphaS |       |                      |                           |                    |                      |                                    |
| 1           | 0.904     | 3663                | 7.549                    | 2.297              | 34.4                | 3.5000, 0.0627                     |
| 2           | 0.918     | 3670                | 7.924                    | 2.339              | 32.08               | 3.4933, 0.0707                     |
| 3           | 0.853     | 2615                | 5.324                    | 1.442              | 22.6                | 3.4000, 0.0693                     |

Figure 5.13 Young’s Modulus of matrix I. (BP= blot paper, WCN= whole casein)
If the inherent difficulty in understanding strength as a function of binding in matrix I had to do with the electrostatic repulsion between casein and cellulose, then matrix II was hypothesized to solve this issue. Figures 5.14-5.15 detail representative stress-strain plots for samples from matrix II.

**Figure 5.14** Representative control samples from matrix II and their stress-strain curves to illustrate Young’s Modulus derivation.
Figure 5.15 Representative casein samples from matrix II and their stress-strain curves to illustrate Young’s Modulus derivation.

Table 5.4 details the DMA data for sample matrix II. The average Young’s Modulus for the matrix samples are graphically represented in Figure 5.16.
Table 5.4 Matrix II tension test results

<table>
<thead>
<tr>
<th>Sample Set pH 5.5</th>
<th>Weight (g)</th>
<th>Young’s Modulus (MPa)</th>
<th>Static force at break (N)</th>
<th>Strain at break (%)</th>
<th>Stress at break (MPa)</th>
<th>Dimensions (width, thickness) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% Blot paper (BP)-chitosan(CS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.865</td>
<td>2200</td>
<td>5.325</td>
<td>2.093</td>
<td>18.21</td>
<td>3.8133, 0.0767</td>
</tr>
<tr>
<td>2</td>
<td>1.031</td>
<td>2569</td>
<td>4.950</td>
<td>1.346</td>
<td>19.40</td>
<td>3.6600, 0.0697</td>
</tr>
<tr>
<td>3</td>
<td>0.998</td>
<td>3853</td>
<td>7.749</td>
<td>2.490</td>
<td>33.99</td>
<td>3.1533, 0.0723</td>
</tr>
<tr>
<td>10% CaCO3-BP/CS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.145</td>
<td>2876</td>
<td>8.490</td>
<td>1.981</td>
<td>24.83</td>
<td>3.7700, 0.0907</td>
</tr>
<tr>
<td>2</td>
<td>0.844</td>
<td>3077</td>
<td>5.698</td>
<td>1.888</td>
<td>23.62</td>
<td>3.1867, 0.0757</td>
</tr>
<tr>
<td>3</td>
<td>0.731</td>
<td>2255</td>
<td>4.903</td>
<td>1.997</td>
<td>21.79</td>
<td>3.6700, 0.0613</td>
</tr>
<tr>
<td>10% CaCO3-Whole Casein-BP/CS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.028</td>
<td>2826</td>
<td>9.002</td>
<td>3.072</td>
<td>30.74</td>
<td>3.3167, 0.0883</td>
</tr>
<tr>
<td>2</td>
<td>0.897</td>
<td>2639</td>
<td>5.242</td>
<td>3.215</td>
<td>17.44</td>
<td>3.6533, 0.0823</td>
</tr>
<tr>
<td>3</td>
<td>0.956</td>
<td>2496</td>
<td>6.086</td>
<td>2.223</td>
<td>19.34</td>
<td>3.6600, 0.0860</td>
</tr>
<tr>
<td>10% CaCO3-alphaS-BP/CS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.906</td>
<td>2781</td>
<td>6.775</td>
<td>2.081</td>
<td>25.38</td>
<td>3.5600, 0.0750</td>
</tr>
<tr>
<td>2</td>
<td>0.800</td>
<td>3130</td>
<td>5.467</td>
<td>1.870</td>
<td>26.74</td>
<td>3.1800, 0.0643</td>
</tr>
<tr>
<td>3</td>
<td>1.069</td>
<td>3023</td>
<td>9.503</td>
<td>1.609</td>
<td>27.60</td>
<td>3.5033, 0.0983</td>
</tr>
</tbody>
</table>
Immediately it is evident that the 0.1% blot paper control with the added chitosan had a significant increase in Young’s Modulus as compared to the blot paper control without the added chitosan. This was anticipated due to the strong electrostatic attraction between the negatively charged cellulose and positively charged chitosan. In this case there is not a drastic increase in Young’s Modulus with the calcium carbonate-loaded blot paper which is in agreement with literature studies that filler material weakens pure cellulose composites. It is notable that the standard deviation precludes definitive assessment concerning whether or not there is a significant difference between the controls and protein-loaded samples of matrix II; statistical studies were subsequently employed. Again, at the 95% confidence level it was found that there was no statistical difference in Young’s Modulus between the mineral control and the casein-loaded samples in matrix II. Additionally, it was found that there was no significant difference in strength between the casein samples in matrix I and their corresponding samples in matrix II.
Therefore, none of the null sub-hypotheses from Hypotheses IIIA and Hypothesis IIIB could be rejected.

When working with composites incorporating proteins it is also desirable to look at the strain imparted on the composite. Proteins have a tendency to make composite materials more brittle, manifest quantifiably as a lower strain value. Figure 5.17 compares the strain (%) between the samples in matrices I and II.

![Figure 5.17 Comparing strain at break (%) between matrices I and II. (BP=blot paper, CS=chitosan, WCN=whole casein)](image)

Unfortunately the variability found in the whole casein samples precluded conclusions about the effect of this protein system on the overall brittleness of the composite. It appears evident that the alphaS system does not render the composite any more brittle than its non-
protein counterparts, but again, care must be taken when drawing any conclusions based on the variability of the datasets.
CHAPTER VI

CONCLUSIONS AND FUTURE WORK

Objective I was accomplished with the successful separation of whole casein using a simplified reversed-high pressure liquid chromatography method. The difficulty in fraction collection and the limited amount of protein eluted off of the column per run made it an unviable method for scale-up from the laboratory and precluded its use even within the laboratory setting. The improved separation of the four subunit peaks through use of a different solvent system or further optimized gradient are two avenues for future work. RP-HPLC is a repeatable, quick, and relatively cheap method for separation of casein protein. If further peak resolution can be achieved with the implementation of the automated fraction collection system, a scaled-up production of the purified casein subunits is feasible. However, separation of the protein from the harshly acidic solvent system must be performed prior to further applications.

Objective II regarding the quantification of the two casein systems (whole and alphaS) for the cellulosic and mineral substrates had mixed success. Ultraviolet spectroscopy is a well-known method for quantification of protein concentration by quantifying absorbance at 280 nanometers. Accurate and repeatable, this method quantified casein protein concentration both before and after binding to the cellulose and mineral substrates. The sporadic nature of the results is concluded to be a function of the protein behavior with a variety of factors: itself, the substrate, and the solvent. Further elucidation of the binding mechanism of casein to its substrates was attempted with isothermal titration calorimetry (ITC), absorbance readings using Bradford assays, and through gel electrophoresis (results not shown). None of these mechanisms
were effective to attain clear and repeatable results within themselves or as companion methods to UV spectroscopy. There is the hope that lower concentration points of whole casein can be tested to establish a Langmuir binding curve since it appears that at the lowest concentration point tested the substrate was fully saturated. This however will require a sensitive method that goes beyond that of the UV spectrophotometer as interference occurred between the amounts of casein used and the solvent background (specifically the reducing agent 2-betamercaptoethanol). AlphaS casein data was too sporadic to generate any quantitative binding conclusions, however, future work on this particular casein system may involve increased concentrations of initial casein amount since in all sample sets with all substrates tested the binding saturation point was never reached. While there was no significantly differences at the 95% confidence level between alphaS and whole casein binding to cellulose or mineral, there was also no significant difference in the binding to micron-sized plant fibers versus bacterial nanofibers. This may necessitate revisiting the initial hypothesis about cellulose-casein binding or to characterize the structures of the two cellulose substrates to identify if a high structural similarity precludes preference to one versus the other.

Objective III was successfully achieved through the generation and implementation of a reproducible paper composite creation method. Additionally, the binding affinity data was used as a launch point to determine the amount of casein to add to a ten percent calcium carbonate loading into the composite sheet. The paper composite method generated repeatable results in composite sample appearance and strength for the controls and the mineral-loaded samples. The average Young’s Modulus for both matrices used to assess composite strength is shown in Table 6.1.
Table 6.1 Average Young’s Modulus of matrices I and II.

<table>
<thead>
<tr>
<th></th>
<th>Matrix I Avg YM (GPa)</th>
<th>Matrix II Avg YM (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% BP</td>
<td>1.02</td>
<td>2.87</td>
</tr>
<tr>
<td>10% CaCO3-BP</td>
<td>2.86</td>
<td>2.74</td>
</tr>
<tr>
<td>WCN-10% CaCO3-BP</td>
<td>3.12</td>
<td>2.65</td>
</tr>
<tr>
<td>alphaS-10% CaCO3-BP</td>
<td>3.32</td>
<td>2.98</td>
</tr>
</tbody>
</table>

Statistically, Hypothesis IIIA, that the paper composites containing the casein with the higher binding affinity for calcium-containing material and cellulose would be stronger was not accepted at the 95% confidence level. According to the binding affinity data the alphaS system would be the clear winner for binding to calcium-containing mineral and cellulose (outlined in Hypothesis IIIB). ANOVA tests on the DMA Young Modulus results for composites showed that there was no significant difference when switching from the whole casein system to the alphaS system.

Objective IV was successfully achieved by the creation of a second matrix of composite samples including a cellulose-chitosan as opposed to just a pure cellulose base (matrix I). However, the alternate hypothesis in Hypothesis IIIC, that the chitosan matrix would exhibit a significant difference in strength from the binding enhancement between the polymer matrix and the protein-mineral addition due to electrostatic attraction, could not be accepted for any of the samples. The only statistical significant increase in strength was between the blot paper control and the chitosan-blot paper control. Inability to reject the null may be due to the fact that material loadings of the chitosan, casein, and mineral for optimal interaction may not have been
tested. Future work could perform more fundamental studies of the interactions between the various components in order to optimize a loading that would enhance binding and translate to improved mechanical properties. It is worth noting however, that the addition of whole casein and alphaS casein did not significantly reduce the strength as measured by the Young’s Modulus of any composite samples and did not appear to increase the brittle nature of the composites to a significant degree.

Historic use of casein in binding with lignocellulosic materials was the motivation behind the generation of new composite materials that are biocompatible. It is heartening that the addition of casein to the cellulose composite did not compromise strength and optimization of concentration or processing conditions could lead to the realization of a marked improvement in paper properties; the aim of this particular research project. While use of a completely “green” processing method was realized in tandem with wholly environmentally compatible materials, it is important that the “greening” of: cellulose extraction from lignocellulosic materials, the purification of casein from bovine milk, the acquisition of calcium-containing material, also must occur so that the entire system of manufacturing and production minimally impacts the environment. In order for a completely sustainable process to be realized, careful analysis and monitoring of each resource acquisition should be in put for future work, not just final materials.

There are still a variety of tools to be explored that could provide data to quantitatively support generalizations about the mechanism of interaction and binding between casein and cellulose. Information gleaned from complications with use of the Langmuir equation, isothermal titration calorimetry (results not shown), and DMA tests at the loadings used in this research may help to whittle away some components of the engineering toolbox used to understand these mechanisms. In future work it is hoped that it will be possible to quantitatively
show the hypothesized multiple molecular events which are thought to occur between casein, calcium-containing mineral, chitosan, and cellulose components upon mixing and to draw subsequent conclusions about the mechanism behind the binding interactions. Ultimately future work should realize a novel biocompatible composite material with various useful and environmentally-friendly applications on the industrial scale.
REFERENCES


