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Sustained release of lysozyme encapsulated in zein micro- and nanocapsules

Minfeng Jin
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Minfeng Jin entitled "Sustained release of lysozyme encapsulated in zein micro- and nanocapsules." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Qixin Zhong, Major Professor

We have read this thesis and recommend its acceptance:

P. Michael Davidson, Arnold M. Saxton

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Sustained Release of Lysozyme Encapsulated in Zein Micro- and Nanocapsules

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Minfeng Jin
August 2008

Dedication

I would like to dedicate this thesis to my mom, Jinfang Shen, my grandma, Miaoying Zhang, my sister Weihong Jin and her family, my relatives and my friends, and especially in loving memory of my dearest father, Mingzu Jin, for always believing in me, inspiring me, supporting me and encouraging me to pursue my career goal.

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Abstract

A hydrophobic biopolymer, corn zein, was studied as a carrier for manufacturing particulate delivery systems of antimicrobials with sustained release. Three techniques, i.e., solvent attrition, supercritical anti-solvent and spray drying, were investigated to produce lysozyme-loaded zein micro- or nanocapsules. The work was focused on particle synthesis and *in vitro* release kinetics as affected by formulations and processes.

The size (100-200 nm) and morphology (separated or connected) of the zein nanoparticles produced using solvent attrition were significantly affected by shear force, ethanol and zein concentrations in stock solutions during synthesis. Zein nanoparticles showed gradual release of lysozyme at pH 7 and 8 but no sustained release at lower pHs. Further, the impact of adding 1% zein nanoparticles in model carboxymethylcellulose solutions (adjusted to pH 3 to 9) was studied for viscosities that increased with pH.

Microcapsules produced from supercritical anti-solvent showed a continuous matrix with internal voids. Sustained release of lysozyme at pH 2 to 8 was observed over 36 days at room temperature, with slower release at higher pH. At pH 4, release kinetics was further slowed by addition of sodium chloride.

Spray drying was studied as one commercially feasible process. To further reduce the material cost, partial purification of lysozyme from hen egg white was studied using binary aqueous alcohol. Extraction with 50% ethanol at pH 3.5 for 6 h enabled high lysozyme activity and relatively high purity. Lysozyme precipitated after increasing the ethanol concentration from 50% to 90% in the extract. The precipitates were resolubilized by dilution to 50% ethanol. Slurries after increasing ethanol concentration from 50% to 60%-90%, with or without additives

of Tween 40 or thymol, were spray dried. Capsules without additives were porous and did not show sustained release of lysozyme. The addition of Tween 40 changed the capsule microstructure to packed nanoparticles but did not achieve sustained release of lysozyme. Thymol facilitated the formation of a continuous capsule matrix and allowed sustained release of lysozyme at near neutral pH.

Findings from this work demonstrated the possibility of using zein as a carrier biopolymer to deliver antimicrobials in food matrices for sustained release.

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Chapter 1 . Introduction

1.1. Role of Antimicrobials in Food Safety and Quality

Food products may lose some quality due to chemical, physical, enzymatic, and microbiological changes during the production and storage (Davidson and Branen 2005). Among these changes, microbiological problems receive considerable attention from consumers, manufacturers and regulatory agencies because microbial contamination is dynamic and not easily controlled. Growth of microorganisms in foods could accelerate the deterioration of foods, resulting in off-colors, off-flavors or -orders, texture changes, or slime, and hazards to the health of consumers due to the presence of pathogenic microorganisms or production of microbial toxins in food products (Davidson and Branen 2005).

Traditionally, foods were preserved by use of heat, cold, drying and/or fermentation (Davidson and Branen, 2005). Currently, both natural and synthesized food antimicrobials are widely added to foods to prevent problems caused by microbial growth. Antimicrobials delay the spoilage of foods and potentially inhibit the growth of foodborne pathogens. The application of antimicrobials, along with preservation treatments including chilling or freezing, thermal processing, non-thermal processing (e.g., high pressure), dehydration or reducing water activity, nutrient restriction, acidification, fermentation, and modified atmospheres may effectively form barriers or hurdles to inhibit or destroy microorganisms in food products. Thus, these treatments enhance food microbial safety to ensure or extend the shelf life of food products.

1.2. Demands and Challenges of Antimicrobial Delivery Systems

The inactivation and inhibition effects of antimicrobial agents are strongly dependent on many factors, including intrinsic environmental factors in foods (e.g., pH, salt concentration and other reactive chemical components), extrinsic environmental factors (mainly storage

temperature and relative humidity), and types of target microorganisms in food products (Davidson and Branen 2005). Enzymatic inactivation, diffusion of antimicrobial agents into food components and binding or reaction of these compounds with food or cell matrices decreases the bioavailability of antimicrobials (Chi-Zhang et al., 2004, Hoffman et al. 2001). Additionally, antimicrobial resistance may develop, causing the loss of inhibitory effects on spoilage microorganisms or foodborne pathogens. Consumer demand for a reduction in "food preservatives" is increasing. Thus, research has focused recently on developing delivery systems for antimicrobials that involve "natural antimicrobials" and sustained release (Bezemer et al. 2000, Gouin 2004, Salmaso et al. 2004), which could potentially decrease the usage of synthetic antimicrobials as well as reduce the concentration required while increasing their bioavailability.

1.2.1. Purpose and Usage

Edible or non-edible packaging films with incorporated with antimicrobials, coatings and microcapsules or microparticles entrapped with antimicrobials are the main methods to deliver antimicrobials to foods. Packaging films and coatings with antimicrobial agents have been applied to solid foods (i.e., meat, cheese/ham, beef, poultry, etc.) and it has been demonstrated that the agents were released and diffused onto the foodstuff to inhibit the growth of microorganisms (Ming 1997, Scannel 2000, Cutter and Siragudsa 1996, Natrajan and Sheldon 2000, Meyer 1959, and Ouattara 2000). Microcapsules may be applied in semi- and/or liquid foods to increase the fresh bioavailability and inhibition efficacy of antimicrobial agents for longer times, or to protect volatile antimicrobials, such as plant essential oils. While antimicrobial packaging films have been studied to a great extent, particulate delivery systems for antimicrobial agents have received less attention.

Controlled release systems composed of microparticles have been mainly studied and developed in drug delivery for pharmaceutical applications (Bezemer et al. 2000, Kuijpers et al. 1998). Salmaso et al. (2004) showed that nisin-loaded poly(-L-lactide) (PLA) nanoparticles produced by CO_2 anti-solvent precipitation had sustained release of nisin over 45 days, whereas non-encapsulated nisin lost its antimicrobial properties after 10 days. However, in this successful investigation of nisin-loaded PLA capsules, the material of the delivery vehicle, PLA, is not allowed to be directly added in food products. Many polymers and solvents used in the production of drug delivery systems are questionable for food applications due to the safety restrictions on ingredients and solvents used. Further, most processes cannot be scaled up to meet the capacity of food production. Identification of food grade ingredients as carriers, solvents and low-cost and scalable processes remains a challenge for developing food grade antimicrobial delivery systems.

1.2.2. Carrier Materials and Antimicrobial Agents

Carrier or film materials of delivery systems normally are biopolymers (polysaccharides and proteins), lipids, or combinations of lipids and biopolymers (Gibbs et al. 1999, Gharsallaoui et al. 2007, Taylor et al. 2005). Most food grade carbohydrate and protein ingredients are water soluble so that the release of encapsulated antimicrobials is rapid in aqueous media. Because microorganisms grow in aqueous or high water content foods, water soluble materials are questionable as carrier materials for long-time release purposes. Conversely, water insoluble materials may be used for development of particulate delivery systems whose stabilities maybe maintained in long term when dispersed in foods. As for the types of antimicrobials, weak organic acids (e.g. lauric acid), enzymes (e.g., lysozyme), bacteriocins (e.g., nisin), and essential

oils, etc. (Joerger 2007; Hoffman et al. 2001; Janes et al. 2002, Gaysinsky et al. 2007, Taylor et al. 2008) are common antimicrobial agents incorporated or entrapped in delivery systems.

1.2.3. Techniques for Developing Antimicrobial Delivery Systems

Various manufacturing techniques have been applied for encapsulation in food industry, including spray drying, spray chilling or spray cooling, spinning disk and centrifugal co-extrusion, extrusion coating, fluidized bed coating, coacervation, liposome entrapment, alginate beads, rapid expansion of supercritical solutions (RESS)/supercritical anti-solvent (SAS) techniques and inclusion encapsulation (Gouin 2004). However, due to the nature/properties of the ingredients, cost-of-use, production capacity and/or controlled release mechanism, not all techniques are feasible for development of antimicrobial delivery system. Identification of appropriate techniques for appropriate antimicrobials is critical for successful delivery of antimicrobials.

1.3. Scope of the Work

The overall goal of this work was to investigate the potential application of corn zein, prolamines, as carrier materials for manufacturing particulate antimicrobial delivery systems. Three techniques, i.e., solvent attrition, supercritical anti-solvent and spray drying, were investigated, producing a range of antimicrobial delivery systems with sizes of micro- and nanoscales. The size of particles is important for ingredients applied in food products so that these particles entrapped with antimicrobials will not affect the sensory property of food products, e.g., mouthfeel. The effect of zein nanoparticles on rheological properties of a model biopolymer solution was also studied.

Lysozyme, a widely used naturally-occurring enzyme that has antimicrobial properties, was selected as a model antimicrobial in this study. Purified, lyophilized commercial lysozyme was used for the techniques of solvent attrition and supercritical anti-solvent. For spray drying (a more established technique), lysozyme was directly extracted from hen egg white and used for particle production to decrease the eventual cost of antimicrobial delivery systems. *In vitro* release kinetics of lysozyme from these particles was evaluated. The structures of synthesized microparticles were determined using surface scanning electron microscopy to study the correlation between microstructure and release kinetics.

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Chapter 2 . Literature Review

2.1. Characteristics of Zein

Corn is one of the largest and most important food and industrial crops in America. Zein is the major storage protein of corn, representing 45-50% of proteins in corn (Shukla and Cheryan, 2001) and mainly distributed in the endosperm. Gorham (1821) first isolated zein from maize and named this protein “zeine”. Osborne (1891) classified zein as a prolamine, which is water-insoluble but soluble in aqueous alcohols. Some of the important works on zein were studied more than 100 years ago, such as extraction of zein (Osborne 1891, 1902) and uses of zein. Commercial zein is produced as the major protein co-products during corn processing.

Commercial zein production dropped from a peak around 15 million pounds a year in the US in the late 1950s, to one million pounds per year in 1978 and has remained constant since then (Shukla and Cheryan, 2001). This drop was mainly due to the high cost of production and the emerging of cheaper alternatives in industrial applications (Lawton 2002). Recently, interest in zein utilization is growing again because of new demands for environmentally friendly materials. Zein is annually renewable and biodegradable and can be directly extracted from corn as one of the few natural cereal proteins in a relatively pure form. Potential applications of zein are applied in fiber, adhesive, coating, ceramic, ink, cosmetic, textile, chewing gum and biodegradable plastics (Shukla and Cheryan, 2001). Unfortunately, the relatively high cost (~$10-40 per kilogram, depending on purity) and the hygroscopic property of zein limited some applications. To achieve its full potential utilization, new purification methods should be established to greatly decrease the cost of manufacturing (Lawton 2002) and more potential applications should be investigated, which may eventually increase the needs of zein and thus further decrease its cost.

As a selected carrier material in developing antimicrobial delivery systems, the composition, solubility, structure, extraction condition, manufacture method, and industrial, health and research applications of zein are reviewed in this section to further understand the chemical and functional properties of zein.

2.1.1. Composition and Solubility

Zein is a mixture of different proteins with various molecular sizes, solubilities and amino acid sequences (McKinney 1958), with an average molecular weight of 44 kDa (Pomes 1971). Based on the solubility and related structures, zein can be classified into four distinct types: α, β, γ, and δ (Coleman and Larkins 1999). α-Zein accounts for the largest amount (~70%) of corn zein (Thompson and Larkins 1989) and can be extracted using only aqueous alcohol, whereas β, γ, and δ-zeins need a reducing agent in the solvent during extraction. α-Zein has two major bands with molecular weights of 19 kDa and 22 kDa. Interestingly, Pomes (1971) suggested that β-zein could be disulfide-linked by α-zein molecules. Recent research indicated that β-zein fraction contains α-zeins and β, γ, and δ-zeins (Lawton 2002).

Commercial zein is slightly yellow and exists in the form of fine powders. It is insoluble in water but soluble in alcohol. For example, zein is soluble in 85% aqueous isopropanol (McKinney 1958) or 55%-95% aqueous ethanol (Figure 2.1). (Figures and tables are located in the appendix to each chapter.) As exemplified in Figure 2.1 for aqueous ethanol, ~55% and ~95% are the critical concentrations of ethanol for the solubility of zein, and ~65% zein is the maximum concentration for the solubility in aqueous ethanol. Zein easily denatures in low concentrations of ethanol (Swallen 1941) and or at high zein concentrations even without heating. Low zein concentrations (<10% w/v) and high ethanol concentrations (>90% v/v) could prevent the denaturation effectively (Shukla and Cheryan 2001).

Kim and Xu (2008) showed that although zein was dissolved in 70%-90% aqueous ethanol, it still had different degrees of aggregation depending on the composition of the binary solvent mixture (ethanol and water); the minimum aggregation number of zein molecules and lowest viscosity of zein solution were observed in 89.7% ethanol solution. In addition, zein is soluble in high concentrations of urea, high concentrations of alkali (≥ pH 11) or anionic detergents (Shukla and Cheryan, 2001).

Shukla and Cheryan (2001) suggested that water insolubility of zein was due to its amino acid composition, that has a high proportion of non-polar amino acid residues (>55 g/100g zein) and a deficiency in basic and acidic amino acids. Savich (1991) proposed that the hydrophobicity of zein was primarily due to the large molecular weight peptides besides the large amount of non-polar amino acids. On the other hand, the absence of some essential amino acids, such as lysine and tryptophan, leads to a poor nutrition value of zein, which limits its applications in human food products as a protein diet.

2.1.2. Structure of Zein

Argos et al. (1982) proposed a helical structure model that described the secondary structure of zein as homologous repeating units of primary sequences (Figure 2.2). Nine adjacent, topologically anti-parallel helices were joined by glutamine-rich “turns” or “loops” to clusters within a distorted cylinder. The polar and hydrophobic residues distributed along the helical surfaces developed intra- and intermolecular “hydrogen bonding” so that zein molecules could be arranged in planes (Figure 2.3). A ~35-60% helical content of zein was measured in 50-80% ethanol (Argos et al. 1982, Momany et al. 2006).

Matsushima et al. (1997) further developed this helical structural model that proposed reduced-zeins as asymmetric particles with the length of about 13 nm and an elongated prism-

like shape with an approximate axial ratio of 6:1 in the 70% (v/v) aqueous ethanol solution. Similarly, Bugs et al. (2004) proposed that zein had an elongated molecular structure of approximately a prolate ellipsoid composed of ribbons of folded α-helical segments with a length of about 14 nm. Recently, Momany et al. (2006) reviewed this molecular model and propesed a new three-dimensional (3D) structure for α-zein with a molecular weight of 19 kDa (Z19). α-Zein has a tendency to form coiled-coil structures resulting in α-helices with about four residues per turn in the central helical sections with the non-polar residue side chains formed a hydrophobic regime inside a triple super-helix. The nine helical segments of Z19 were modeled as three sets of three interacting coiled-coil helices with segments positioned end to end. The incorporation of natural carotenoids and/or lutein into the core of the triple-helical segments helps stabilize the configuration.

Kim and Xu (2008) suggested a concept of structural inversion of zein in aqueous ethanol and 90% ethanol was regarded as the structural inversion point. Since zein has amphiphilic characteristics, micelle-like structures formed in lower than 90% ethanol have polar moieties oriented toward the solvent medium, whereas those formed in greater than 90% ethanol have non-polar moieties oriented toward the solvent medium.

2.2. Manufacture of Zein

The properties of zein are strongly dependent on the composition of zein in the resources and the processes of production. The manufacture of zein is thus important to understand how different processes affect the composition of commercial zein. Normally, zein production includes dissolving zein from raw materials, removal of fats and oils, extraction and/or elimination of pigments, extraction of zein, and recovery of zein (Shukla and Cheryan, 2001).

Raw materials, solvents used during extraction, purification methods, and recovery methods are important for zein manufacture.

2.2.1. Raw Materials

Raw dry-milled corn, corn gluten meal (CGM), or distillers dried grains with solubles (DDGS) are three common raw materials applied in zein production (Shukla and Cheryan, 2001). Zein content, in a native form without denaturation, in raw dry-milled corn is low (~4%). This limits the yield and increases the cost for extraction and recovery due to the increased cost from the solvent used. DDGS has a medium zein content (27-30%) when compared with CGM and raw corn, but DDGS is not an idealraw material for zein production due to low yields and low protein purities of extracts (Shukla and Cheryan, 2001). The most commonly used raw material in commercial zein production is CGM due to its low cost and high protein content (>60% on a dry basis, because almost all of the zein in the raw corn ends up in CGM; Shukla and Cheryan, 2001).

2.2.2. Extraction of Zein

Extraction of zein from raw materials is the first step to manufacture zein. To achieve high extraction efficiency, a solvent with a high solubility of zein should be used. Since zein is a mixture of proteins with different solubilities, a mixture of non-aqueous solvents is possible to dissolve zein. Shukla and Cheryan (2001) suggested two types of non-aqueous solvents for zein extraction; one is a mixture of an organic compound with water, the other is a mixture of two anhydrous organic compounds. Ethanol (55-95%), isopropyl alcohol (IPA, 55-88%), ketones (e.g., methyl ethyl ketone, acetone), amide solvents (e.g., acetamide), high concentrations of salt (NaCl, KBr), and esters and glycols are the possible solvents for zein (Shukla and Cheryan 2001). Appropriate heating can cut solvent losses, reduce the extraction time, and increase the

extraction efficiency (Shukla and Cheryan 2001). Generally, the temperature for zein extraction is below the boiling point of the non-aqueous solvent. In addition, acidic and alkali treatments with HCl (pH<1) or NaOH (pH>11.5) and enzymatic hydrolysis are the possible methods to increase the solubility of zein in water so that aqueous solvents are still possible for zein extraction (Payne and Tyrpin 1990, Mannheim and Cheryan 1993).

2.2.3. Commercial Production of Zein

Current commercial zein production follow the processes (Figure 2.4) of Corn Products Corporation (CPC), which was the largest manufacturer of zein from 1939 to 1967 (Shukla and Cheryan 2001). Modification of CPC processes has also been used (Figure 2.5). In CPC processes, CGM is dispersed in 86%-88% IPA or 95% ethanol for extraction, adjusted for pH and heated to 50-60°C for 30 min to 2 h, followed by filtration or centrifugation of the mixture. The filtrate or supernatant (~6% of zein) is clarified by vacuum filtration, followed by extracting fats, oils, and colorants using toluol, hexane or benzene. Next, zein is precipitated by chilled water at low temperature, and the final product, light yellow powdered zein, is yielded by vacuum drying and grinding.

Alkali treatment (pH 12) and re-extraction with 88% IPA are used in the modified CPC processes (Figure 2.5) (Swallen 1941). The modification improves the purity of zein and removes oil to approximately 2% in the zein product. The re-extraction process can reduce the oil content further to 1.4%. In the modified procedures, low temperature is used to protect against the denaturation or gelation of zein (Shukla and Cheryan 2001).

2.3. Applications of Zein

The deficiency of lysine and tryptophan and water-insolubility of zein limit direct applications in food products (Shukla and Cheryan, 2001). As a result, major interests in zein have been in the utilization as an industrial polymer. Corn zein has been commonly used in the manufacture of paper coatings, plastics, adhesives, substitutes for shellac, laminated board, and solid color printing films, and in commercial coating formulations for shelled nuts, candies, and pharmaceutical tablets (Gennadios et al. 1997).

2.3.1. Non-Food Applications

The most extensive application of zein is the tablet-coating in the pharmaceutical industry. The unique solubility and excellent film-forming properties of zein, originating from its amino acid composition and molecular structure, are bases of commercial developments. About 75% of the annually produced 500 tons of zein was used in coating medicine tablets (Reiners et al., 1973). One potential application for zein is the use as a carrier of controlled release for drugs (Hurtado-Lopez and Murdan 2005). Zein microspheres incorporated with drugs delay the release of drugs until the drug reaches the gastrointestinal tract. This application indicates the potential application of zein as carriers of food antimicrobial delivery systems.

Other applications count for a small part of the total zein uses, including zein-based textile fibers, coating in cosmetic products, paper coatings for glossy magazine covers, toilet cleansing blocks, composite wound dressings, phonograph records, and artificial jewellery (Lawton 2002). As a low-valued protein, zein still has some special nutraceutical or pharmaceutical value in health-care application. Ariyoshi (1993) reported that enzyme-hydrolyzed α-zein contained peptides inhibitory again angiotensin-converting enzyme (ACE).

2.3.2. Food Applications

Application of zein in the food industry is the main interest in this study. It has been long known that zein can form tough, glossy, hydrophobic coatings on solid food surfaces or films that provide barriers to water, oxygen and microbes (Pol et al. 2002, Hargens-Madsen 1995, Herald et al. 1996, Torres et al. 1985). In recent years, the increasing concerns on environmentally friend disposals have rekindled research interests in biodegradable packaging. Zein, as a renewable and biodegradable biopolymer, has great potential for producing edible coatings and films to enhance food quality, food safety, and product shelflife. Coatings/films act as barriers against mass diffusion (moisture, gases, and volatiles) and serve as carriers for many food additives, including flavoring agents, antioxidants, vitamins, and colorants (Cagri et al. 2004).

Physically, corn zein coatings provide a good barrier to water vapor, oxygen, and diffusion of other chemicals. The hydrophobic nature of corn zein improves water vapor barrier properties of the laminated films (Pol et al. 2002). Application of corn zein as edible coatings or packaging films decreases lipid oxidation for cooked meat and cooked turkey (Hargens-Madsen, M. R. 1995, Herald et al. 1996). Zein coatings or films are also an acceptable edible diffusion barrier to control the distribution of surface sorbic acid (Torres et al. 1985). Park et al. (1994) reported that corn zein films had an effect on delaying ripening and color changes in tomatoes during storage, and the degree of color change was mainly dependent on the thickness of zein coating. Furthermore, zein films incorporated with antimicrobial agents have the ability to extend the lag phase and reduce the growth rate of microorganisms, which may extend shelf life and maintain product quality and safety (Han 2000).

Padgett et al. (1998) studied the inhibitory effect of lysozyme, nisin, and EDTA incorporated in corn zein film produced by heat-press and casting. The cast films exhibited larger inhibition zones compared to the heat-press films against *Lactorbacillus plantarum*. The cast corn zein films had a lower nisin diffusivity, higher nisin retention, and higher activation energy for nisin diffusion than heat-pressed corn zein films, wheat gluten cast films, and heat-pressed wheat gluten films (Teerakarn et al. 2002). Corn zein films incorporated with nisin alone and in combination with lauric acid affected the water permeability and inhibition of bacterial growth (Padgett et al. 2000). Del Nobile et al. (2008) suggested that the studied antimicrobial films could be advantageously used for extending the shelf life of packed foods. In their investigation, zein films loaded with 20% and 35% thymol inhibited the growth of the selected spoilage food borne microorganisms (cells and spores of *Bacillus cereus*, *Candida lusitaniae* and *Pseudomonas spp*.) during the entire observation period; whereas films loaded with 5% and 10% thymol only slowed down the growth cycles of the tested microbes. Moreover, the thymol concentration did not affect the thymol diffusion coefficient to a significant extent, which means that zein matrix had similar release properties for thymol at different concentrations.

With the growing interests in utilizing unique properties of zein as an industrial and specialty polymer, more technologies may be developed. The potential application of producing zein particles with controlled release of antimicrobials was investigated in this study.

2.4. Properties and Functionality of Lysozyme

Lysozyme (EC 3.2.1.17) is widely present in tears, saliva, nasal secretion, mucus, milk of humans and other animals, and plants (Johnson and Larson 2004, Safarik et al. 2007). It was discovered by Alexander Fleming in 1921 (Fleming 1922). The increased research interest in lysozyme is because hen egg lysozyme is one of the few naturally-occurring antimicrobials

approved as GRAS by FDA for use in foods (Federal Register 1998). In addition, the majority of the lysozyme used both in research and in food products is purified from hen egg white (type C lysozyme) due to the relative ease of purification, low toxicity, low effective usage levels and low interference on sensory qualities of foods (Johnson and Larson 2004). Lysozyme can be added directly into food products to inhibit the growth of undesired bacteria in traditional food applications and be incorporated into plastic (e.g., crosslinked polyvinylalcohol (PVOH)) or biopolymer edible films (e.g., whey protein films and chitosan films) to produce antimicrobial packaging materials as emerging food applications (Buonocore et al. 2003, Min et al. 2005, and Park et al. 2004).

2.4.1. Physicochemical Properties of Lysozyme

Lysozyme is a single polypeptide chain composed of 129 amino acids (Figure 2.6) with a molecular weight of ~ 14.4 kDa (Johnson and Larson 2004). It is amphiphilic, as a protein, having embedded hydrophobic amino acids and outer hydrophilic amino acids. Four pairs of cysteines (amino acids No. 6 and 127, 30 and 115, 64 and 80, 76 and 94) form 4 disulfide bonds, which gives lysozyme a compact globular tertiary structure with a long cleft on the protein surface. This structure is responsible for the function of lysozyme. Johnson and Larson (2004) mentioned that if more than 2 of the 4 disulfide bonds are disrupted, lysozyme would lose its bioactivity, i.e., the cell-lysing ability.

2.4.2. Antimicrobial Mechanism of Lysozyme

Lysozyme has a broad spectrum of antimicrobial activities against Gram-positive bacteria and fungi such as *Bacillus stearothermophilus, Micrococcus spp., Clostridium tyrobutyricum,* and *Listeria monocytogenes* (Johnson and Larson 2004). The antibacterial property comes from catalyzing hydrolysis of β-1, 4-glycosidic bonds between the C-1 residue of N-acetylmuramic

acid and the C-4 residue on N-acetylglucosamine of bacterial peptidoglycan in the cell membrane (Johnson and Larson, 2004), which increases the bacteria's permeability and causes the bacteria to burst. The cell membranes in Gram-positive bacteria contain around 40-90% of peptidoglycan, but only 10% for Gram-negative bacteria (Islam et al. 2006). Thus, this enzyme has broad lysis effects against Gram-positive bacterial but seldom inhibits Gram-negative bacteria. However, with EDTA, *E. coli* could be sensitive to lysozyme. The use of lysozyme in combination with nisin has synergic effects in inhibition property, which also broadens the spectrum of antimicrobial activity for lysozyme (Johnson and Larson 2004).

The antimicrobial mechanism has been also related with the physical structure of lysozyme. Philips (Blake et al. 1965) determined lysozyme's structure using x-ray crystallography and provided an explanation for how the enzyme facilitates a chemical reaction due to a substrate distortion in the physical structures. Recently, Vocadlo et al. (2001) revised the mechanism of the hydrolysis for lysozyme proposed by Philips and formulated a general catalytic mechanism for all enzymes containing β-glycosidase that includes substrate distortion, formation of a covalent glycosyl-enzyme intermediate (Asp52 of lysozyme), and the electrophilic migration of C1 along the reaction coordinate.

2.5. Techniques of Developing Particulate Antimicrobial Delivery Systems

Investigation of delivery systems with sustained release of drug proteins or food antimicrobials has been greatly advanced in recent years due to the potential benefits of sustained release of the encapsulated compounds. The common methods of obtaining sustained release are to entrap, coat or microencapsulate antimicrobials into special food biopolymers to develop

delivery systems, such as films directly coated with antimicrobials or microcapsules (Gouin 2004).

Particulate delivery systems with controlled release have mainly been studied and developed for pharmaceutical applications using techniques such as emulsification, phase separation, coacervation, liposome entrapment and freeze drying (Bezemer et al. 2000, Gibbs et al. 1999, Hurtado-Lopez and Murdan 2005, 2006, Liu et al. 2004, Parris et al. 2005). Bezemer et al. (2000) encapsulated lysozyme in poly(ethylene glycol) / poly(butylene terephthalate) using a water-in-oil-in-water (w/o/w) emulsion method to prepare bilayer microspheres, followed by filtration and freeze-drying. The system showed zero-order release kinetics of the encapsulated lysozyme. Liu et al. (2004) investigated a novel microsphere drug delivery system of ivermectin using hydrophobic protein corn zein by the phase separation method. A coacervation method was applied to study zein microspheres as drug/antigen/vaccine carriers (Hurtado-Lopez and Murdan 2005, 2006). These methods are efficient but expensive due to a set of processes, including emulsification, evaporation of alcohol, concentration by centrifugation or filtration, and spray or fluidized bed drying (Gibbs et al. 1999).

Meanwhile, techniques with fewer steps were also studied for producing delivery systems. Nanoemulsions of essential oils were produced by the solvent attrition technique, using high speed homogenization, to encapsulate three essential oils (oregano, red thyme, and cassia) into zein nanocapsules (Parris et al. 2005). Salmaso et al. (2004) microencapsulated nisin in poly(L-lactide) using a supercritical anti-solvent technique (SAS). The encapsulated nisin showed sustained release over 45 days. Good miscibility of supercritical CO_2 and co-solvent is important for the particle synthesis using the SAS technique. The simple “one-step” , low temperature process makes SAS attractive for food applications.

The above solvent attrition, spray drying and SAS techniques are feasible for microencapsulating antimicrobials in biopolymers. Development of these techniques for manufacturing antimicrobial delivery systems should also consider the properties of carrier materials and antimicrobials, in addition to processing conditions. This work utilized zein as the carrier material and lysozyme as an antimicrobial to study the feasibilities of the above three processes.

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Appendix

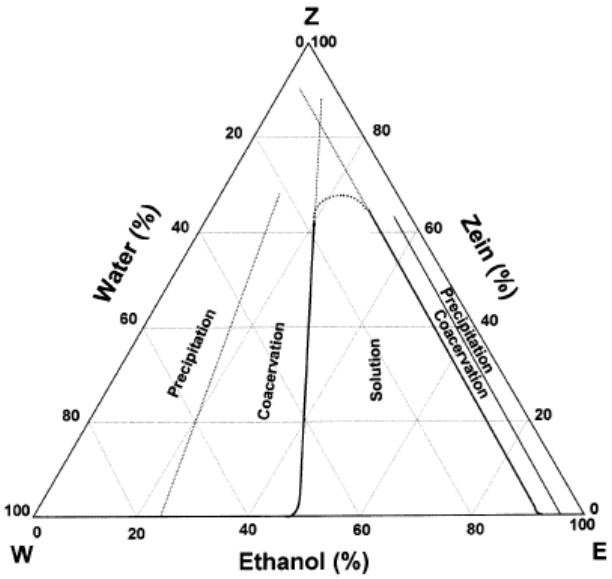

Figure 2. 1. Ternary phase diagram showing the solubility of zein in binary solvent mixtures of ethanol and water.
Adapted form Shukla and Cheryan (2001).

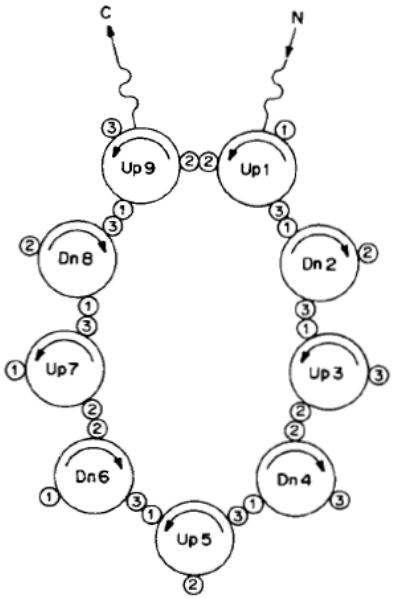

Figure 2. 2. A possible nine-helical zein protein structural model shown in projection with the helical axes orthogonal to the figure plane.
The hydrogen-bonding polar residue segments are shown as small circles. Adapted from Argos et al. (1982) © the American Society for Biochemistry and Molecular Biology.

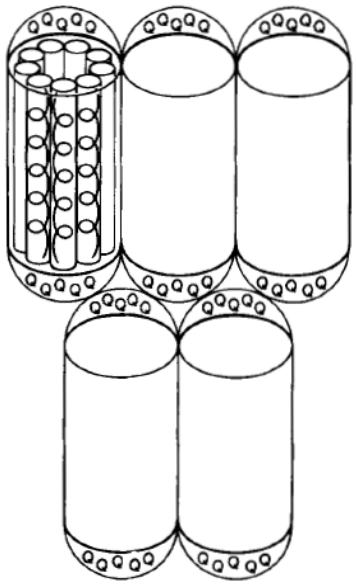

Figure 2. 3. A possible model for the arrangement of zein proteins within a plane as well for the stacking of molecular planes.
The glutamine residues (Q) would allow hydrogen bond interactions among molecules in neighboring planes. Adapted from Argos et al. (1982) © the American Society for Biochemistry and Molecular Biology.

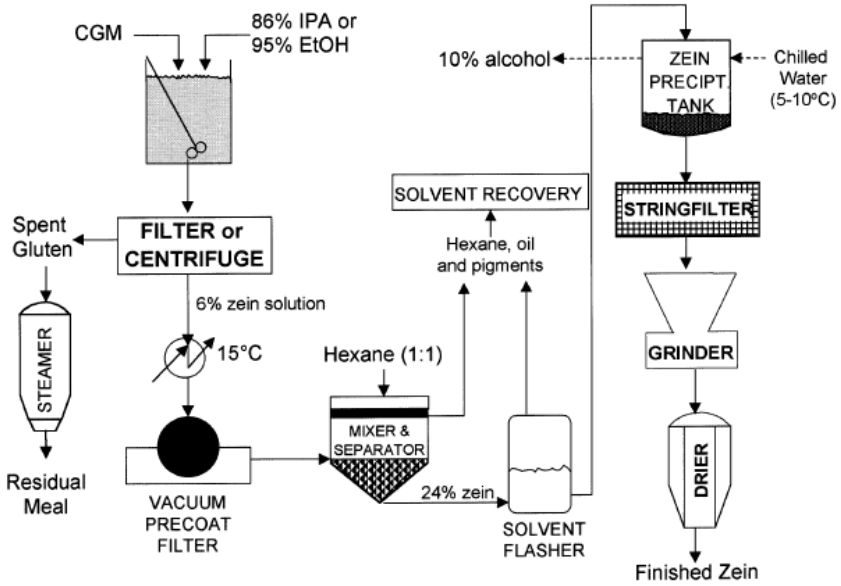

Figure 2. 4. Corn Products Corporation (CPC) processes for production of zein from corn gluten meal.
Adapted from Swallen. (1941).

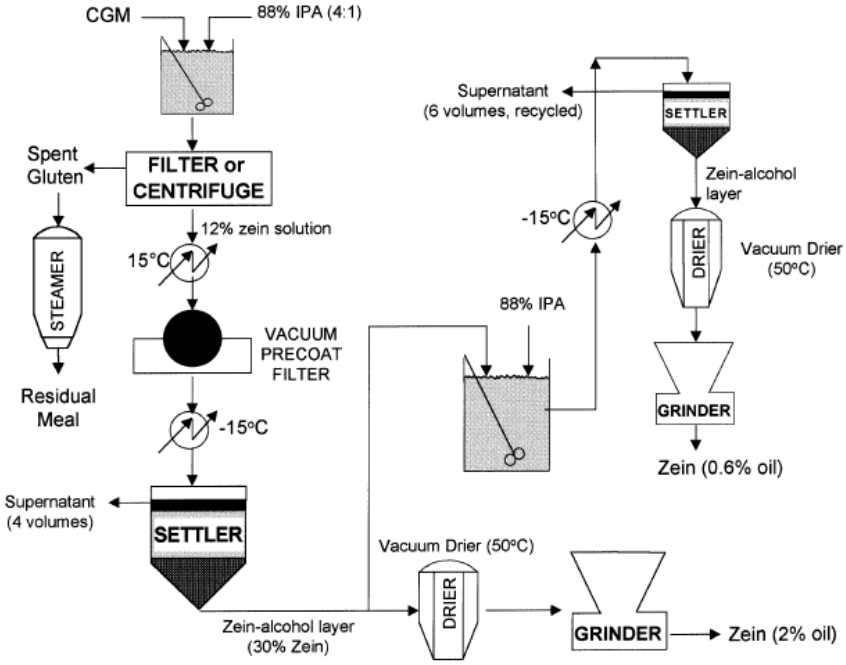

Figure 2. 5. Nutrilite processes for production of zein from corn gluten meal. Adapted from Carter and Reck (1970).

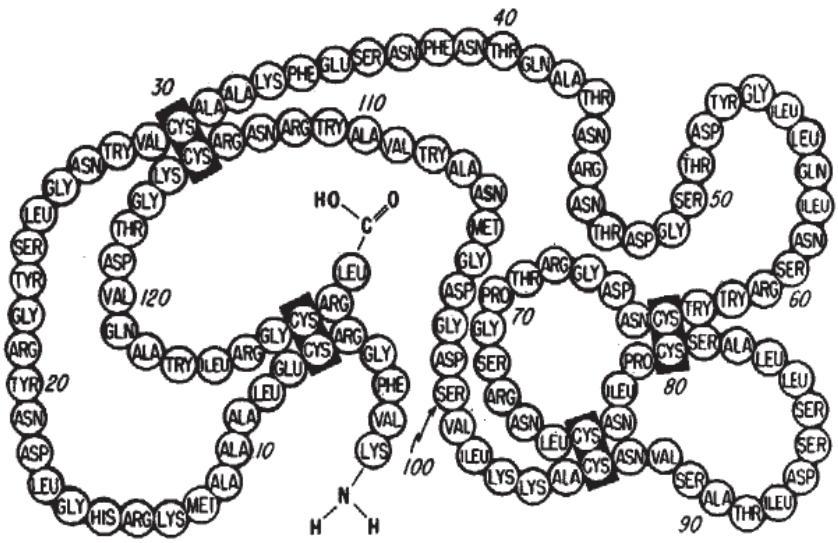

Figure 2. 6. Sequence of amino-acid residues in hen egg white lysozyme. Adapted from Blake et al. (1965).

Chapter 3 . Production of Zein Nanoparticles as Delivery Systems by Solvent Attrition

3.1. Abstract

The first objective of this work was to explore processing variables that affect the nano- or microparticle production using zein during the process of solvent attrition. The second objective was to encapsulate lysozyme in zein particles, a water-soluble antimicrobial, and evaluate in vitro lysozyme release kinetics. Because eventually, these nano-delivery systems will be incorporated in consumer products that have other ingredients, such as thickening agents, our third objective was then to study rheological properties of a model system containing carboxymethylcellulose (CMC) solutions with dispersed nanoparticles. The process was based on dissolving zein in a stock solution of 55-90% aqueous ethanol, which was then subjected to shearing in deionized water using a high speed blender. The size and morphology of zein nanoparticles were significantly affected by the variables during synthesis, including shear rate, ethanol concentration and zein concentration in stock solutions. Both separated or connected particles were observed due to two competing processes, fragmentation of stock solution droplets and solidification of zein. Diameters of zein nanoparticles were typically between 100-200 nm. The addition of 1% zein nanoparticles into 0.5% carboxymethylcellulose solutions (adjusted to pH 3 to 9) increased the viscosities of the polymer solutions. Viscosities increased at higher pHs. Lysozyme was demonstrated to be encapsulated in zein nanoparticles by the solvent attrition process, with sustained release at pH 7 and 8 but no sustained release at lower pHs of 2 to 6. Under basic conditions, release was much less and slower due to greater electrostatic and hydrophobic attractions when the pH was closer to the pI of lysozyme (10.5-11.0). The results showed that solvent attrition was a simple and scalable process to manufacture delivery systems of lysozyme. The materials used in this work are generally-recognized-as-safe and may find unique applications for food systems.

3.2. Introduction

The promise of nanometer-sized food delivery systems has been recently discussed (Weiss et al. 2006). Most studies thus far have utilized colloid-based structures, such as liposomes and microemulsions (Were et al. 2004, Gaysinsky et al. 2005a, Were et al. 2003, Gaysinsky et al. 2005b, Taylor et al. 2005). Much work is needed to develop scalable processes using low-cost, abundant food ingredients as carrier materials, e.g., food biopolymers.

One practical application of nanodelivery systems involves their use in beverages because of the advantages of enhanced dispersibility. To maintain the delivery functions during the long shelflife of beverages (weeks or months), it is preferable that delivery systems can sustain the structure integrity. The stability of delivery systems may be achieved by application of water-insoluble food biopolymers or self-organized structures that are stable in aqueous media (for example, microemulsions). One such system that could function successfully would be zein. Zein (corn prolamines) is a group of water-insoluble but alcohol-soluble storage proteins that are predominantly present in the endosperm of corn kernels (Shukla and Cheryan 2001). Further, zein is considered odorless and tasteless (Park 1999) and thus may potentially mask undesirable aroma and taste of some bioactive compounds.

Zein is produced in large quantities - more than 7000 tons per year before 1970s and ~500 tons in 2001; fluctuation was dependent largely on the demand (Shukla and Cheryan 2001). Commercial zein is currently separated from corn gluten meal, a co-product of corn wet milling, and is a mixture of at least four types of proteins: α-, β-, γ-, and δ- zein, each with a different amino acid sequence, molecular weight, and solubility (Shukla and Cheryan 2001, Zhu et al. 2007). Although the majority of zein is the α- and β-zein (Shukla and Cheryan 2001), the

variation in proportions of different types of zein is expected to be a function of raw materials and conditions during purification. Highly-purified zein is currently relatively expensive - $10-40 per kg or ~$5-20 per pound, depending on purity (Shukla and Cheryan 2001). Improvement in separation technology or use of less pure zein (assuming the controlled delivery of bioactive compounds can be achieved) can further reduce the cost of zein. Further, recovering zein as a co-product from the ethanol industry can decrease the cost of zein to $4.4 per kg or ~$2 per pound with ~90% purity (Kale et al. 2007). Approximately 13,000 tons of zein could be recovered in the production of 50 million gallons of ethanol annually (Kale et al. 2007), which also increases the profitability of the biorefinery (Shukla and Cheryan 2001, Kale et al. 2007, Xu et al. 2007).

Several studies have used zein to produce edible capsules (Liu et al. 2005, Hurtado-Lopez and Murdan 2006) or films (Dawson et al. 2003, Wang et al. 2005). Controlled release of heparin, a water-soluble drug, was observed *in vitro* over 20 days from the films made of microspheres with encapsulated heparin (Wang et al. 2005). To prepare microspheres, zein was first dissolved in aqueous ethanol and then water was added to precipitate zein and drug simultaneously by coacervation (Liu et al. 2005, Hurtado-Lopez and Murdan 2006). *In vitro* release of a water-soluble drug, ivermectin was reported over 9 days from the produced microspheres (Liu et al. 2005). These studies suggested the potential of developing delivery systems of water-soluble antimicrobials using zein as a carrier biopolymer.

In the present study, we used a process that manipulated the solubility characteristics of zein in different concentrations of ethanol which was first reported for pharmaceutical coatings application (Oshlack et al. 1994, O'Donnell et al. 1997). Nanoparticles were produced by shearing zein, dissolved in a 70:30 ethanol/water mixture, into bulk water phase. Nanoparticle production was based on the solvent-attrition mechanism in which the ethanol diffuses out of the

sheared droplets into the continuous phase water during shearing. The process has been used to microencapsulate spice essential oils, oregano, red thyme, and cassia (Parris et al. 2004). This technology is very simple and scalable and may be used to microencapsulate a variety of compounds.

The first objective of this work was to explore processing variables that affect the particle production during solvent-attrition. The second objective was to encapsulate lysozyme, a water-soluble antimicrobial, and evaluate *in vitro* lysozyme release kinetics. Because eventually, these nano-delivery systems will be incorporated in consumer products that have other ingredients such as thickening agents, our third objective was then to study rheological properties of a model system containing carboxymethylcellulose (CMC) solutions with dispersed nanoparticles. The CMC was used because the hydrocolloid is extensively used in food and non-food products for various functions such as thickening and stabilizing agents (BeMiller and Whistler 1996).

3.3. Materials and Methods

3.3.1. Materials

Purified zein and ethanol (200 proof) were purchased from Acros Organics (Morris Plains, NJ). The CMC sample (Ticalose® CMC 2500 Powder) was a free sample from TIC Gums, Inc. (Belcamp, MD). Lyophilized hen egg white lysozyme (product # L-6876) and the test microorganism *Micrococcus lysodeikticus* were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were from Fisher Scientific (Pittsburgh, PA).

3.3.2. Protocol of Producing Nanoparticles

The protocol followed that of a literature method (Parris et al. 2005), with slight modification. Stock solutions were prepared by dissolving different amounts of zein in 15 ml 55-

90% ethanol (rest being deionized water). The stock solution was sheared into 40 ml of de-ionized water for 2 min while a high speed blender (Cyclone I.Q.^{2}, The VirTis Compary, Inc., Gardiner, NY) was running at a fixed speed from 5,000 to 15,000 rpm. Particle size distributions of fresh dispersions were measured by dynamic light scattering (DLS) (model ZetaPALS, Brookhaven Instruments Corporation, Brookhaven, NY). All operations were at room temperature. Dispersions were freeze-dried to prepare the powder-form nanoparticles. Powdered samples were stored at -20 °C until for morphological or rheological tests.

3.3.3. Scanning Electron Microscopy (SEM)

The surface morphology of microparticles was imaged using a LEO 1525 microscope (LEO Electron Microscopy, Oberkochen, Germany). The microparticles were loosely attached onto a black adhesive tape mounted on a stainless steel stub and sputter-coated with a gold layer of ~5 nm thickness to prevent the charging on protein samples. Selected images were also analyzed for particle sizes using the ImageJ software available from the National Institute of Health (Bethesda, MD).

3.3.4. Production of Zein Nanoparticles with Encapsulated Lysozyme

A stock solution was prepared by dissolving 5 g of zein and 0.015 g lysozyme in 30 mL 60% ethanol. The stock solution was then blended into 300 mL deionized water at 10,000 rpm for 2 min. The dispersion was immediately placed in a -40 °C freezer and then freeze-dried (Model 12EL, The Virtis Company, Inc., Gardiner, NY). Particles were stored in -20°C.

3.3.5. Evaluation of *in vitro* Release Kinetics

To evaluate lysozyme *in vitro* release kinetics, 27 mg of lysozyme-loaded zein particles were dispersed in a microcentrifuge tube filled with 1.5 mL of a 66 mM potassium phosphate

buffer (adjusted to a pH between 2 and 8 with 1 N HCl or 1 N KOH, respectively). The dispersions were continuously mixed using an end-to-end shaker (Lab Industries Inc., Berkeley, CA) at room temperature. At a preset time point, the dispersions were centrifuged at 5,000 × *g* for 5 min (MiniSpin Personal, Eppendorf, Westbury, NY). One mL of each supernatant was transferred for measurement of the released lysozyme using a Sigma method (for product L-6876). The remainder sample was supplemented with 1.0 mL of the fresh corresponding buffer, redispersed and mixed for continued release studies. The total amount of lysozyme in unit mass of powders was measured by dissolving 27 mg of powders in 15 mL 80% ethanol that dissolved both zein and lysozyme completely.

Calculation of accumulative lysozyme release was as follows:

Let the lysozyme volumetric concentration (U/ml) at the first sampling time, t_1 to be a_1, the accumulative release at time t_1 is:

$$R_{t_1}(\%) = \frac{1.5\,a_1}{U_o} \times 100\% \tag{2}$$

where U_o is the total lysozyme units included in the dispersion before release tests, and the prefix 1.5 before a_1 is the total volume of dispersion.

After removing 1 mL of supernatant, there are 0.5 a_1 units of lysozyme due to the release after time t_1, which carries to the second sampling at time t_2. Similarly, if the volumetric concentration of supernatant from the second sampling is a_2 (U/ml), the total lysozyme released up to this point is a_1 + 1.5 a_2, which gives an accumulative release at time t_2:

$$R_{t_2}(\%) = \frac{a_1 + 1.5\,a_2}{U_o} \times 100\% \tag{3}$$

Therefore, at the i^{th} sampling, the accumulative release is:

$$R_{t_i}\ (\%) = \frac{\sum_{n=1}^{i-1} a_n + 1.5\, a_i}{U_o} \times 100\% \qquad (3)$$

3.3.6. Measurement of Lysozyme Activity

The activity of lysozyme was measured according to the protocol provided by Sigma for hen egg white lysozyme (product number L-6876). The test microorganism was *Micrococcus lysodiekticus*, and measurements were based on the reduction rate of absorbance at 450 nm due to the lysis of cells by lysozyme at pH 6.24 and 25 °C. The lysis kinetics was measured using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA).

3.3.7. Rheological Measurements

3.3.7.1. Sample preparation

Nanoparticles were prepared using a stock solution of 1 g zein dissolved in 15 mL 85% ethanol, emulsified into 40 mL deionized water at 10,000 rpm and freeze-dried as above. The CMC powder and zein nanoparticles were redispersed in deionized water and stirred for 16 h overnight to hydrate at room temperature. The dispersion was then adjusted to pH 3, 5, 7, or 9 with 1 N HCl or 1 N NaOH. The final concentration of CMC was 0.5% (w/v) and that of zein was 1% (w/v). Controls were the 0.5% (w/v) CMC solutions without zein nanoparticles.

3.3.7.2. Rheological tests

Each sample was measured in triplicate and the averages from three tests were reported. Tests were performed with an AR2000 rheometer (TA Instruments, New Castle, DE) using a Searle set up (bob OD = 28 mm; cup ID = 30 mm). After positioning the bob and removing the excess sample, a layer of mineral oil was applied onto the top of the sample to minimize the moisture loss during measurements. Viscosity tests followed two subsequent steps: (1) a shear

rate ramp from 1 to 1000 s^{-1} at 20 °C and (2) a heating ramp from 20 to 90 °C at 2 °C/min, with a shear rate of 1 s^{-1}.

3.4. Results

3.4.1. Effect of Blending Speed

The stock solution with 85% ethanol was used to study the effect of blending speed during particle production. Diameters of particles in fresh dispersions were not greatly different at different blending speeds based on DLS (Figure 3.1.). SEM images showed the formation of spherical nanoparticles (Figure 3.2). The measured diameters based on SEM images showed a decreased diameter at a higher blending speed (Figure 3.1.). Because the lowest speed (5000 rpm) enabled the production of spherical nanoparticles, the following studies were performed at this shearing speed.

3.4.2. Effect of Ethanol Concentration

Results from DLS showed the smallest diameter of zein nanoparticles when the ethanol concentration in stock solution was 80% (Figure 3.3). With an increased ethanol concentration from 55% to 80%, the hydrodynamic diameter measured from DLS decreases; the opposite was true when the ethanol concentration was increased from 80% to 90%. SEM results showed that not all particles are separated for the 60% and 90% ethanol treatments (Figure 3.4); particles are connected more extensively for the 90% ethanol treatment. Estimations of particle size based on diameters of identifiable partial spherical structures showed the opposite trend to the data from DLS (Figure 3.3), although the numbers are of the same magnitude.

3.4.3. Effect of Zein Concentration

Dissolving a smaller amount of zein in stock solutions resulted in smaller diameters based on DLS (Figure 3.5). However, more than 2 g of zein dissolving into the stock solution did not enable the formation of a homogenous dispersion because agglomerates of unemulsified zein were visibly observed during experiments.

3.4.4. Lysozyme *in vitro* Release Kinetics

In vitro release kinetics of lysozyme is plotted in Figure 3.6. At pH 2-6, the release reached equilibrium in 30 min. At pH 7, gradual release was observed up to 24 h. Gradual release of lysozyme was also observed at pH 8, but a much lesser amount of lysozyme was released when compared to other pH conditions.

3.4.5. Rheological Properties

Shear rate ramp results are plotted in Figure 3.7. For CMC solutions without nanoparticles, viscosity increased monotonically with pH until pH 7, further increasing the pH to 9 reduced the viscosity. Addition of zein nanoparticles into CMC solutions further increased the viscosity, and the degree of increase was more apparent at a higher pH condition. In all samples, shear-thinning was observed.

When heating the CMC solutions (samples without zein nanoparticles), the viscosity decreases with temperature except for the sample adjusted to pH 3 at a temperature above ca. 80°C (Figure 3.8). The addition of zein nanoparticles did not change the overall characteristics of CMC solutions, except for the sample adjusted to pH 7 that showed fluctuations above a temperature of approximately 35 °C, although the overall trend showed a decrease with temperature.

3.5. Discussion

3.5.1. Particle Synthesis

The principle of solvent attrition during synthesizing zein nanoparticles is presented in Figure 3.9, similar to a "liquid-liquid dispersion" technique (Alargova et al. 2006, Alargova et al. 2004). The zein sample in this work is soluble at an ethanol concentration range of ~55-90% ethanol (v/v), based on visual absence of particulates. When a stock solution of zein is sheared into bulk deionized water, the stock solution is emulsified to smaller droplets. Due to the excellent miscibility of ethanol and water, ethanol in the emulsified droplets partitions into the bulk water – solvent attrition. When the ethanol concentration in the emulsified droplets decreases to a level low enough, zein becomes insoluble and precipitates to form nanoparticles.

During shearing, there are two competing mechanisms. The first one is the breakup of the stock solution droplets by the shear force applied. The second one is the simultaneous solidification of zein during the solvent attrition. If the time scale for breakup is shorter than zein solidification, individual zein nanoparticles may be formed. However, if zein solidifies before breakup of droplets, connected spherical particles or even nonspherical structures may be present (Figure 3.9). The observations in Figures 3.1-3.5 are qualitatively discussed below.

3.5.1.1. Effect of Blending Speed

Theoretically, stock solutions are sheared to smaller droplets at a higher blending speed that should correspond to smaller particles after zein solidification. The trend was observed based on SEM images (Figures 3.1 and 3.2). However, DLS results showed no difference in diameters for samples produced at three blending speeds (Figure 3.1.).

3.5.1.2. Effect of Ethanol Concentration

The stock solution with 85% ethanol produced individual spherical nanoparticles (Figure 3.2A). When the ethanol was lowered to 60% or increased to 90%, the majority of spheres seemed to be "bonded" to each other (Figure 3.4). The observations can be explained by the two competing processes during zein precipitation into particles. On one hand, the stock solution is being sheared and extended, facilitating the breakup of droplets and thus formation of individual particles. On the other hand, ethanol diffuses from the emulsified droplets to the continuous medium, and this lowers the overall ethanol concentration to a limit where zein starts to precipitate. At a lower ethanol concentration, the time scale for zein precipitation may be shorter than that of droplet breakup; zein precipitates prior to the complete droplet breakup, generating predominantly spherical structures with some bonding structures (Figure 3.4A). At 90% ethanol, the time scale for zein precipitation is relatively long because it takes a longer time for ethanol diffusing out of the droplets (to decrease ethanol concentration to induce zein solidification); this may have enabled the coalescence of initially broken-up "soft" particles whose ethanol concentration was still sufficiently high to maintain zein unsolidified. The "partially coalesced" stock solution droplets eventually precipitated into many particles with irregular structures, as seen in Figure 3.4B. At intermediate ethanol concentrations, the balance of two time scales may correspond to conditions for formation of separated spherical particles (Figure 3.2A).

Because DLS measures the hydrodynamic radius from the overall structures, the interconnected structures at lower and higher ends of ethanol concentrations may have resulted in larger measured particle sizes (Figure 3.3). Smaller spherical diameters measured from the SEM image of the 60% ethanol sample (Figures 3.3 and 3.4A) may have been caused by the faster solidification of zein, which may be true until an ethanol concentration of 80%. Above

80% ethanol, “partial coalescence” and breakup of stock solution droplets occur simultaneously; solvent attrition gradually reduces ethanol concentration in the “partially coalesced” droplets. Eventually, the “spherical structures” formed are smaller in characteristic “diameters” because of the longer shearing time (before zein eventually loses solubility) allowed for 85% and 90% ethanol treatments.

Although connected structures of zein nanoparticles were observed at both ends of ethanol concentrations, it however should be noted that plasticizers may be used to manipulate the solidification of zein during solvent attrition (O'Donnell et al. 1997). This will be our future research topic.

3.5.1.3. Effect of Zein Concentration

A monotonic increase of particle sizes measured from DLS corresponds to an increase in zein concentration or solution viscosity (Figure 3.5). When the zein concentration increases to above a certain value (e.g., the sample with 2 g in 15 mL 80% ethanol), the viscosity of stock solution generates a stronger resistance for deformation, leading to larger droplets and thus particle sizes (Dixon et al. 1993). A higher zein concentration also corresponds to a shorter time scale for polymer solidification because of the larger amount of nuclei created for growth of precipitates (Kashchiev and van Rosmalen 2003). Therefore, at high zein concentrations, incomplete emulsification was observed (Figure 3.5).

3.5.2. *In vitro* release Kinetics of Lysozyme

The dependence of release characteristics on pH (Figure 3.6) may be explained by molecular interactions between zein and encapsulated lysozyme. The isoelectric point (pI) of zein is approximately 6.8 (Cabra et al. 2005); therefore zein is positively charged at pH 2-6 and negatively charged at pH 7 and 8. On the other hand, lysozyme has a pI of 10.5-11.0

(Cunningham et al. 1991) and is always positively charged at pH 2-8. Therefore, the electrostatic interactions are repulsive at pH 2-6 (both zein and lysozyme are positively charged) and attractive at pH 7 and 8 (zein and lysozyme are oppositely charged). In addition, because zein is hydrophobic (water insoluble), hydrophobic interactions should theoretically become stronger at a higher pH closer to the pI of lysozyme. Summation of these two forces gives overall stronger attractive forces at a higher pH, which corresponds to (1) a quick release of lysozyme at pH 2-6, (2) gradual release of lysozyme at pH 7 and 8, and (3) a smaller amount of lysozyme release at a smaller rate (a smaller increase at a same release time period) at pH 8 than at pH 7.

3.5.3. Rheological Properties

For CMC solutions without zein nanoparticles, the monotonic increase in viscosity at a higher pH condition until pH 7 (Figure 3.7) may be explained by a larger hydrodynamic radius of polyelectrolytes. The carboxymethyl group has the pKa of 3.65 (King and Smibert, 1963). The CMC is thus more negatively charged at a higher pH and has a bigger hydrodynamic radius. A bigger hydrodynamic radius of polymers corresponds to a higher solution viscosity because of the higher degree of polymer chain entanglement (Morris et al. 1981). However, when polyelectrolytes are extensively charged (at pH 9), polymer chains become rigid, that may have accounted for the reduced viscosity when pH was increased from 7 to 9 (Figure 3.7). A similar trend (increase in viscosity from pH 4 to pH 8, followed by a decrease at higher pH values) was also observed for hydrophobically modified CMC (Cohen-Stuart et al. 1998).

The increase of CMC solution viscosities due to the addition of zein nanoparticles is expected, as the case of dispersing colloidal particles (fillers) into polymer solutions (Gupta 2000). A greater degree of increase in viscosity by zein nanoparticles at a higher pH may be explained by the effective diameters of zein nanoparticles at different pH values. Zein is soluble

in highly alkaline solutions (pH ≥ 11) (Shukla and Cheryan 2001). At a higher pH, the interactions between zein nanoparticles and water should be strengthened (although may still be insoluble at the tested pH range of 3-9), which may increase the effective diameter of zein nanoparticles in solutions.

Finally, the abnormity of viscosity during heating of the pH 7 sample with added zein nanoparticles may be caused by strengthened hydrophobic interactions and reduced electrostatic repulsion because the pH is close to the pI of zein, 6.8 (Cabra et al. 2005). The hydrophobic interactions between zein nanoparticles however may not have been strong enough to induce aggregation but have resulted in weak flocculation. The weakly flocculated zein nanoparticles may have been instantaneously dissociated by the continuous shear during heating, which caused the abnormally fluctuating viscosities in Figure 3.8.

3.6. Conclusions

Solvent attrition was studied for the formation of zein nanoparticles with diameters between 100-200 nm, and used to encapsulate water-soluble antimicrobial lysozyme. The size and morphology of zein nanoparticles were significantly affected by the variables during synthesis. Separated or connected particles were both observed due to two competing processes: breakup of stock solution droplets and solidification of zein. The encapsulated lysozyme showed *in vitro* sustained release at pH 7 and 8 up to 24 h, but a complete release within 30 min at pH 2-6. The pH-dependent release profiles were affected by the type and strength of electrostatic interactions, as well as hydrophobic interactions. When zein nanoparticles were dispersed in CMC solutions at different pH conditions, the viscosities of dispersions increased monotonically with pH, possibly due to an increased effective diameter of nanoparticles. This work

demonstrated that solvent attrition is a simple and scalable process that may find applications to develop delivery systems of many bioactive compounds, including antimicrobials. However, the effects of these potential delivery systems on physical properties of eventual food products need to be examined.

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Appendix

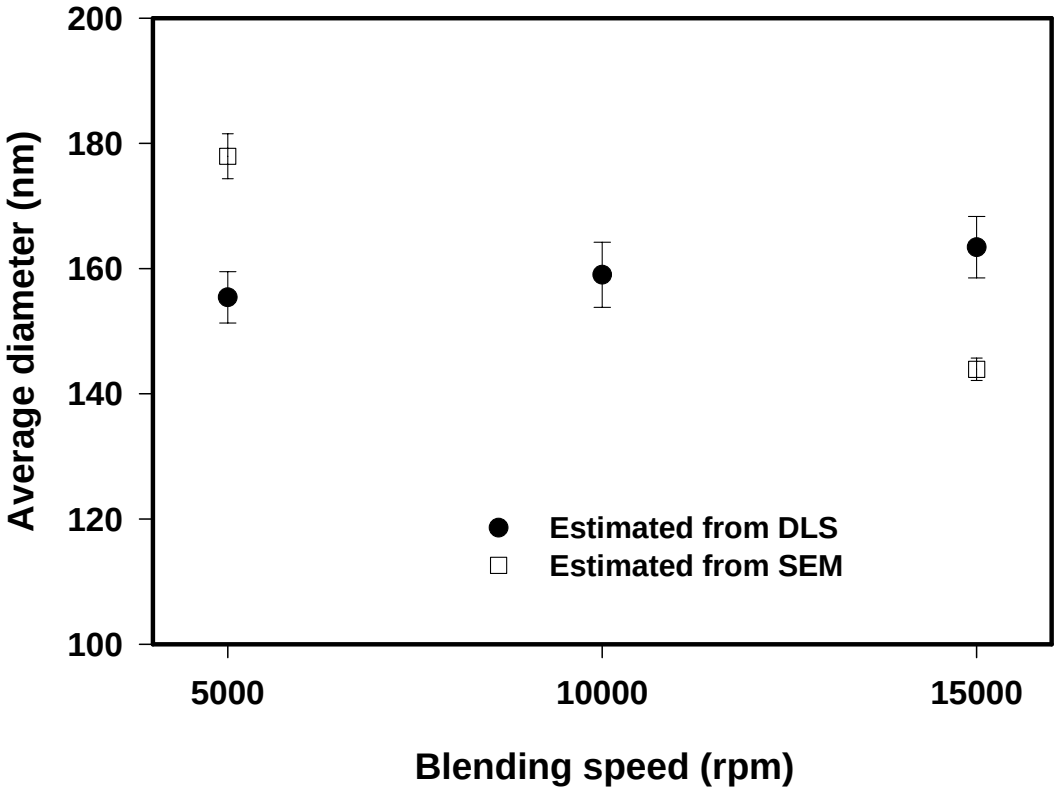

Figure 3. 1. Effect of blending speed on the sizes of zein nanoparticles prepared from a stock solution with 1 g zein dissolved in 15 ml of a 85: 15 (v:v) ethanol : deionized water mixture, blended into 40 ml deionized water.
Results of dynamic light scattering are surface average diameters ($D_{3,2}$) from 10 runs and those of SEM are estimates from sphere diameters based on SEM images. Error bars are 95% confidence intervals.

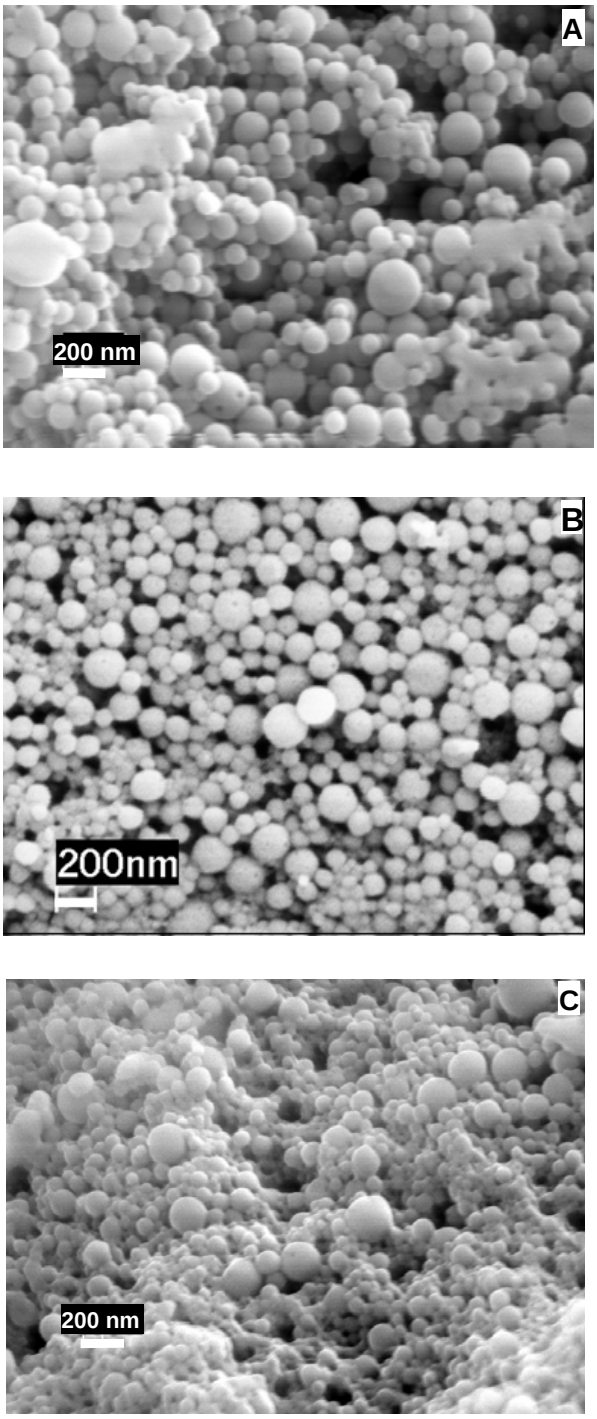

Figure 3. 2. SEM images of zein nanoparticles prepared with a stock solution with 1 g zein dissolved in 15 ml of a mixture of 85: 15 (v:v) ethanol : deionized water, blended into 40 ml deionized water at 5,000 (A), 10,000 (B), or 15,000 rpm (C).

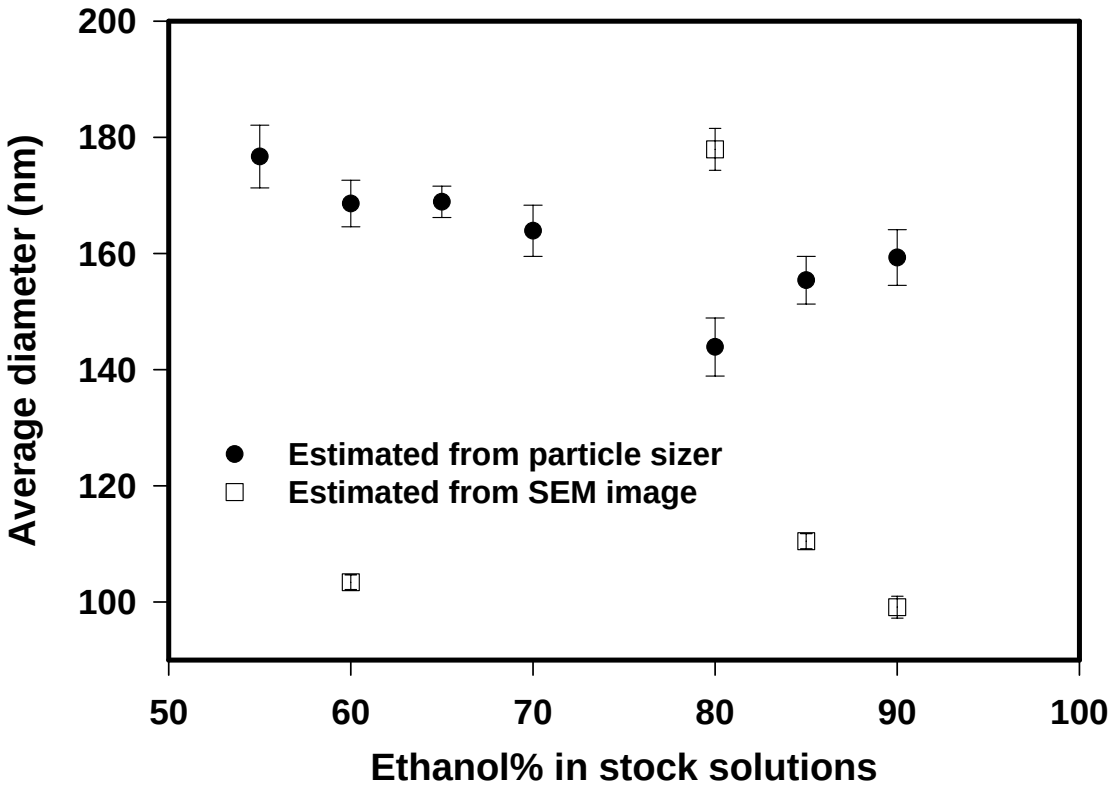

Figure 3. 3. Effect of ethanol concentration in stock solutions on the sizes of zein nanoparticles prepared from stock solutions of 1 g zein dissolved in 15 mL of different ethanol concentrations, blended at 5,000 rpm into 40 mL deionized water.
Results of dynamic light scattering are surface average diameters ($D_{3,2}$) from 10 runs and those of SEM are estimates from sphere diameters based on SEM images. Error bars are 95% confidence intervals.

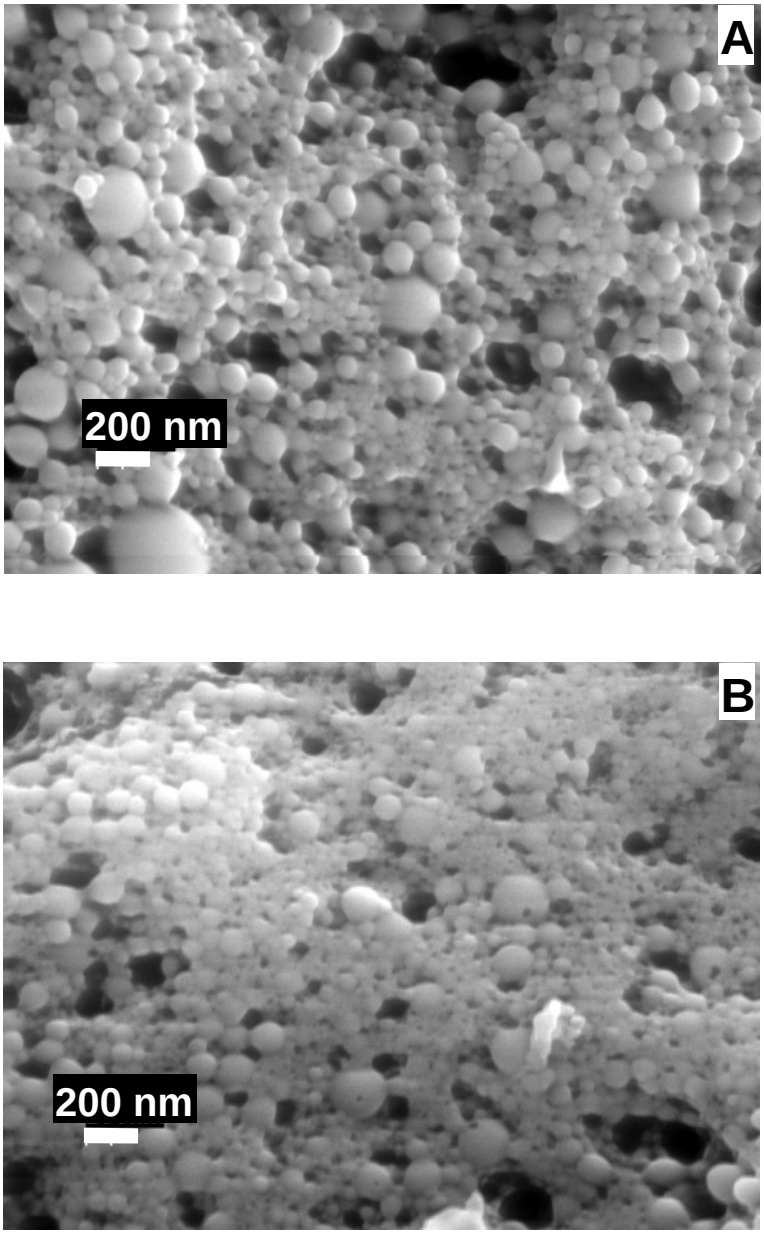

Figure 3. 4. SEM images of zein nanoparticles prepared with a stock solution with 1 g zein dissolved in 15 ml 60% v/v ethanol (A) or 90% ethanol (B), blended into 40 ml deionized water at 5,000 rpm.

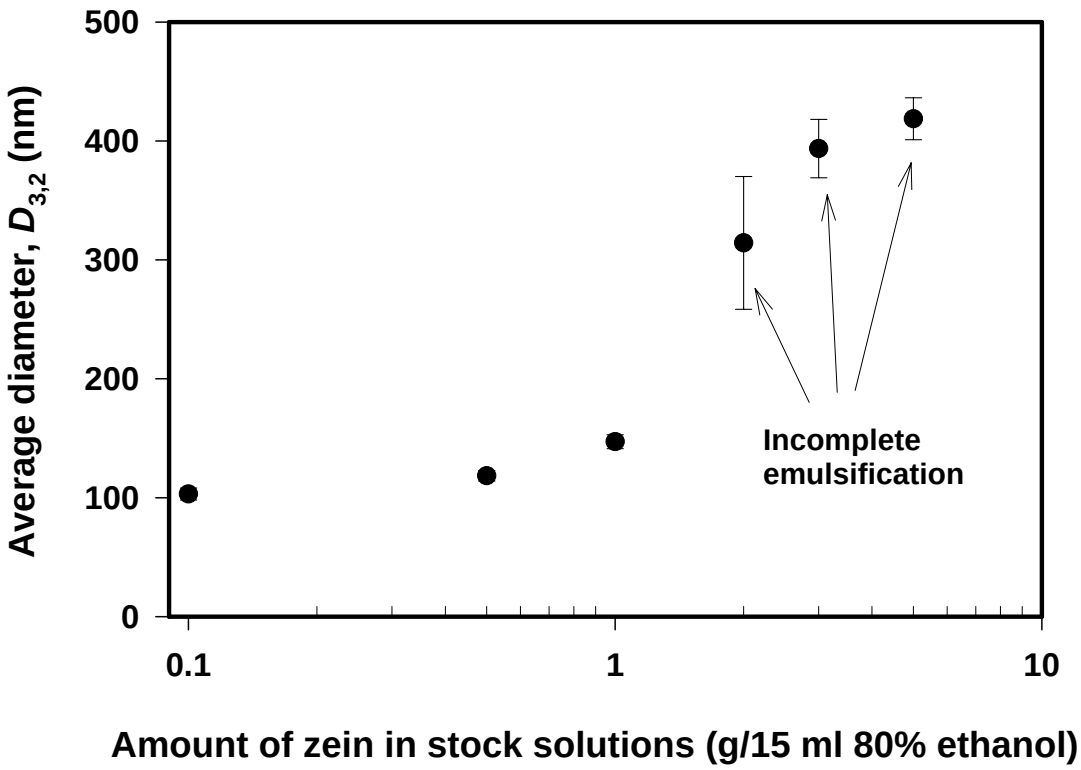

Figure 3. 5. Effect of zein concentration in stock solutions on the sizes of zein nanoparticles, prepared from stock solutions with different amounts of zein dissolved in 15 mL 80% ethanol, blended into 40 mL deionized water at 5,000 rpm.
Results of dynamic light scattering are surface average diameters ($D_{3,2}$) from 10 runs. Error bars are 95% confidence intervals.

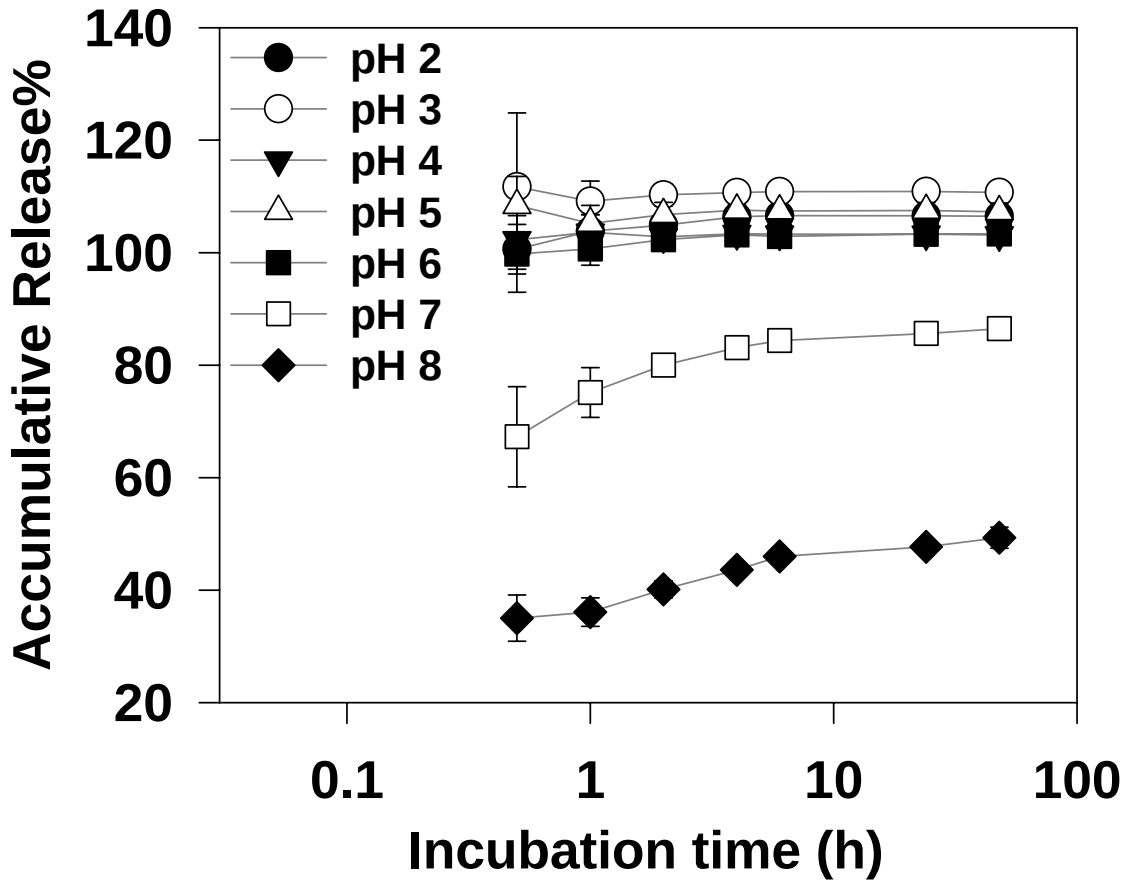

Figure 3. 6. Lysozyme in vitro release kinetics from zein nanocapsules in buffers at pH 2-8 at room temperature.
Capsules were prepared by blending a stock solution (5 g of zein and 0.015 g lysozyme in 30 mL 60% ethanol) into 300 mL deionized water at 10,000 rpm for 2 min. Error bars are 95% confidence intervals.

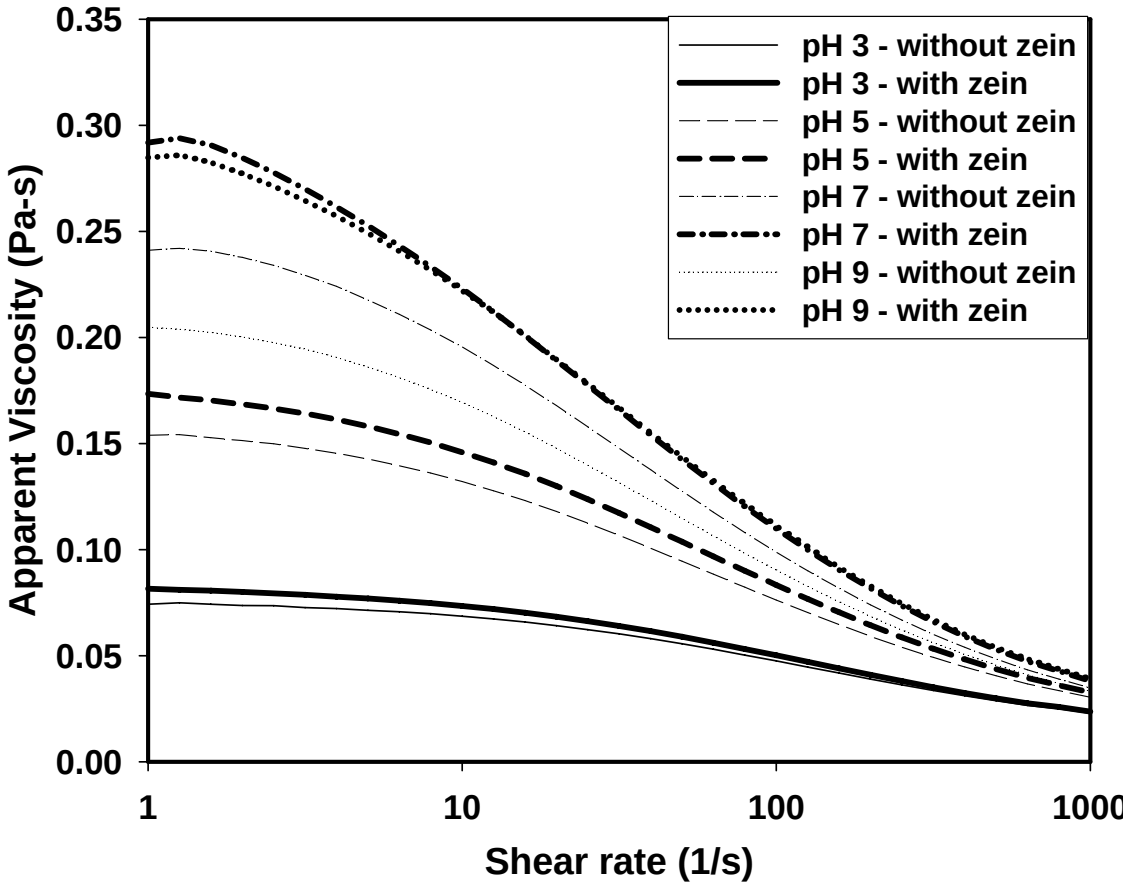

Figure 3. 7. Comparison of viscosities of 0.5% (w/v) CMC solutions at 20°C before and after addition of 1% (w/v) zein nanoparticles, adjusted to pH 3-9.

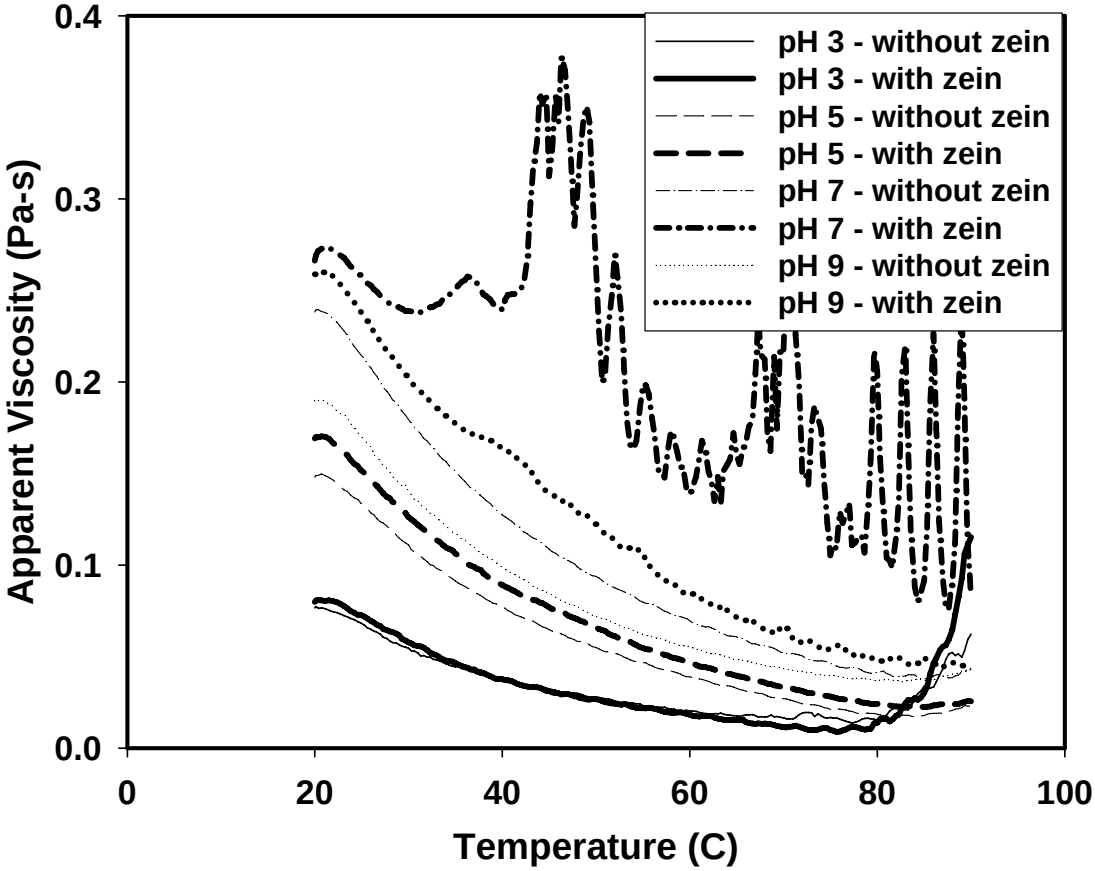

Figure 3. 8. Comparison of viscosities of 0.5% (w/v) CMC solutions before and after addition of 1% (w/v) zein nanoparticles, adjusted to different pH values, during heating from 20 to 90°C at a heating rate of 2 °C/min.

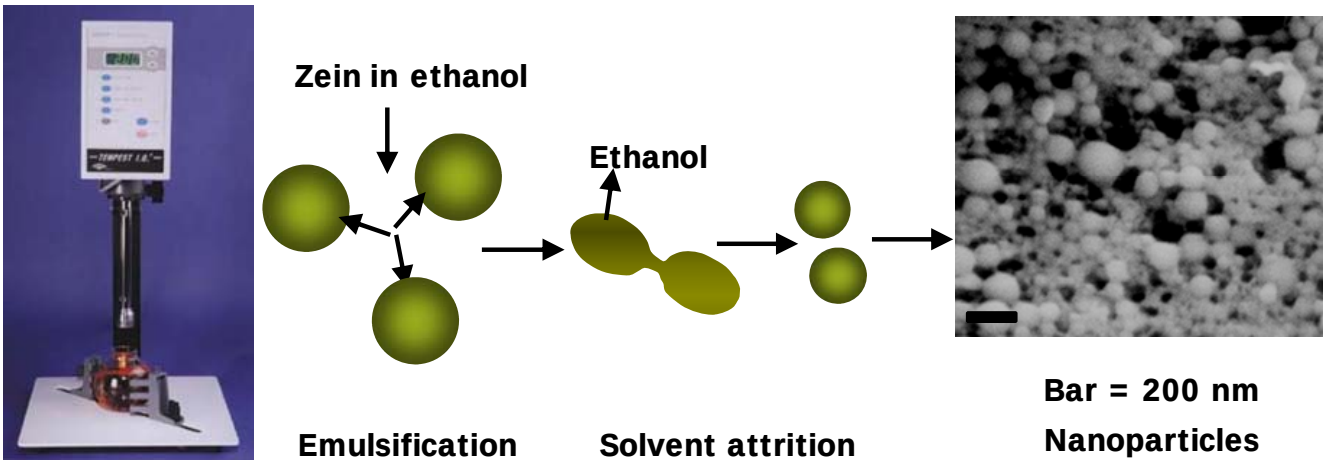

Figure 3. 9. Principle of the solvent-attrition process to synthesize zein nanoparticles. Zein dissolved in an ethanol/water mixture is sheared into smaller droplets (left-most drawing), the inter-diffusion of ethanol and water (solvent attrition, center) reduces the solubility of zein, precipitating into nanoparticles (right).

Chapter 4 . Sustained Release of Lysozyme from Zein Microcapsules Produced by Supercritical Anti-Solvent Technology

4.1. Abstract

Sustained release has been shown to be a possible solution to minimize the binding between antimicrobials and food matrices, thereby enhancing the efficacy of antimicrobials during shelf-lives of foods. Supercritical anti-solvent is a technology that has been researched to produce delivery systems with sustained release of antimicrobials, based on synthetic polymers and non-food grade solvents. However, for food applications, regulations require solvents and ingredients are either generally-recognized-as-safe or approved by FDA. In this work, GRAS corn zein was used as a carrier material and 90% aqueous ethanol was used as a solvent to microencapsulate GRAS hen egg white lysozyme. The microcapsules produced using supercritical anti-solvent showed a continuous matrix with internal voids. The release of lysozyme was observed over 36 days at room temperature, with slower release at a higher pH between pH 2-8. At pH 4, release kinetics was further controlled by addition of sodium chloride. The results were discussed based on the strengths of molecular interactions as affected by pH and ionic strength. Because sustained release is required to inhibit microorganisms over a long period of time to minimize the loss of antimicrobial efficacy, supercritical anti-solvent may be a viable process for microencapsulation. The carrier material, zein, may also be used to manufacture food grade antimicrobial delivery systems using other processes such as spray drying.

4.2. Introduction

Recent developments in drug delivery and nanotechnology revived interests in controlled delivery of bioactive food components, with nutraceuticals and antimicrobials receiving most attention (Desai and Park 2005, Gaysinsky et al. 2005a, b, Setchell et al. 2005, Were et al. 2003, 2004). The motivations for developing techniques to achieve controlled delivery of these components are to improve their availability or activity, either in food matrices or surfaces, or at the absorption sites of the gastrointestinal tract. For antimicrobials, it is essential to ensure their availability during the shelf-life of a food product, which may be achieved via the incorporation of antimicrobials into edible films or coatings or as capsules dispersed in food matrices.

Many antimicrobials are hydrophobic or amphiphilic (Davidson 2001) and they may bind hydrophobic food components such as lipids, hydrophobic proteins, and cell walls, which limits their availability (Chi-Zhang et al. 2004). As a result, traditional and naturally occurring food antimicrobials are used at high concentrations to achieve even moderate reductions in growth rates of pathogenic microorganisms in food products (Davidson 2001). The incorporation of large amounts of antimicrobials may compromise the sensory properties of products, not to mention their high costs (Sofos et al. 1998). Several antimicrobial delivery systems have been studied based on surfactant micelles and liposomes (Gaysinsky et al. 2005a, b, Laridi et al. 2003, Were et al. 2003, 2004), but much work is needed before the application of these delivery systems into foods.

Formulating delivery systems and developing microencapsulation techniques may be the key to solving the above challenges. Recent research in controlled drug delivery has provided many promising techniques that can be similarly used for controlled delivery of food antimicrobials. This provides tremendous opportunities for food science research. On the

negative side, many of these techniques require stringent conditions or have other limitations that make application in the giant food industry problematic.

One promising category of scalable techniques applicable to food systems is the production of micro- and nanoparticles with supercritical carbon dioxide ($scCO_2$) as a solvent or an anti-solvent (non-solvent). Supercritical antisolvent (SAS) is one example that involves continuous spraying of a solution into $scCO_2$. The technique, and its variations, requires polymers dissolved in a solvent or solvent mixture (called co-solvent) miscible with CO_2. With extraction of co-solvent out of the atomized droplets by CO_2, polymers lose solubility and precipitate into micro- and nanoparticles.

The SAS process operates at a mild temperature and readily-achievable pressure as the critical temperature and pressure of CO_2 is 31.1 °C and 7.38 MPa, respectively. The micro- and nanoparticles formed are dry and can be stored and directly added into any type of food products. When bioactive compounds co-precipitate with carrier polymers, these compounds are microencapsulated into the polymer matrix with preserved activities (Elvassore et al. 2001a, Falk and Randolph 1998, Moshashaée et al. 2000, Randolph et al. 1993). Further, because a co-solvent (alcohol or other organic solvent) of a good miscibility with non-polar CO_2 is used, the SAS may be particularly attractive for microencapsulating hydrophobic or amphiphilic antimicrobials that have limited solubility in water.

The potential of applying SAS in food applications has recently been shown for nisin microencapsulated in poly(L-lactide) (PLA) nanoparticles (Salmaso et al. 2004). The hydrophobic nature of L-PLA enabled intermediate hydrophobic interactions with nisin, causing release in a controlled manner over 45 days. The gradually released nisin was effective in inhibiting the growth of microorganism *Lactobacillus delbrueckii* spp. *bulgaricus* for more than

40 days. In comparison, free nisin was only efficacious for 4 days. The sustained release of antimicrobials is particularly attractive for products that have a long shelf-life. However, PLA is not a food ingredient, and the organic solvents used, dichloromethane and dimethylsulfoxide, are of toxicity concerns, although their concentrations in capsules produced from SAS can be reduced to be below standards for pharmaceutical products (Elvassore et al. 2001a, b, Moshashaée et al. 2000, Winters et al. 1997, Winters et al. 1996, Yeo et al. 1994).

The objective of this work was to explore the possibility of using the SAS to manufacture food grade antimicrobial delivery systems. Corn zein (prolamines) was used as a carrier polymer for formation of capsules matrix, and lysozyme was used as a model antimicrobial. The GRAS solvent, 90% aqueous ethanol was used as a co-solvent.

4.3. Materials and Methods

4.3.1. Materials

Purified zein and ethanol (200 proof) was purchased from Acros Organics (Morris Plains, NJ). Hen egg white lysozyme, in lyophilized form, and the microorganism (*Micrococcus lysodiekticus*) used to measure the lysozyme activity were purchased from Sigma-Aldrich Co. (St. Louis, MO). Chemicals were used directly without purification.

4.3.2. Apparatus and Particle Production Protocol

The SAS-50 supercritical particle design system (Thar Technologies, Pittsburgh, PA) is schematically presented in Figure 4.1. Strictly, the system should be called an “aerosol-solvent extraction system,” based on the nomenclature used in supercritical fluids technologies (Jung and Perrut 2001). The description of the apparatus and particle production procedures was detailed previously (Zhong et al. 2008). Briefly, the system has a polymer feed stream and a CO_2 stream

connected to a high pressure vessel. Within the pressure vessel, a sample collection basket with a 5 μm frit at the bottom is placed. During an operation, the pressure cell was filled with CO_2 and equilibrated to and maintained at a set temperature (40 °C) and pressure (10 MPa). The zein solution (5% w/v in 90% aqueous ethanol, with 0.1% lysozyme) was injected via the polymer feed stream by a high pressure pump through a silica nozzle (inner diameter of 100 μm) into the pressure cell at 1 ml/min. The total solution volume injected was 15 mL. The CO_2 stream had a continuous flow of CO_2 (grade CD-50S, 99.9% pure, Airgas, Inc., Chicago, IL) at 50 g/min during equilibrium and spraying. After spraying, CO_2 continued to flow through the pressure cell for 30 min to dry the particles and extract residual solvent in the particles. The pressure cell was then gradually depressurized to the atmospheric pressure, followed by opening the pressure cell to harvest particles.

4.3.3. Measurement of Encapsulation Efficiency

To estimate the microencapsulation efficiency, 9 mg of zein capsules was dissolved in 5 mL 80% ethanol where zein and lysozyme were completely solubilized. The solution was then diluted to an appropriate ratio during the enzymatic assay. The encapsulation efficiency (η) was calculated based by:

$$\eta\,(\%) = \frac{\text{Lysyzome units per mg of capsule} \times \text{Total mass of capsules}}{\text{Total lysozyme units injected}} \times 100\% \qquad (1)$$

4.3.4. Evaluation of *in vitro* Release Kinetics

To characterize release kinetics, 27 mg capsules were dispersed in a microcentrifuge tube filled with 1.5 mL of a 66 mM potassium phosphate buffer (adjusted to a pH between 2 and 8 with 1 N HCl or 1 N KOH). The microcentrifuge tubes were continuously agitated on an end-to-end shaker (Lab Industries Inc., Berkeley, CA) at room temperature. At a predetermined time

interval, the dispersion was centrifuged at 5,000 × *g* for 5 min (MiniSpin Personal, Eppendorf, Westbury, NY), and 1 mL of supernatant was sampled for assays. The remaining dispersion was supplemented with 1 mL of the fresh corresponding buffer and particles were re-dispersed for continued tests. The total amount of lysozyme in unit mass of powders was measured by dissolving 27 mg of powders in 15 mL 80% ethanol that dissolved both zein and lysozyme completely. The samples were tested up to a release time of 36 days.

Calculation of accumulative lysozyme release was as follows:

Let the lysozyme volumetric concentration (U/ml) at the first sampling time, t_1 to be a_1, the accumulative release at time t_1 is:

$$R_{t_1}(\%) = \frac{1.5\,a_1}{U_o} \times 100\% \tag{2}$$

where U_o is the total lysozyme units included in the dispersion before release tests, and the prefix 1.5 before a_1 is the total volume of dispersion.

After removing 1 mL of supernatant, there are 0.5 a_1 units of lysozyme due to the release after time t_1, which carries to the second sampling at time t_2. Similarly, if the volumetric concentration of supernatant from the second sampling is a_2 (U/ml), the total lysozyme released up to this point is a_1 + 1.5 a_2, which gives an accumulative release at time t_2:

$$R_{t_2}(\%) = \frac{a_1 + 1.5\,a_2}{U_o} \times 100\% \tag{3}$$

Therefore, at the i^{th} sampling, the accumulative release is:

$$R_{t_i}(\%) = \frac{\sum_{n=1}^{i-1} a_n + 1.5\,a_i}{U_o} \times 100\% \tag{3}$$

4.3.5. Measurement of Lysozyme Activity

The activity of lysozyme was measured according to the protocol provided by Sigma for hen egg white lysozyme (product number L-6876). The test microorganism was *Micrococcus lysodiekticus*, and measurements were based on the reduction rate of absorbance at 450 nm due to the lysis of cells by lysozyme at pH 6.24 and 25 °C. The lysis kinetics was measured using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA).

4.3.6. Scanning Electron Microscopy (SEM)

The SEM tests were performed with a LEO 1525 SEM microscope (LEO Electron Microscopy, Oberkochen, Germany). Besides surface morphology of microcapsules, the inner structures were observed after fracturing capsules by a razor blade, following a literature method (Lee and Rosenberg 2000). The specimen was sputter-coated with a ~5 nm-thick gold layer.

4.4. Results and discussion

4.4.1. Particle Structures

SEM images of microcapsules are shown in Figure 4.2. Capsules are heterogeneous in size, ranging from as small as submicrometers to as big as ~50 micrometers in diameter (Figure 4.2A). Big particles are red-blood-cell shaped but have smooth surfaces. The internal structure showed a continuous network with many heterogeneously-sized voids (Figure 4.2B), but the outer particle surface is generally continuous and compact.

The non-uniform size and irregular shape are similar to the observations from our previous study based on zein alone (Zhong et al. 2008). This may have been caused by the co-solvent used. Because water is a very polar solvent that has low miscibility with CO_2, the

evaporation of the co-solvent (90% ethanol) may be relatively slow, resulting in size variation and shape irregularity. When 95-100% aqueous methanol was used as co-solvents in our previous report (Zhong et al. 2008), all particles were observed to be spherical and much more uniform. Decreased average diameters (500 - 80 nm) and narrower distributions were observed when the zein solution had a higher methanol content and/or a lower zein concentration. Lysozyme is insoluble in 95-100% methanol; production of zein nanocapsules with encapsulated lysozyme may not be feasible. However, these high methanol concentrations are capable of extracting another antimicrobial, nisin, from a 2.5% commercial nisin preparation (Taylor et al. 2007). Encapsulation of nisin in zein nanocapsules is currently being investigated in our group.

4.4.2. Encapsulation Efficiency and Release Kinetics

Approximately 0.5 g of powders was collected, which accounted for 65% of the non-solvent mass injected. One mg of powder contained 1063 U lysozyme, which gave an encapsulation efficiency of:

$$\eta\,(\%) = \frac{1063\ \text{U/mg} \times 500\ \text{mg}}{76133\ \text{U/mg} \times 15\ \text{mg}} \times 100\% = 46.5\% \qquad (2)$$

where 76133 U/mg in the denominator is the activity of pure lysozyme, 15 mg is the amount of lysozyme sprayed.

The low encapsulation efficiency may arise from two factors. One is the difficulty to collect all particles (some stick to the wall of sample basket and frit), which will give a significant error for such a small amount of material processed. The other factor may be the limitation of the frit used. The frit has a nominal opening of 5 μm, but the SEM image shows many particles smaller than 5 μm (Figure 4.2A). A finer frit may be used in the future to improve the yield.

In vitro release kinetics in phosphate buffers at pH 2-8 are presented in Figure 4.3. Lysozyme showed sustained release at all pH conditions; slower release was observed at higher pH. For pH 2-5, it took a longer time to reach 100% release at a higher pH. At pH 6-8, continued release was still detected after incubation for 36 days (864 h). A monotonic decrease of nisin release kinetics at a higher pH was also observed for nisin encapsulated in PLA nanoparticles, which was caused by stronger hydrophobic interactions between nisin and PLA at a higher pH (Salmaso et al. 2004). In our case, hydrophobic interactions may have also contributed to slower release at higher pH conditions because zein is water-insoluble (hydrophobic) and a higher pH closer to the isoelectric point of lysozyme, 10.5-11.0 (Cunningham et al. 1991), increases the hydrophobicity of lysozyme (a protein). In addition, because zein is also a protein and has an isoelectric point of 6.8 (Cabra et al. 2005), zein is positively charged at pH 2-6 and negatively charged at pH 7 and 8. Therefore, electrostatic interactions are repulsive between zein and lysozyme at pH 2-6. However, the release profiles do not show a quick equilibrium of lysozyme release at pH 2-6 (Figure 4.3), indicating the significance of attractive forces, most likely hydrophobic interactions. At pH 7 and 8, electrostatic interactions become attractive, which, together with stronger hydrophobic interactions, resulted in slower lysozyme release than those at lower pH conditions.

The effect of ionic strength on lysozyme was studied for buffers at pH 4, after adding 0.1, 0.5 and 1.0 M NaCl (Figure 4.4). Without NaCl, the accumulative release reached 100% in approximately 192 h (8 days). Addition of 0.1 M NaCl did not change too much release characteristics. When the salt concentration was increased to 0.5 M NaCl, continued release of lysozyme was measured even after 48 days (1152 hours). Even slower release was observed when the NaCl concentration was increased to 1.0 M.

Increasing ionic strengths in buffers has two effects on molecular interactions between lysozyme and zein, both are proteins and positively charged at pH 4. An increase in ionic strength suppresses the Debye length and thus the effective distance of electrostatic repulsion (Israelachvili 1992). On the other hand, a higher ionic strength increases hydrophobicity of lysozyme. Combination of weaker electrostatic repulsion and stronger hydrophobic attraction contributed to slower release kinetics at higher ionic strengths in Figure 4.4.

4.5. Conclusions

Microcapsules of zein with encapsulated lysozyme were produced using SAS. The microcapsules had a large variation in particle sizes based on SEM, possibly due to the co-solvent (90% ethanol) used. Although the capsule internal structure was porous, the surface of capsules was smooth. The encapsulated lysozyme gradually released from microcapsules when suspended in aqueous buffers. A faster and more complete release was observed at a lower pH between 2 and 8. Addition of salt into buffers at pH 4 further slowed release kinetics. The correlation between molecular interactions and release profiles revealed that interactions were more attractive at a higher pH or at a higher ionic strength at the same pH. This work demonstrated the feasibility of using zein as a carrier material to develop food grade delivery systems of antimicrobials such as lysozyme, using SAS or other processes.

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Appendix

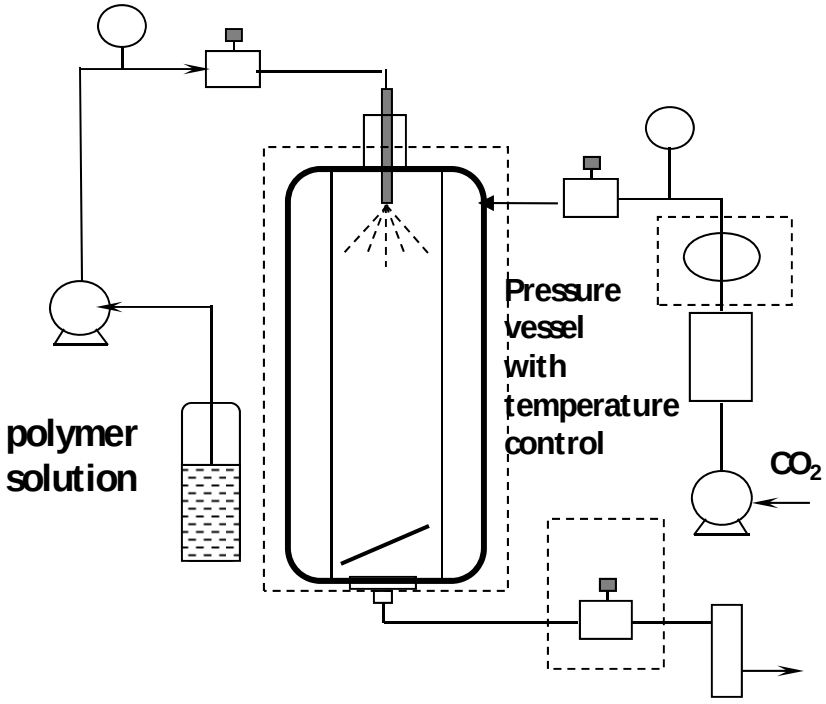

Figure 4. 1. Schematic of the supercritical anti-solvent system (aerosol-solvent extraction system).

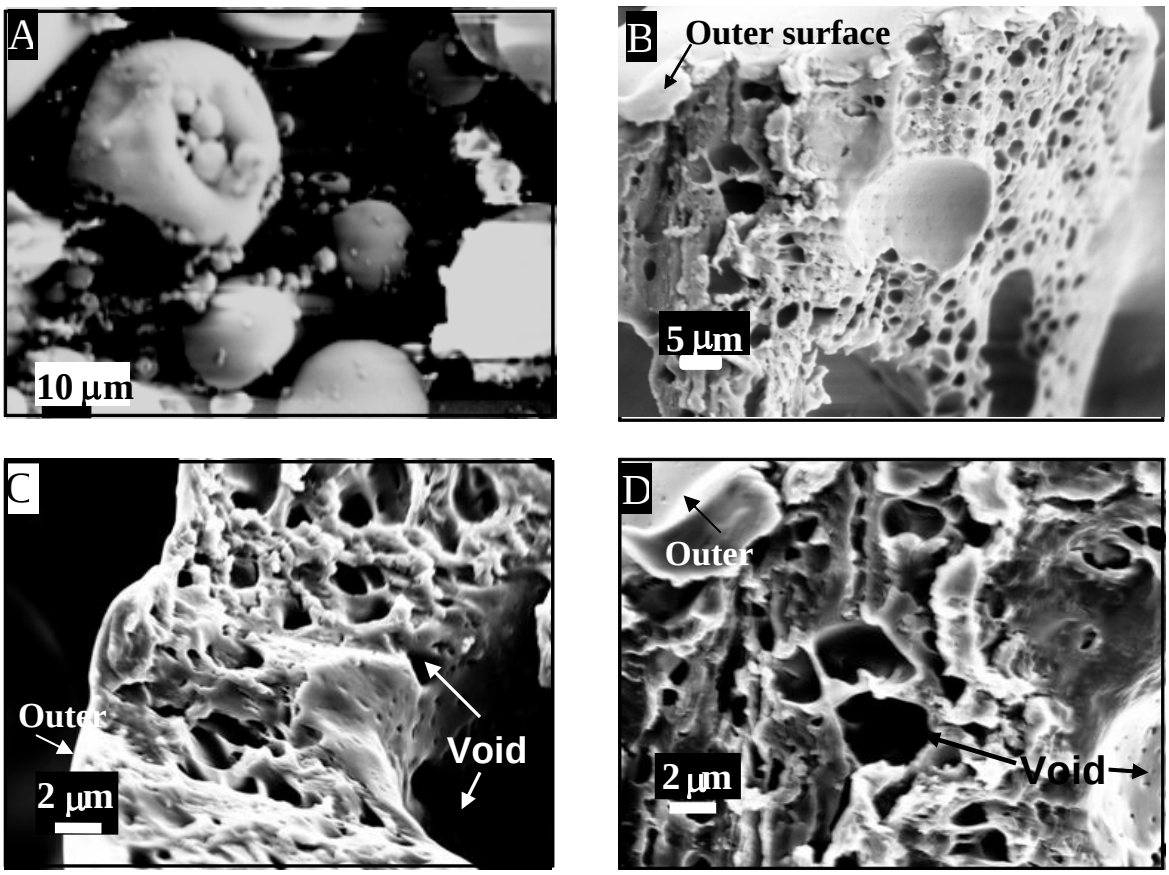

Figure 4. 2. SEM images of representative lysozyme-loaded zein microcapsules: surface morphology (A) and internal structure (B, C, D).

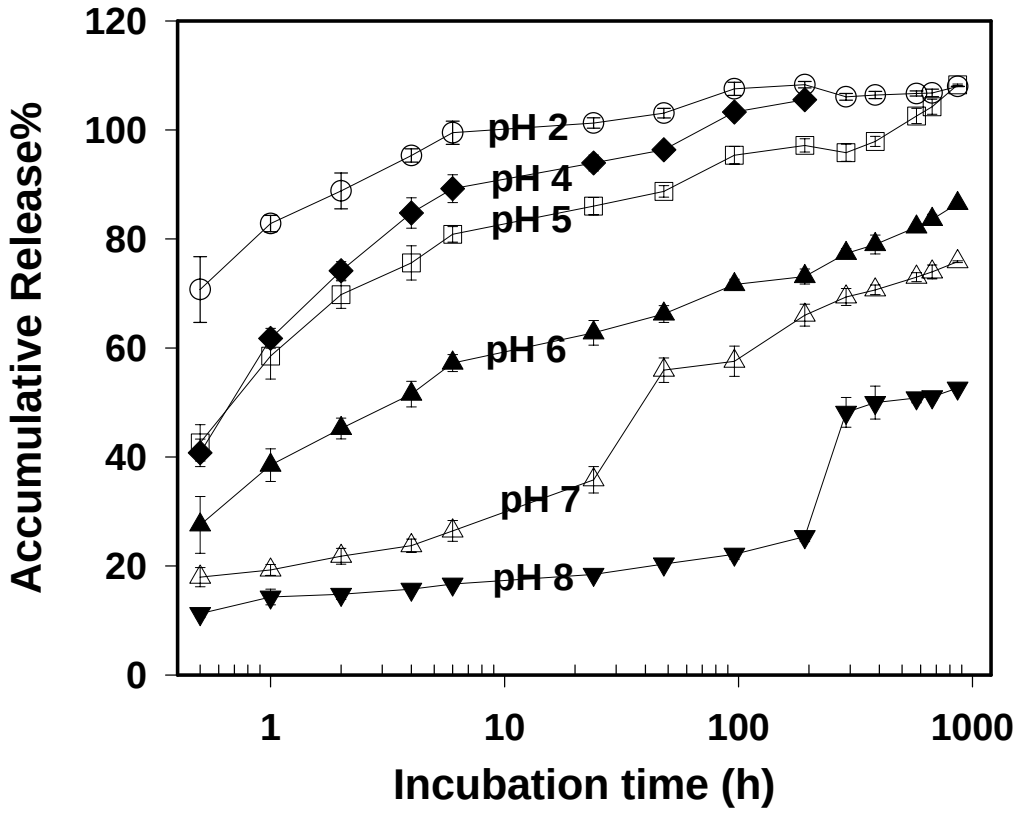

Figure 4. 3. *In vitro* release kinetics of lysozyme from zein microcapsules suspended at room temperature in 66 mM potassium phosphate buffers at pH 2-8. Error bars are 95% confidence intervals.

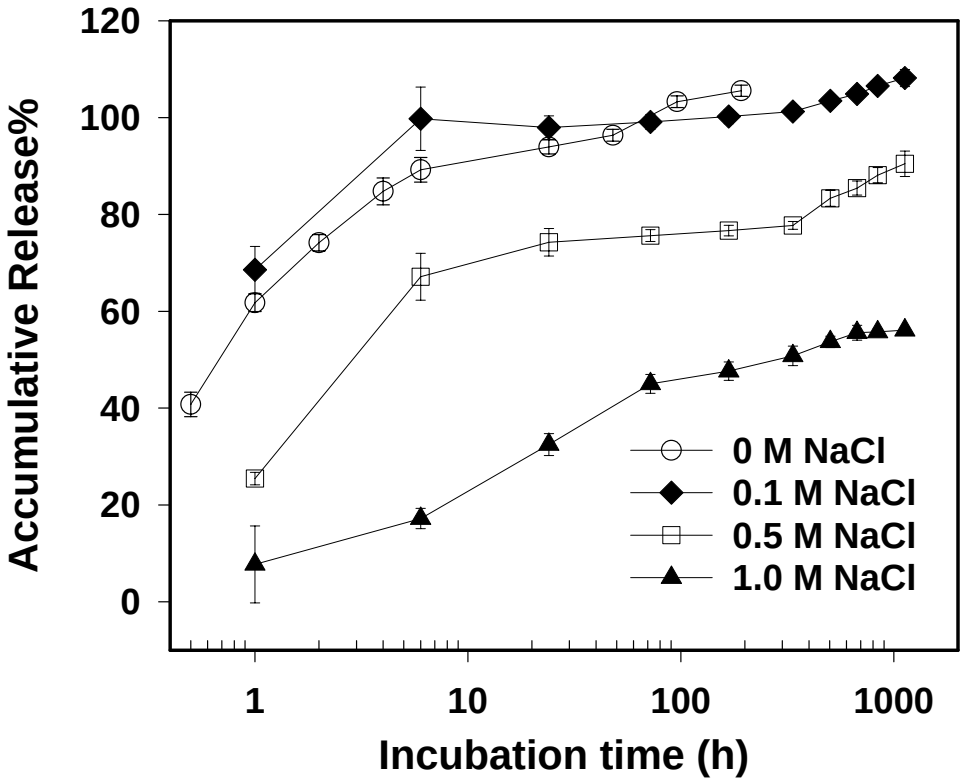

Figure 4. 4. *In vitro* release kinetics of lysozyme from zein microcapsules suspended at room temperature in 66 mM potassium phosphate buffers at pH 4 with different concentrations of NaCl. Error bars are 95% confidence intervals.

Chapter 5 . Production of Corn Zein Microparticles with Loaded Lysozyme Directly Extracted from Hen Egg White Using Spray Drying. Part I. Extraction Studies

5.1. Abstract

In this paper series, our objective was to study the feasibility of achieving sustained release of lysozyme by encapsulation in corn zein microparticles using spray drying. To reduce the material cost, this part of work focused on partially purifying lysozyme from fresh hen egg white (HEW) by extraction with aqueous alcohol, which was then dissolved with corn zein and spray-dried directly in the second part of our paper series. Specifically, the HEW was mixed with 30-90% v/v ethanol and adjusted to pH 3.0-9.24 and then extracted up to 24 h. Following centrifugation of slurries, the purification performance was evaluated based on the detectable amount of lysozyme units, total protein content and specific activity (units of lysozyme per unit mass of proteins) in the supernatant. Kinetics was concluded to be an insignificant parameter, although a slight increase in lysozyme activity was observed up to 24 h. Conversely, the pH value and aqueous ethanol concentration were critical for extraction. The extraction was inefficient at pH above 5.0. An ethanol concentration between 30-50% was effective for extraction as indicated by the number of lysozyme units in the supernatant, while poorer yields were achieved at higher than 60% ethanol. Among the purification parameters studied, extraction with 50% ethanol at pH 3.5 enabled a good extraction and relatively high specific activity. Further, lysozyme almost completely precipitated from the supernatant prepared with 50% ethanol (at pH 3.5) after the ethanol concentration was increased to 90%, but dilution of the precipitates using deionized water back to 50% ethanol enabled a complete recovery of the precipitated lysozyme. Findings from this part of the work may lead to low-cost encapsulation technologies using partially-purified lysozyme, exemplified by spray drying in the second part of our paper series.

5.2. Introduction

Development of delivery systems for drugs and food antimicrobials has been active in recent years due to the benefits such as sustained release of drugs or antimicrobials, which may improve the bioactivity or bioavailability, reduce the interaction with other components in the microenvironment, and thus prolong the efficacy or shelf life (Al-Nabulsi and Holley 2007, Bezemer et al. 2000, Del Nobile et al. 2008, Mecitoglu et al. 2006). Lysozyme is one of the extensively used model antimicrobials due to its general stability under a variety of conditions and a broad spectrum of antimicrobial activities against bacteria such as *Bacillus stearothermophilus*, *Micrococcus spp.*, *Clostridium tyrobutyricum*, and *Listeria monocytogenes* and fungi (Johnson and Larson 2004).

Lysozyme is a single polypeptide chain of 129 amino acids cross-linked by four disulfide bridges, with a molecular weight of ~14.4 kDa and isoelectric point of ~10.5-11.0 (Johnson and Larson 2004). The antibacterial property of lysozyme originates from the ability of the polypeptide to cleave the β-1, 4-glycosidic bonds between the C-1 of N-acetylmuramic acid and the C-4 on N-acetyl-glucosamine of bacterial peptidoglycan in the cell membrane (Johnson and Larson, 2004). Among several types of naturally-occurring lysozyme (type G, C, etc.), only the type C enzyme from hen egg white (HEW) is currently used in food preservation (Johnson and Larson 2004) due to the relative ease of purification, low toxicity, low effective usage levels and low interference on sensory qualities of foods.

Different procedures or methods have been developed to isolate or extract lysozyme from HEW, including ion exchange chromatography (Banka et al. 1993 and Jiang et al. 2001), gel-filtration chromatography (Islam et al. 2006), dye-binding chromatography (Tejeda-Mansir et al. 2003), membrane separation (Chiu et al. 2007), ultrafiltration (Lee et al. 2003), reverse micelles

(Noh and Imm 2005), magnetic cation exchange (Safarik et al. 2007), and ethanol precipitation (Mecitoglu et al. 2006, Gemili et al. 2007). Among these techniques, partial purification by the ethanol precipitation method has the advantages of low-cost, easy availability and convenience for processing. Partially purified HEW lysozyme, compared to the more expensive purified, lyophilized product, can be used to reduce the materials cost for manufacturing delivery systems of lysozyme.

The overall goal of this work was to investigate the feasibility of manufacturing corn zein-based antimicrobial delivery system of lysozyme using spray drying. Corn zein, prolamines, is a group of alcohol-soluble storage proteins existing mostly in the endosperm fraction of corn kernels (Shukla and Cheryan 2001). To prepare solutions used for spray drying, both zein and lysozyme need to be dissolved in ethanol/water mixtures. Thus, the ethanol precipitation method offers feasibility to partially purify lysozyme from HEW for subsequent steps of dissolving the carrier biopolymer, corn zein, in aqueous ethanol extracts and spray drying.

The ethanol precipitation method was used to partially purify lysozyme from HEW in a few studies for preparation of antimicrobial-loaded films (Mecitoglu et al. 2006, Jiang et al. 2001). In these studies, HEW proteins were precipitated at pH 4, further facilitated by 30% ethanol that acted as a non-solvent for some HEW proteins. However, 30% ethanol is not a solvent for zein. This paper was thus focused on extraction of lysozyme from HEW at various conditions, i.e., kinetics, pH, and ethanol concentrations to optimize lysozyme extraction and removal of HEW proteins. The information from this part of work was used to prepare samples by directly dissolving zein in HEW extracts for spray drying, discussed in the second part of this paper series.

5.3. Materials and Methods

5.3.1. Materials

Fresh hen eggs were obtained from a grocery store. Ethanol (200 proof) was the product from Acros Organics (Morris Plains, NJ). Purified lyophilized HEW lysozyme (catalog number L6876) and *Micrococcus lysodeikticus*, the indicator microorganism for measuring lysozyme activities, were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin and Coomassie® Plus Reagent (product 23236) were purchased from Pierce Biotechnology (Rockford, IL). Polyacrylamide gels, 15% Tris-HCl Ready Gel® Precast gels, were ordered from Bio-Rad Laboratories (Hercules, CA). Salts, base, acids and other chemicals were from Fisher Scientific (Pittsburgh, PA).

5.3.2. Extraction Protocol

The extraction protocol was a slight modification from a literature method (Mecitoglu et al. 2006, Jiang et al. 2001). Egg white was carefully separated from hen eggs and mixed with a 0.05 M NaCl solution at a volume ratio of 1:2 to three-fold dilution. The pH of suspension was adjusted from 9.24 initially to 7.0, 6.0, 5.0, 4.5, 4.0, 3.5 and 3.0 with 1 M acetic acid. An appropriate amount of ethanol was added to obtain a final ethanol concentration between 30% and 90% (v/v). While being continuously agitated, 1 ml of the suspension was sampled at a predetermined time point (i.e., shortly after adding ethanol and after mixing for 1, 2, 3, 4, 5, 6, or 24 h) and centrifuged at 14,500 × *g* for 5 min (model MiniSpin Personal, Eppendorf, Westbury, NY). The supernatant was transferred for further analyses. The experimental design to study extraction kinetics and pH used the completely randomized design (CRD), with one replicate of the full factorial of 8 time points by 5 pH values at ethanol concentration of 30%, 40% and 50%.

5.3.3. Determination of Lysozyme Activity

Lysozyme activities were determined according to a method of Sigma-Aldrich (St. Louis, MO) for product HEW lysozyme (catalog number L6876), with slight modification in the measurement period and sample and reagent volumes. *Micrococcus lysodeikticus* was used as the test microorganism, suspended in a 66 mM potassium phosphate buffer at pH 6.24. The suspension after addition of samples was monitored the reduction in absorbance at 450 nm for 3 min by using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA) with a thermal jacket set at 25 °C. One unit of lysozyme is defined as the reduction of absorbance of 0.001 per min at the above test conditions. Each sample was tested in triplicate.

5.3.4. Determination of Total Protein Content

The total protein content of samples was determined by the Bradford method with a Coomassie® Plus Protein Assay kit (product 23236, Pierce), and bovine serum albumin was used as a reference. The absorbance was measured at 595 nm, and triplicate tests were performed for each sample.

5.3.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Hen egg white proteins in extracts were separated according to their molecular weights at denatured conditions on a 15% Tris-HCl gel (Ready Gel® Precast Gel from Bio-Rad). Electrophoresis was performed with a Protean® II xi 2-D Cell (Bio-Rad) at a constant voltage of 200 V until the protein marker standards reached the gel bottom. The staining and destaining procedures followed the instruction manual of the Trish-HCl gel. The steps included staining in a mixture of methanol, acetic acid and Coomassie® Blue, destaining in a mixture of methanol and

acetic acid, and rinsing with distilled water until satisfactory visibility of protein bands. The destained gels were dried and photographed.

5.3.6. Data Analyses

Data analyses were carried out by using SAS software (v. 9.1, SAS Institute, Cary, NC). Significant differences were analyzed with a least-significant-difference ($P < 0.05$) mean separation method (LSD). Response surface regression was used to analyze the importance of independent variables (i.e., extraction time, pH, or ethanol concentration) in explaining dependent variables (the extracted lysozyme units, total protein content, etc.) and to predict the optimum extraction conditions.

5.4. Results and Discussion

5.4.1. Extraction Kinetics

Extract kinetics as affected by pH (3.5 - 9.24) and ethanol concentration (30-50%, v/v) are presented in Figure 5.1. During extraction up to 24 h, there were slight fluctuations in detected lysozyme activities in different extraction time points for each pH and each ethanol concentration; however, no obvious conclusion could be made regarding the effect of extraction time.

To statistically understand the effect of kinetics on lysozyme extraction, a response surface analysis was performed using SAS (Figure 5.2). A slight increase in lysozyme extraction up to 24 h was predicted based on the actual experimental data (Figure 5.2A). However, the response surface regression analysis result (Figure 5.2B) showed the insignificance of extraction time ($P = 0.6186$).

Nevertheless, an extraction time of 6 h was sufficient to extract most lysozyme at all studied pH and ethanol conditions, and was chosen to screen extraction conditions in the next step. The selection was similar to the recommendation of Gemili et al. (2007): 4-6 h for 30% ethanol and 6-8 h for 40% ethanol.

5.4.2. Extraction pH

At the three studied ethanol concentrations, i.e., 30%, 40% and 50%, little lysozyme activity was detected in extracts at pH 6.0 and above (Figure 5.1A). The inefficient extraction at pH 6 or higher may have been caused by the co-precipitation of lysozyme with HEW proteins, due to electrostatic interactions. Ovalbumins, the most abundant protein in HEW, have an isoelectric point (pI) of 4.7 (Moritz and Simposon 2005); lysozyme has a pI of ~10.5-11.0 (Johnson and Larson 2004). Therefore, ovalbumins are positively charge below pH 4.7 and negatively charge above pH 4.7, while lysozyme is always positively charged at pH 3.5-9.24. The electrostatic attraction between lysozyme and ovalbumins may have caused the co-precipitation of lysozyme with ovalbumins: a reduced efficiency at pH 5 (ovalbumins are weakly negatively-charged) and inefficiency at pH 6 and above (when ovalbumins become more extensively charged). In addition, some precipitated HEW proteins are hydrophobic and their attraction with lysozyme may also be strengthened when the pH is increased to closer to the pI of lysozyme.

The statistical analysis (Figure 5.2B) showed that pH is a significant independent variable for lysozyme extraction with both significant linear and quadratic effects of pH ($P < 0.0001$). Additionally, the response surface regression predicted higher yields at lower extraction pH conditions (Figure 5.2C). Also, the analysis showed that ethanol concentration (30-50%) and the interaction between pH and ethanol concentration were significant ($P < 0.0001$). However, the

effect of ethanol concentrations in Figure 5.1 was not compared because different batches of HEW were used for each ethanol concentration. We addressed this issue by reinvestigating effects of ethanol concentration and the interaction between ethanol concentration and pH using HEW from a same batch of HEW at a fixed extraction time of 6 h.

5.4.3. Ethanol Concentration

The same batch of HEW was used to investigate the effects of ethanol concentration and interaction between pH and ethanol concentration. Further, ethanol concentrations greater than 60% (v/v) were studied for the possibility of using the centrifuged supernatant to directly dissolve zein for spray drying. A pH value higher than 5.0 was not studied because of the poor extraction (Figure 5.1). The extraction variables were compared using three parameters: number of lysozyme units (indicative of lysome molecules that are active), total protein concentration in extracts (relative indication of amount of impurities), and specific activity (units of lysozyme per unit mass of protein, indication of purity).

Lysozyme activity in extracts

The amount of lysozyme extracted, compared after conversion to lysozyme units per mL HEW, showed that an ethanol concentration between 30-50% was generally effective for extraction (Figure 5.3A). The highest lysozyme activity was detected in extracts prepared at pH 3.5 and 4.0 with 50% ethanol. When the pH was decreased to 3, the extraction became less efficient, especially for the 50% ethanol treatment. The SDS-PAGE experiment showed a band corresponding to lysozyme extracted at pH 3 and 50% ethanol (Figure 5.5A), indicating the presence of lysozyme in the supernatant; the much lower activity tested may have been caused by the denaturation of lysozyme.

When the ethanol concentration was increased to 60 and 70%, lysozyme activities in extracts dramatically decreased at all studied pH values (i.e., pH 3-4.5). When the ethanol concentration was increased to 90%, lysozyme activity in the centrifuged extracts fell below the detection limit of the assay method.

Total protein concentration in extracts

The total protein concentration in centrifuged HEW extracts decreased with an increase in ethanol concentration (Figure 5.3B), while the effect of extraction pH was not significant. SDS-PAGE showed similar band patterns and intensities for 30-50% ethanol treatments (Figure 5.5B, lanes 2-4). The band patterns were similar but the intensity was decreased when the ethanol concentration was increased to 60% (Figure 5.5B, lane 5). For the 70% ethanol treatment, there was only a visible band corresponding to lysozme (Figure 5.5B, lane 6). However, it should be noted that protein concentrations are expected to be lower by simple dilution with a larger amount of ethanol. We further examined the effect of ethanol concentration on precipitating HEW proteins by converting the results to total proteins extracted from each mL HEW (Figure 5.4). The data indicate that more HEW proteins precipitated at a higher ethanol concentration.

Specific activity of extracts

At pH 3.0, the effect of ethanol concentration on specific activity (Figure 5.3C) was similar to that on the extracted lysozyme activity (Figure 5.3A): a higher specific activity at a lower ethanol%. Similar changing trends of lysozyme activity and specific activity were observed at pH 3.5 and 4.0. The specific activity of extracts at pH 4.5 was unchanged when ethanol concentration was increased from 30% up to 60% (Figure 5.3C), different from a monotonic decrease in the extracted lysozyme activity (Figure 5.3A). This indicates that, at pH 4.5, simultaneous precipitation of lysozyme (numerator in the definition of specific activity) and

HEW proteins (denominator in the definition) occurred proportionally at 30-60% ethanol. Comparing all the studied conditions, the highest specific activity (best purity) and most lysozyme activity (best yield) was observed at pH 3.5 with 50% ethanol and was concluded as the recommended conditions for future work.

5.4.4. SDS-PAGE

SDS-PAGE was performed to study the effects of pH and ethanol concentration on protein types in extracts, based on the molecular weight. HEW has 5 major proteins - ovalbumins (64%, MW 45 kDa), conalbumin (12%, MW 76 kDa), lysozyme (3.5%, MW 14.4 kDa), ovomucin (11%) and avidin (0.05%, MW 68.3 kDa) (Li-Chan et al. 1995) . Crude ovomucin has two subunits, α- and β-. The α-ovomucin has two types: α1- and α2- with a molecular weight of 150 kDa and 220 kDa, respectively, based on SDS-PAGE; β-ovomucin has a molecular weight of 400-720 kDa (Hiidenhovi, 2007).

Our SDS-PAGE result showed three protein bands (Figure 5.5), similar to the results of Raikos et al. (2005) who considered these proteins to be conalbumin (76 kDa), ovalbumin (45 kDa), and lysozyme (14.4 kDa). The largest band on the polyacrylamide gels may be ovalbumin after ethanol precipitation, because it is most abundant among the 5 major HEW proteins and has similar molecular weight ~40-50 kDa. However, the exact identification of these bands needs additional techniques such as Western blotting, 2-D gel electrophoresis and MALDI-TOF mass spectrometry (Raikos et al., 2005) and is beyond the scope of this work. Nevertheless, SDS-PAGE helped the understanding of extraction conditions on lysozyme extraction, as discussed in the above relevant sections.

5.4.5. Effect of Ethanol Concentration on the Measured Lysozyme Activity

Mecitoglu et al. (2006) discussed the possibility of activation of lysozyme by ethanol. We addressed this concern by using purified lyophilized HEW lysozyme, dissolved in 0-90% ethanol. The measured specific activities are listed in Table 5.1. The highest lysozyme activity was detected for the 60% ethanol treatment, followed by the 70% ethanol treatment, while there was no statistical difference for other ethanol concentrations (0, 30, 40, 50 and 90%). The exact mechanism of enhanced lysozyme activity in 60% ethanol is beyond the scope of this work. Nevertheless, the effect of ethanol on lysozyme extraction should not affect the conclusions above, because 60% ethanol was observed to be less efficient than other lower ethanol concentrations (Figure 5.3A).

5.4.6. Precipitation of Lysozyme at High Ethanol Concentrations

As shown in Figure 5.3A, the efficiency of lysozyme extraction decreased dramatically when the ethanol concentration was increased to 60 and 70%, and no activity was detectable in extracts when ethanol concentration was increased to 90%. 60-90% ethanol is of interest to our work because zein is soluble in this ethanol concentration range. Because little or no lysozyme activity was detected in the supernatant after centrifugation of extracts prepared with 60-90% ethanol, the direct use of supernatant for microencapsulation is inappropriate. Instead, the slurry with precipitated lysozyme and HEW proteins should be used. This should not affect our eventual goal of microencapsulation, if we can remove substantial amount of HEW proteins, because HEW proteins are GRAS ingredients. The question however remains regarding whether or not the precipitated lysoyzme maintains activity or can be recovered.

To test the lysozyme activity affected by extraction with high ethanol concentrations and to find suitable conditions for later spray drying studies, an extract was prepared by extraction

for 6 h at 50% ethanol and pH 3.5 to remove a portion of HEW proteins. The supernatant after centrifugation (Treatment T0) was transferred and added with ethanol to 90%. The slurry was then centrifuged and a portion of supernatant (Treatment T1) was transferred for measurement of lysozyme activity. The remainder suspension was readjusted to 50% ethanol using deionized water (Treatment T2); the precipitates were completely dissolved after adjusting back to 50% ethanol. A control was prepared using purified lysozyme, processed identically to the HEW extract.

The results, normalized by the lysozyme activity of Treatment T0, are plotted in Figure 5.6. The purified lysozyme did not show big variations after decreasing or increasing ethanol content, consistent with the results in Table 5.1. For the HEW extract, lysozyme activity in the supernatant after adjusting to 90% ethanol (Treatment T1) was below the detection limit of the assay method. However, after adjusting ethanol concentration back to 50% ethanol (Treatment T2), an activity 229% that of the Treatment T0 was measured. Because ethanol content had no effect on lysozyme activity, based on purified, lyophilized lysozyme, the dilution process may have released more lysozyme from the precipitates (pellets corresponding to the Treatment T1). Nevertheless, Figure 5.6 indicates the maintained lysozyme activity after increasing the ethanol concentration in the HEW extract.

The findings suggest that 50% ethanol and pH 3.5 can be used to extract lysozyme from HEW to remove a significant portion of HEW proteins. The extract can be adjusted to 60-90% ethanol to dissolve zein. The slurry can then be used for spray drying, reported in the second part of this paper series.

5.5. Conclusions

The aqueous pH value and ethanol concentration were critical for the extraction of lysozyme from HEW, while kinetics was not an important parameter. At an ethanol concentration of 30-50%, poor extraction was observed at pH 5.0 and above. Poor extraction was also the case for extraction with higher than 60% ethanol. The recommended extraction condition is 50% ethanol at pH 3.5 for 6 h because of a good extraction and relatively high purity. Lysozyme in the extract prepared with 50% ethanol at pH 3.5 precipitated after the ethanol concentration was increased to 90%, but the lysozyme activity was completely recovered after dilution of the precipitates using deionized water to 50% ethanol. Findings from this part of the work may lead to low-cost encapsulation technologies using partially-purified lysozyme, exemplified by spray drying in the second part of our paper series.

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Appendix

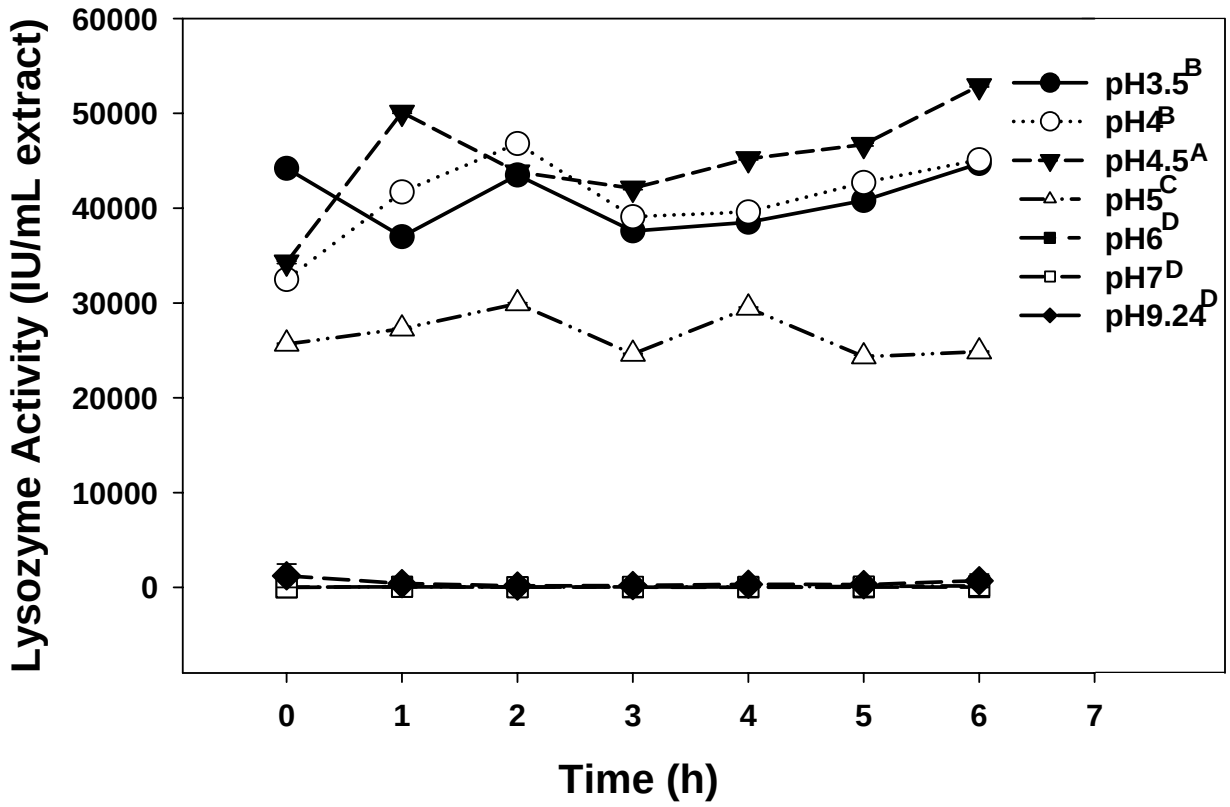

Figure 5.1 A. Kinetics of lysozyme extraction with 30% ethanol at different pH conditions.
Superscripts with different letters in the legend indicate that the results from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% confidence intervals (CI) from three independent measurements.

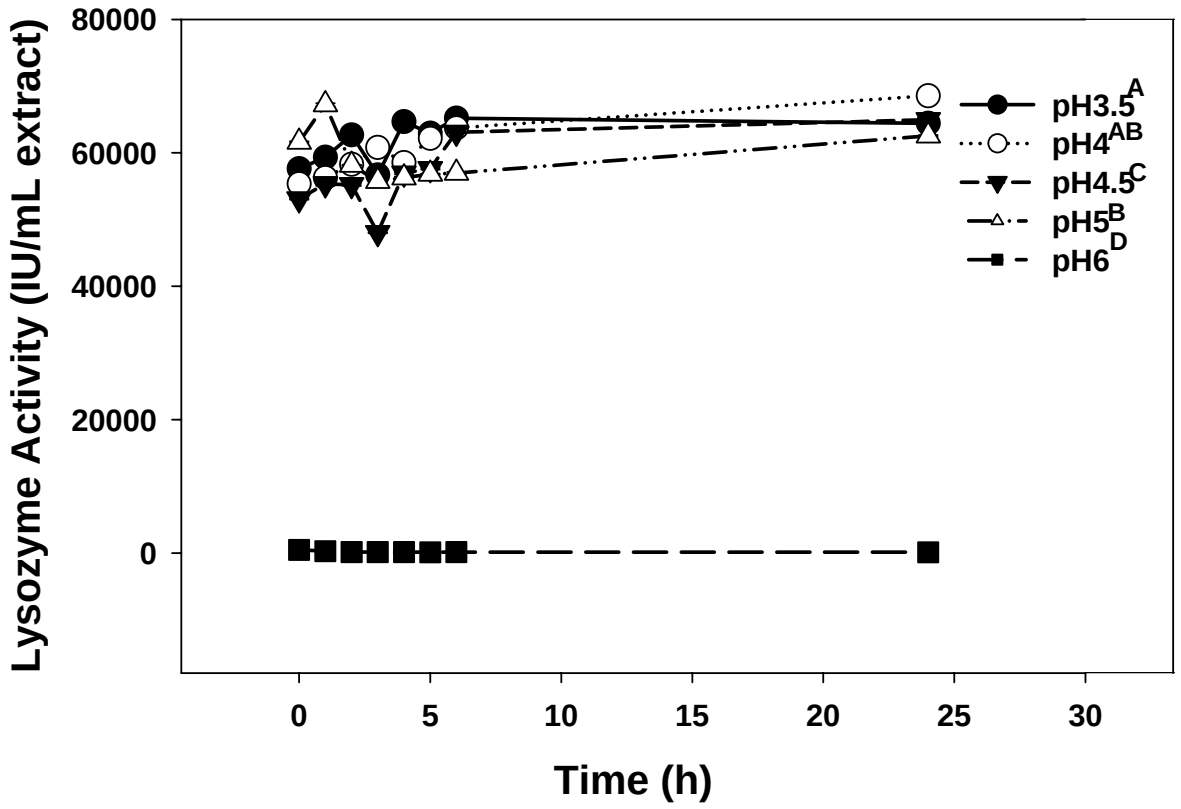

Figure 5.1 B. Kinetics of lysozyme extraction with 40% ethanol at different pH conditions.
Superscripts with different letters in the legend indicate that the results from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% CI from three independent measurements.

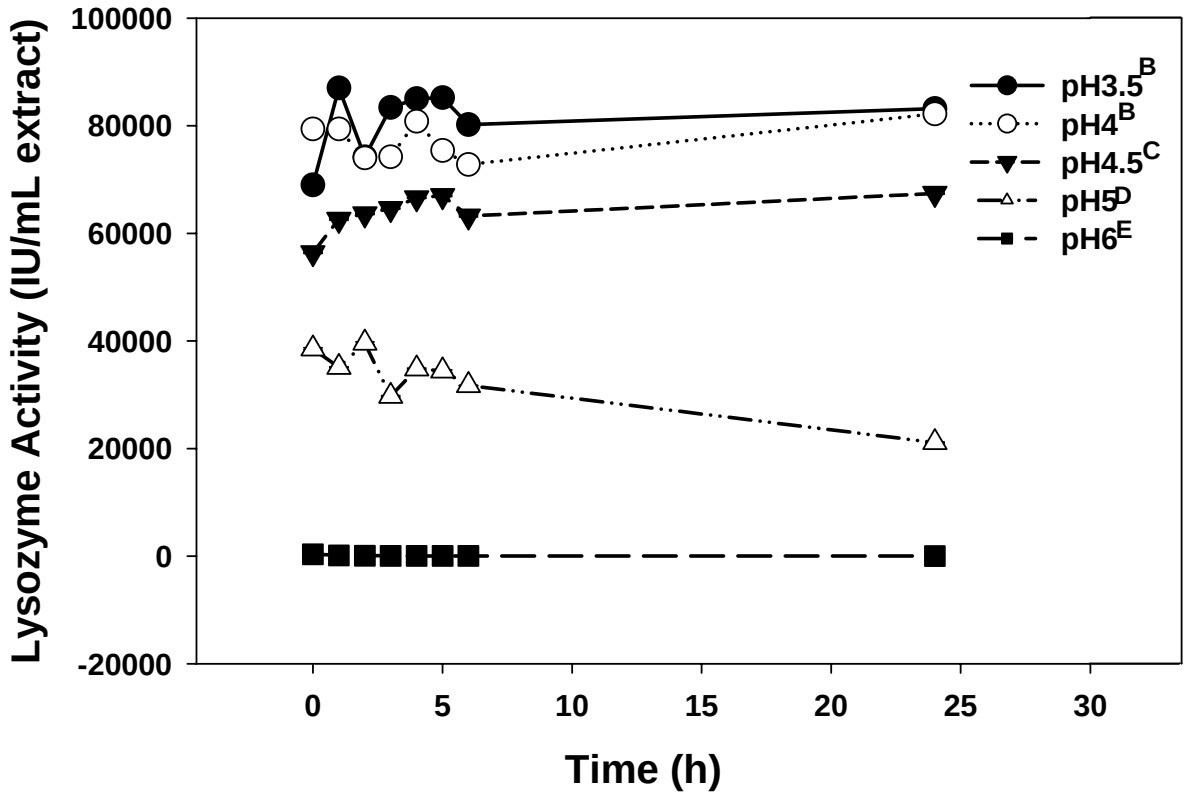

Figure 5.1 C. Kinetics of lysozyme extraction with 50% ethanol at different pH conditions.
Superscripts with different letters in the legend indicate that the results from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% CI from three independent measurements.

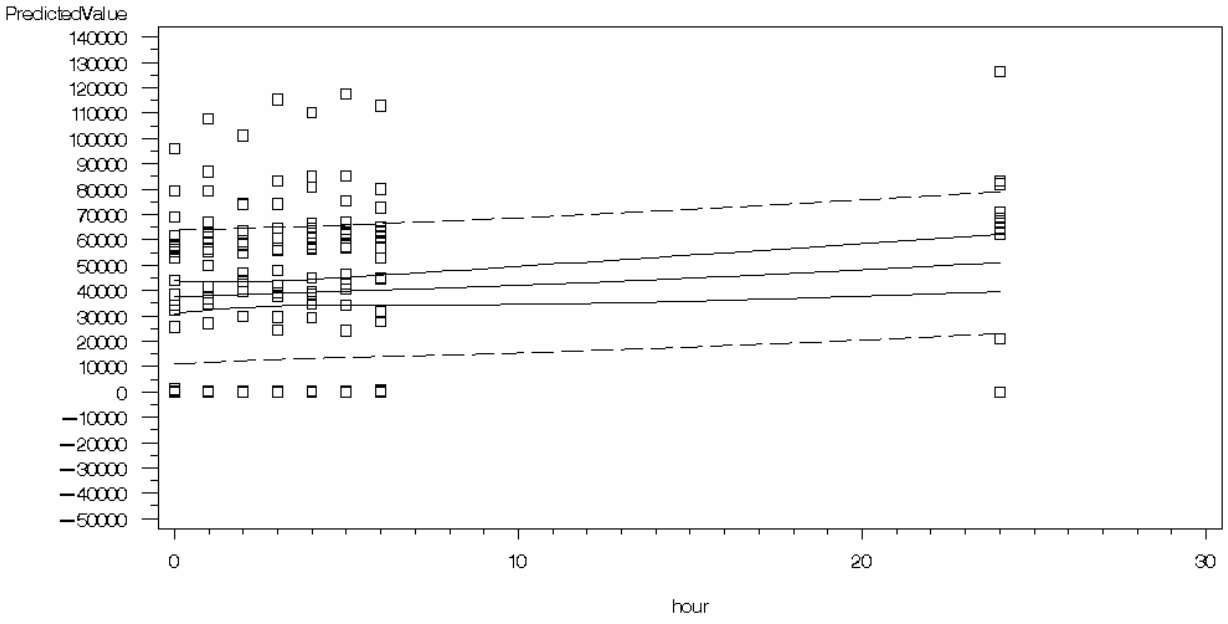

Figure 5.2 A. Response surface analysis to interpret the effects of time, pH, and ethanol concentration on lysozyme extraction.
Symbols indicate the actual experimental data, solid lines indicate the predicted result (center solid line) and 95% CI for mean predictions (two mirrored solid line), and dashed lines indicate 95% CI for individual predictions.

The RSREG Procedure

Response Surface for Variable LysoyzmeActivity

Response Mean	39866
Root MSE	12953
R-Square	0.8545
Coefficient of Variation	32.4920

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	3	128696808487	0.7390	255.68	<.0001
Quadratic	3	13995081867	0.0804	27.80	<.0001
Crossproduct	3	6113543435	0.0351	12.15	<.0001
Total Model	9	148805433788	0.8545	98.54	<.0001

Residual	DF	Sum of Squares	Mean Square
Total Error	151	25335508508	167784825

Parameter	DF	Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	55309	42195	1.31	0.1919
hour	1	1346.983738	1464.972929	0.92	0.3593
pH	1	-30069	4941.926656	-6.08	<.0001
EtOH	1	3865.205814	1876.482763	2.06	0.0411
hour*hour	1	-12.383813	27.933198	-0.44	0.6582
pH*hour	1	-82.069910	89.062006	-0.92	0.3583
pH*pH	1	2711.220513	312.815745	8.67	<.0001
EtOH*hour	1	-9.590271	31.026344	-0.31	0.7577
EtOH*pH	1	-404.052771	70.435519	-5.74	<.0001
EtOH*EtOH	1	-9.472860	22.679337	-0.42	0.6768

Factor	DF	Sum of Squares	Mean Square	F Value	Pr > F
hour	4	444963948	111240987	0.66	0.6186
pH	4	123767625542	30941906386	184.41	<.0001
EtOH	4	13922289887	3480572472	20.74	<.0001

Figure 5.2 B. Significance analysis and coefficient estimates in response surface regression for independent variables of time (hour), pH, and ethanol concentration, with respect to the dependent variable of extracted lysozyme activity.

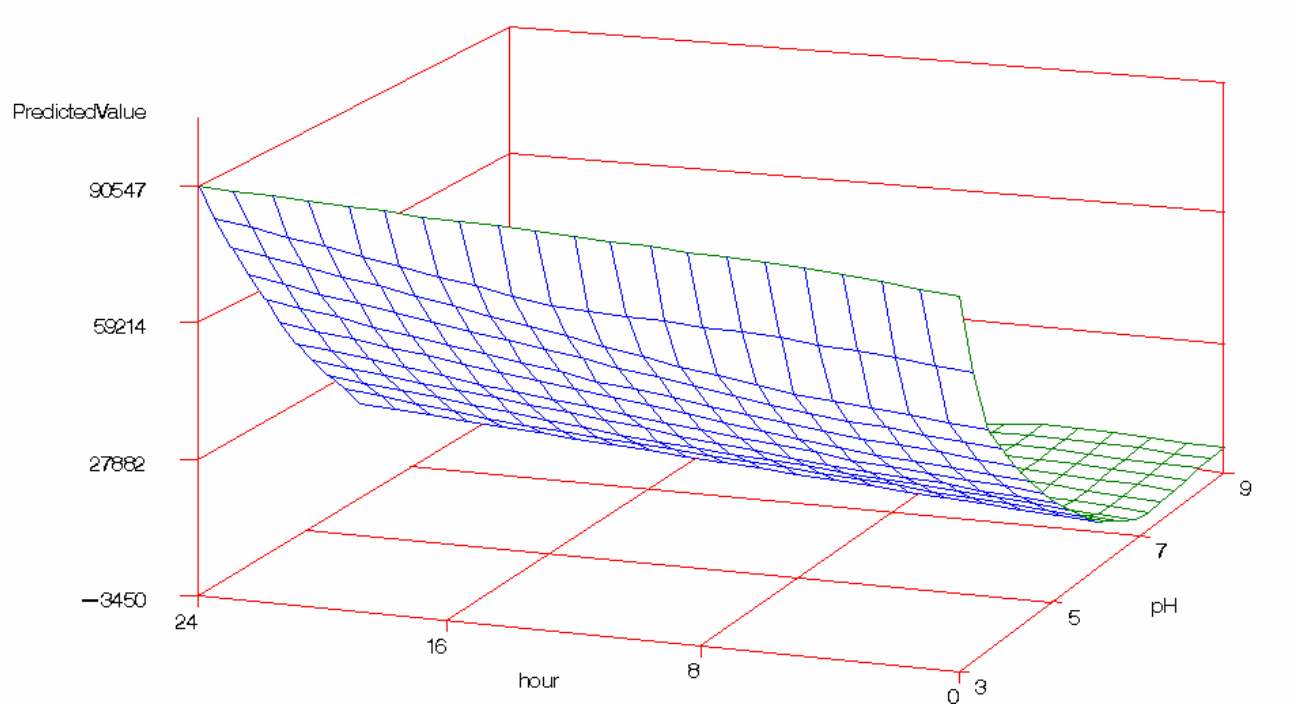

Figure 5.2 C. Contour plot of variables, time and pH, on lysozyme extraction from response surface analysis.

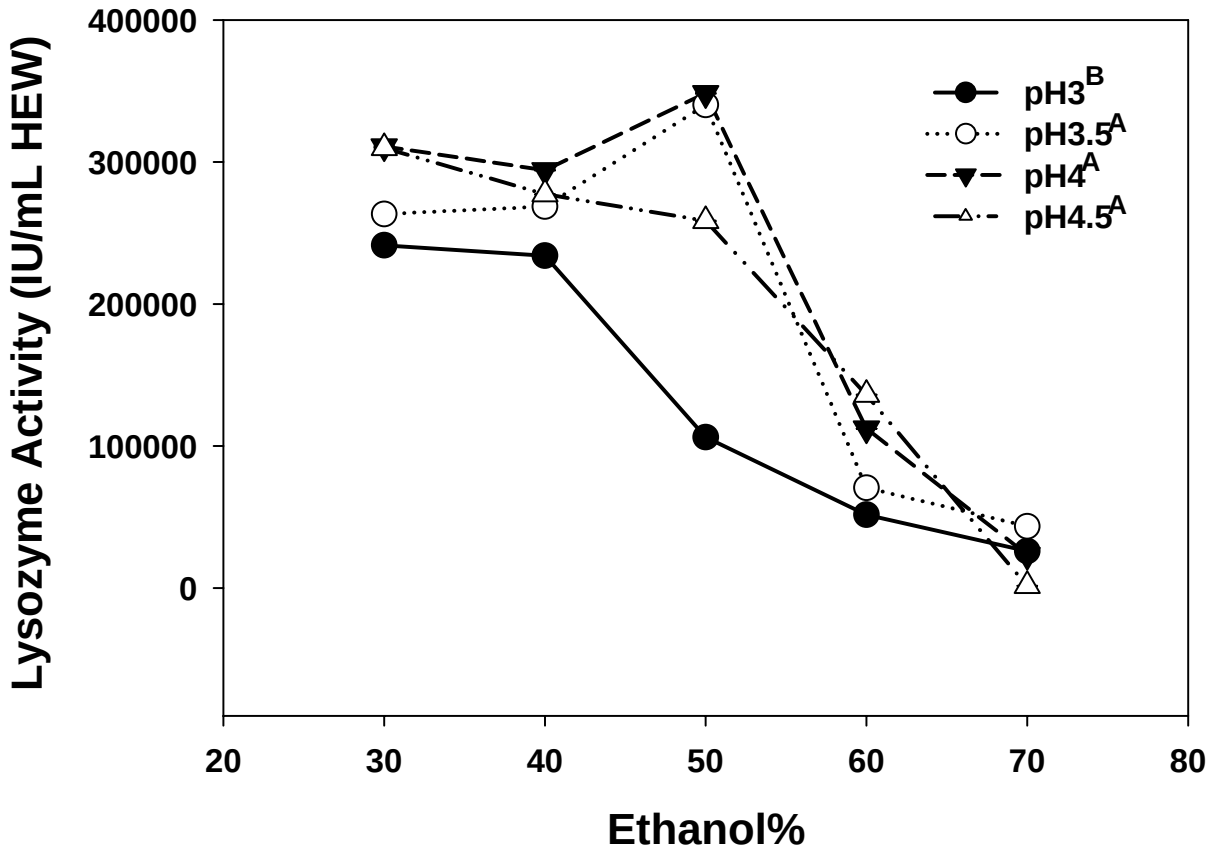

Figure 5.3 A. Comparison of extractable lysozyme units in 6-h extracts, converted to per mL HEW, prepared at various pH and ethanol conditions.
Superscripts with different letters in the legend indicate that the results from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% CI from three independent measurements.

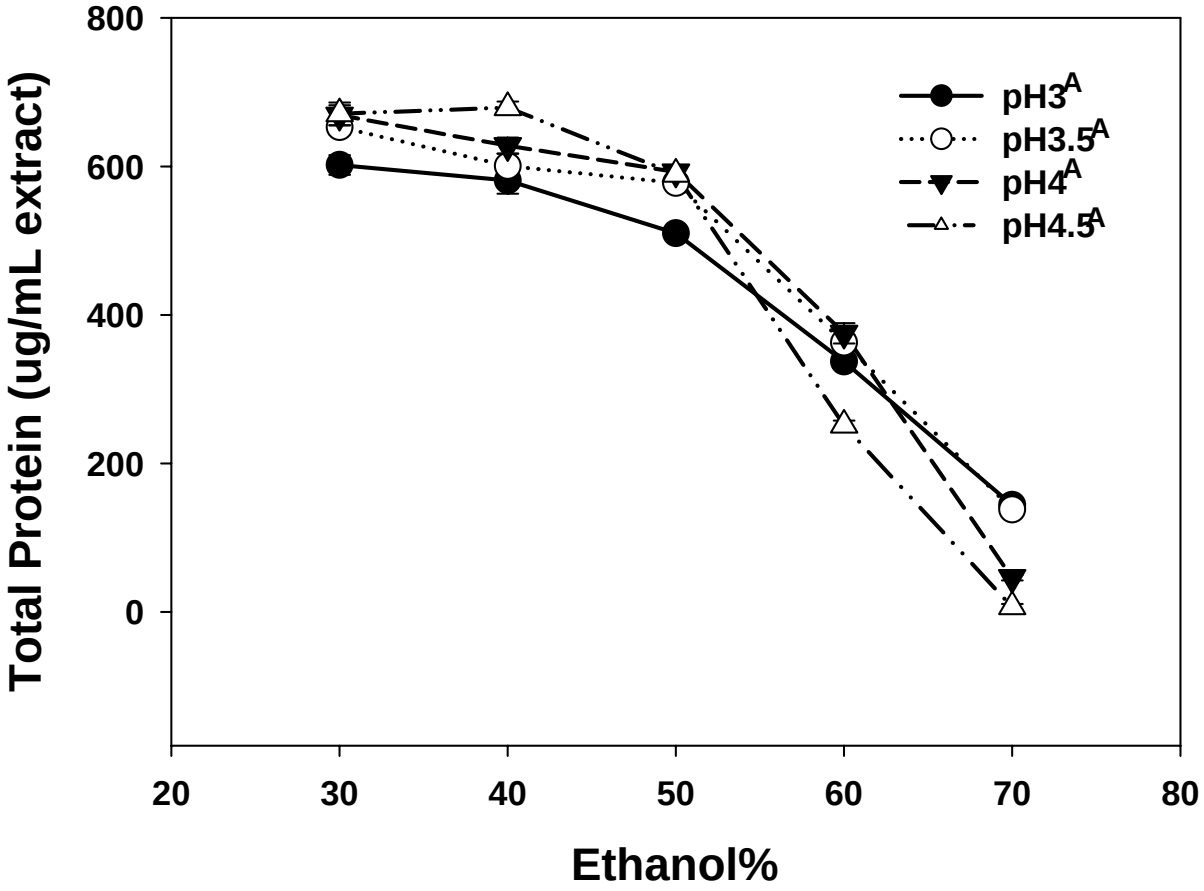

Figure 5.3 B. Comparison of total protein content in lysozyme extracts prepared at various pH and ethanol conditions.
Superscripts with different letters in the legend indicate that the results from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% CI from three independent measurements.

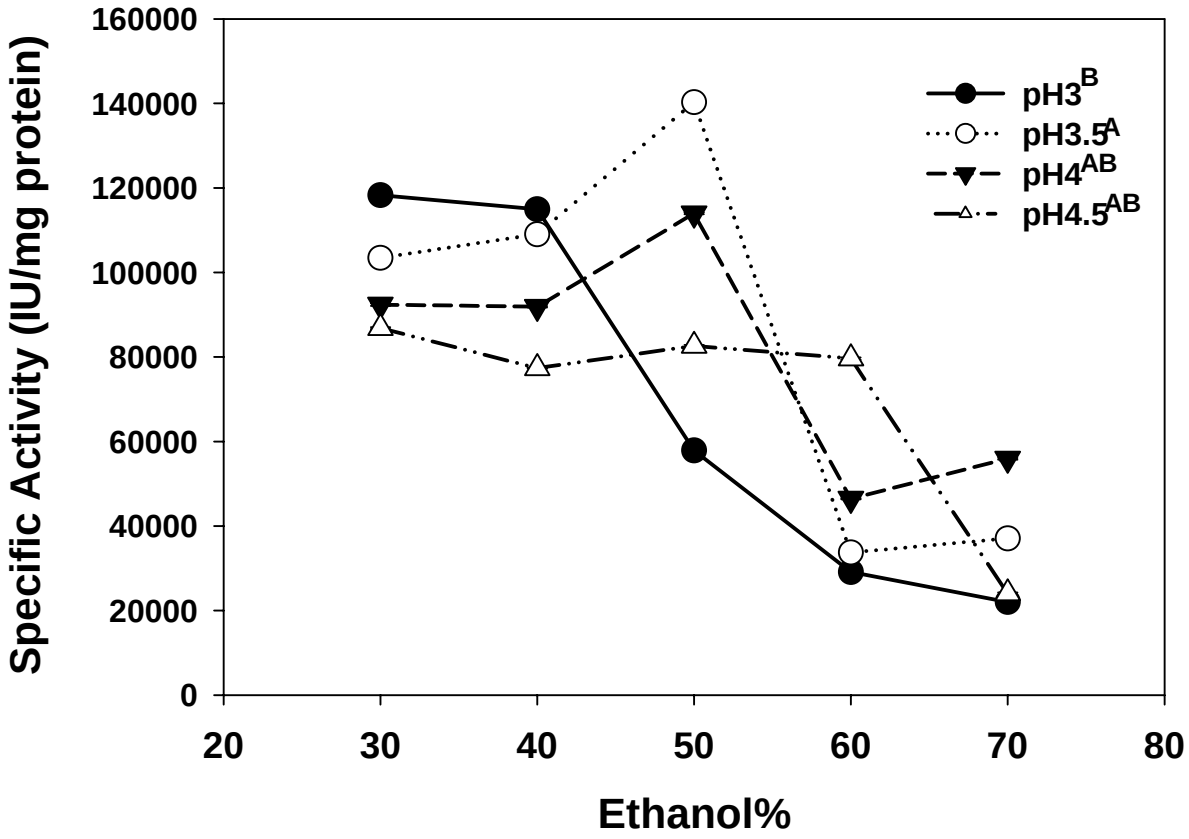

Figure 5.3 C. Comparison of specific activity of lysozyme extracts prepared at various pH and ethanol conditions.
Superscripts with different letters in the legend indicate that the results from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% CI from three independent measurements.

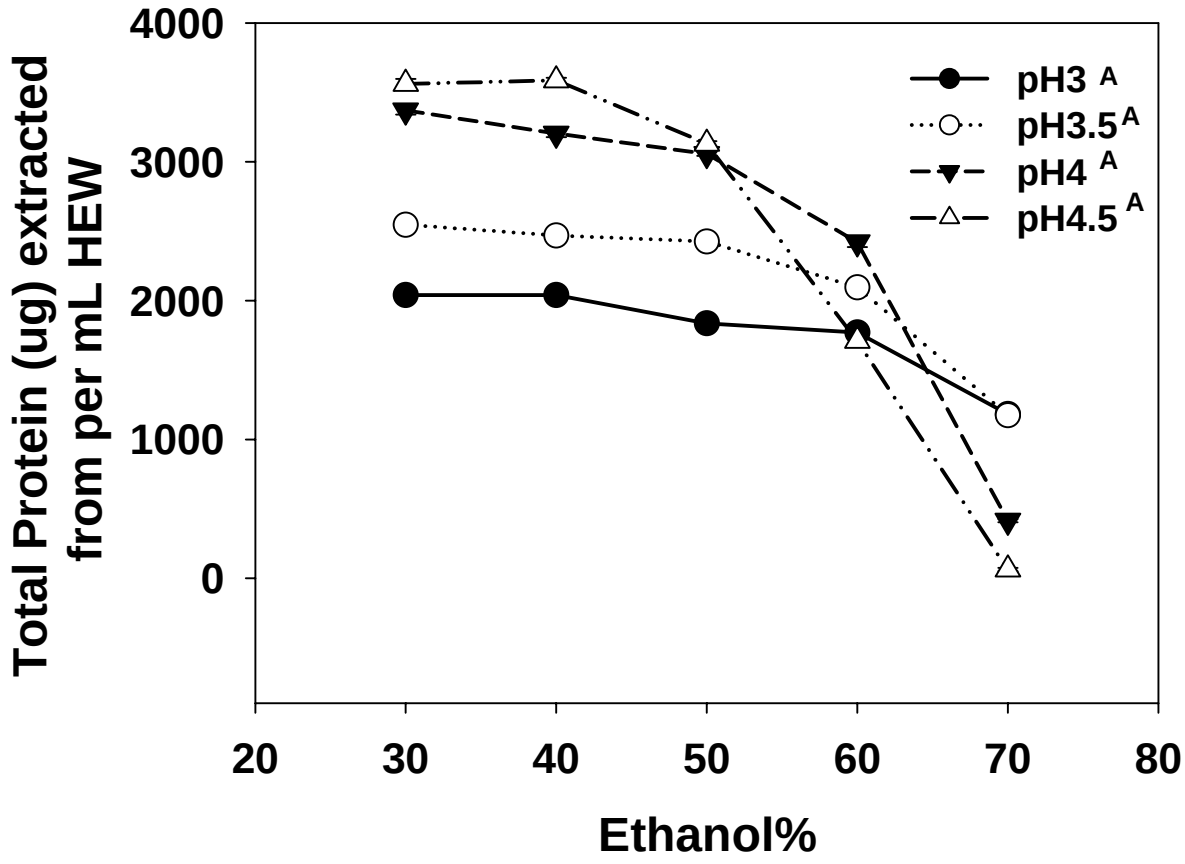

Figure 5.4. 1. Comparison of amount of proteins extracted from per mL HEW at various pH and ethanol conditions.
Superscripts with different letters in the legend indicate that the results from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% CI from three independent measurements.

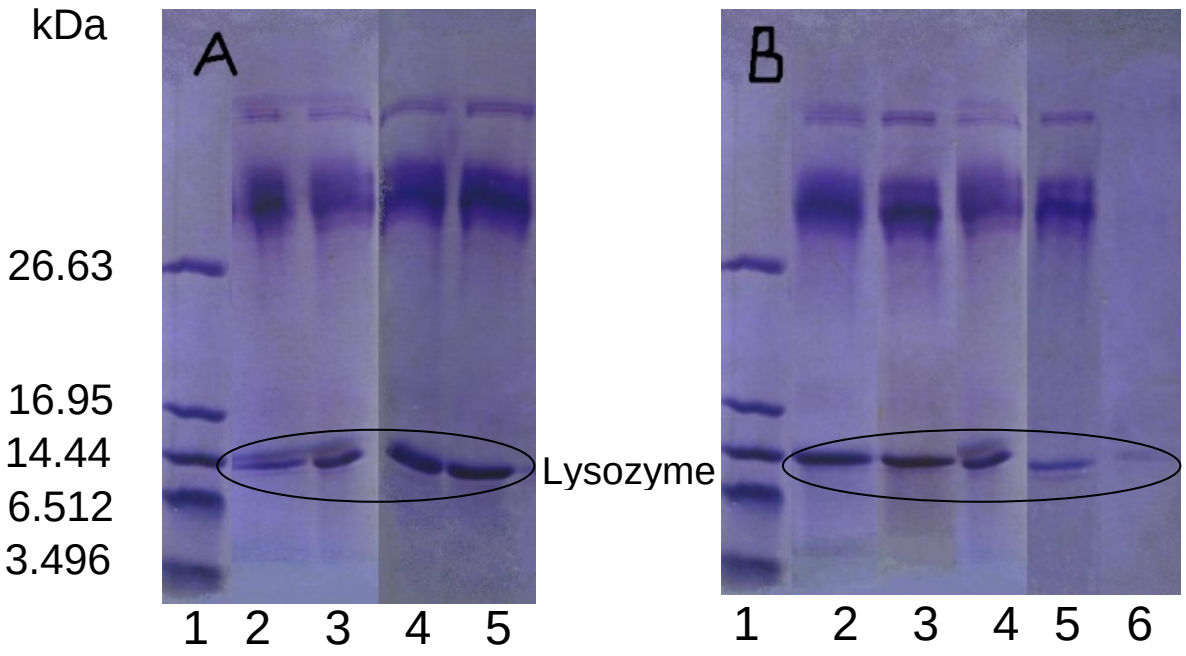

Figure 5.5. 1. Effects of solvent conditions on lysozyme extraction analyzed by SDS-PAGE.
(A) Effect of pH (Lane 1: molecular weight standards; Lanes 2-5: extracts with 50% ethanol at pH3, 3.5, 4, and 4.5, loaded with 1.53, 1.73, 1.78, and 1.77 μg protein, respectively), and **(B)** Effect of ethanol concentration (Lane 1: molecular weight standards; Lanes 2-6: extraction with 30%, 40%, 50%, 60%, and 70% ethanol at pH3.5, loaded with 1.96, 1.80, 1.73, 1.09, and 0.41 μg protein, respectively).

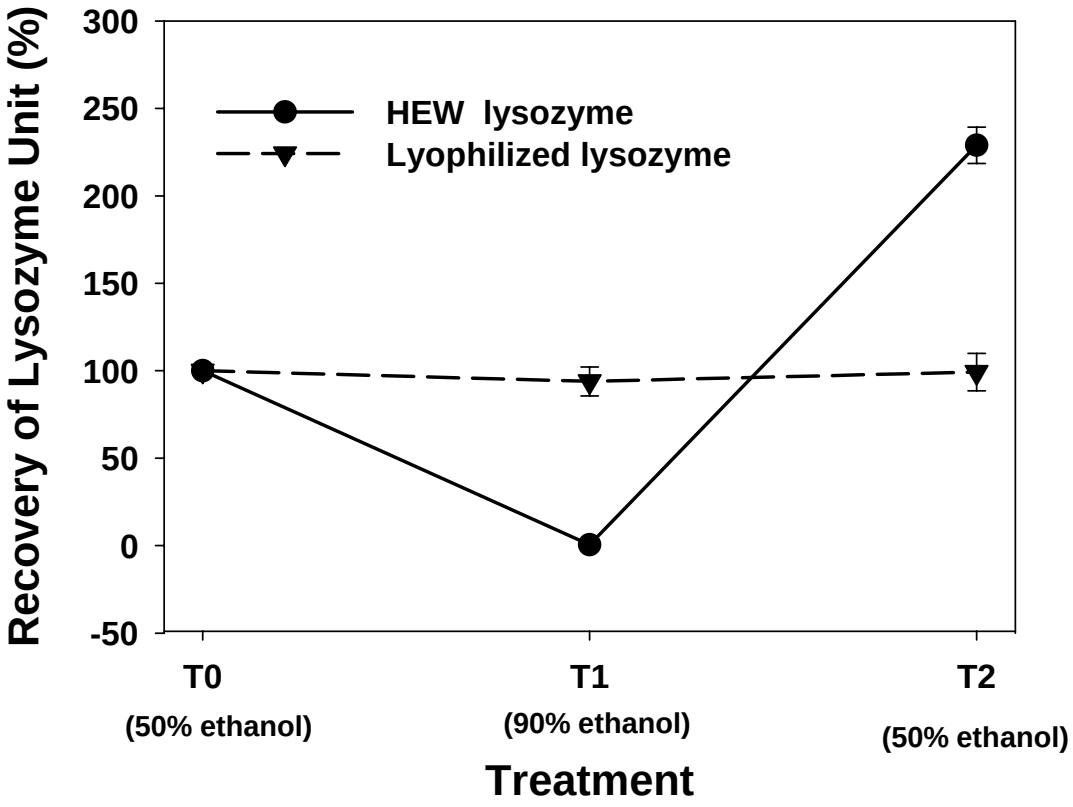

Figure 5.6. 1. Comparison of lysozyme activity in a 6-h extract prepared with 50% ethanol, pH 3.5 (Treatment T0), in the supernatant after centrifuging a sample prepared by adjusting the Treatment T0 to 90% ethanol (Treatment T1), and in a sample after readjusting the pellets from preparation of the Treatment T1 to 50% ethanol (Treatment T2). Error bars are 95% confidence intervals from three independent measurements.

Table 5. 1 Specific activity of purified, lyophilized lysozyme dissolved in different ethanol concentrations

Ethanol concentration (%)	Specific activity (IU/mg)*
0%	76133 ± 1473^{B}
30%	63733 ± 3106^{C}
40%	67333 ± 2893^{BC}
50%	64696 ± 2503^{C}
60%	113103 ± 10027^{A}
70%	75454 ± 1388^{B}
90%	63333 ± 5326^{C}

* Numbers are averages ±standard deviations from three measurements. Numbers with different superscripts are statistically different.

Chapter 6 . Production of Corn Zein Microparticles with Loaded Lysozyme Directly Extracted from Hen Egg White Using Spray Drying. Part II. Particle Properties and Release Kinetics

6.1. Abstract

Microencapsulation of hen egg white (HEW) lysozyme into corn zein was investigated by using spray drying in this study. Several variables, i.e., zein concentration and solvent composition in stock solution (60 - 90% ethanol) and addition of an amphiphilic (Tween 40) or hydrophobic chemical (thymol), were studied for the effect on microcapsule microstructure and lysozyme release characteristics. Physical properties of capsules, i.e., particle size, surface morphology and internal structures, were a strong function of formulations used in spray drying. Porous particles were produced for the formulations without additives. The addition of Tween 40 changed the capsule matrix to packed nanoscale particles. The addition of thymol assisted the formation of a continuous capsule matrix. Besides affecting the detailed microstructures differently, these two additives had different effects on release profiles. Capsules without additives had no sustained release. The added Tween 40 did not change the release characteristics of lysozyme, while thymol resulted in gradual release of lysozyme, especially nearby neutral pH. In all case, less lysozyme was released at higher pH, due to stronger molecular attraction between lysozyme and carrier zein. This work demonstrated that, with appropriate formulations, spray drying can be used to produce food grade antimicrobial delivery systems with sustained release. Our results also showed that release profiles are function of both capsule microstructure and molecular interactions between antimicrobials and the carrier biopolymer.

6.2. Introduction

Microencapsulation of antimicrobials has attracted much interest in recent years due to the fact that sustained release of antimicrobials can potentially improve the antimicrobial bioavailability by reducing the interaction with food matrices, and thus prolong the shelf life of products (Gouin 2004). Sustained release may be achieved by entrapping, coating or microencapsulating antimicrobials in structures formed of certain food biopolymers, in the forms of capsules and films (Bezemer et al. 2000, Gouin 2004).

Particulate delivery systems of antimicrobials with controlled release have been mainly studied and developed for pharmaceutical applications. Bezemer et al. (2000) reported that encapsulating lysozyme in a polymer blend of poly(ethylene glycol) and poly(butylene terephthalate) resulted in zero-order release kinetics of the encapsulated lysozyme. Salmaso et al. (2004) showed that microencapsulation of nisin in poly(L-lactide) using a supercritical anti-solvent technique (SAS) resulted in sustained release of nisin from nanoparticles over 45 days. However, many polymers and solvents used in production of drug delivery systems are questionable for food applications due to the toxicity concerns of ingredients and solvents. In addition, most processes developed for manufacturing drug delivery systems are either too expensive or cannot be scaled up to meet the capacity of food production. Identification of food grade ingredients as carriers, solvents, and low-cost and scalable processes remains a challenge for developing food grade antimicrobial delivery systems.

Our previous study (Jin and Zhong 2007) showed that commercially-available purified lyophilized lysozyme was successfully encapsulated in corn zein by using SAS. Gradual release of lysozyme from zein microcapsules lasted over 40 days, which indicates that corn zein is a potential carrier biopolymer for developing food grade particulate antimicrobial delivery systems.

Because the SAS requires specialized equipment to achieve the supercritical state of carbon dioxide (that is an anti-solvent to biopolymers), the high capital and operational costs may not be currently feasible for food production.

This work focused on replacement of the SAS by spray drying, a low-cost, widely accepted and commercially feasible process, to produce food grade delivery system of a GRAS antimicrobial – lysozyme. Corn zein and aqueous ethanol were selected as the carrier material and solvent, respectively. To further reduce the material cost, partially purified lysozyme directly extracted from hen egg white (HEW) was used, with details reported in the first part of this paper series. Specific tasks were to (1) investigate the effect of formulations on surface and internal microstructure of spray-dried zein microparticles and (2) evaluate release characteristics of lysozyme from synthesized microparticles in model buffers at different pH conditions.

6.3. Materials and Methods

6.3.1. Materials

Fresh hen eggs were obtained from a grocery store. Ethanol (200 proof) and corn zein were products from Acros Organics (Morris Plains, NJ). Thymol and *Micrococcus lysodeikticus*, indicator microorganism for estimating lysozyme activities, were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were from Fisher Scientific (Pittsburgh, PA).

6.3.2. Extraction of Lysozyme from HEW

The extraction condition was established in the first part of this paper series. Briefly, egg white was carefully separated from eggs and diluted three-fold with a 0.05 M NaCl solution, adjusted to pH 3.5 with 1 M acetic acid, and then mixed with an equal volume of ethanol to achieve a final ethanol concentration of 50%. After extraction for 6 h, the mixture was

centrifuged at 5,000 × g for 30 min (Sorvall RC 5B Plus, Newtown, CT). The supernatant was taken for preparation of stock solutions used in spray drying.

6.3.3. Production of Microparticles using Spray Drying

The supernatant with pH 3.5 (~50 v/v% ethanol) was adjusted to 60-90% (v/v) ethanol, followed by dissolving 0.4-6.4%, w/v, corn zein. In two additional trials, 0.05% w/v Tween 40 or 0.032% w/v thymol was supplemented to the HEW extract adjusted to 90% ethanol. The final suspension (feed) was then spray-dried with a B-290 Mini Spray Dryer (BÜCHI Labortechnik AG, Postfach, Switzerland) at inlet and outlet temperatures of 80 and 50-55 °C, respectively, and a feed rate of 6.67 mL/min. The gas applied in the process was compressed air. The collected products were kept in a -20 °C freezer until analyses.

6.3.4. Scanning Electron Microscopy (SEM)

The inner and outer structures of microparticles were imaged using a LEO 1525 microscope (LEO Electron Microscopy, Oberkochen, Germany). The microparticles were loosely attached onto a black adhesive tape mounted on a stainless steel stub. Besides directly imaging surface morphology, the inner structures were observed for microparticles fractured by a razor blade that moved perpendicularly through a layer of sample powder, as used by Lee and Rosenberg (2000). To prevent the charging on protein samples, the specimen was sputter-coated with a gold layer of ~5 nm thickness. Particle diameters were estimated based on SEM images, with the ImageJ software available from the National Institute of Health (Bethesda, MD).

6.3.5. Determination of Yield and Encapsulation Efficiency

The particle yield was defined as the ratio of collected powder mass to that of the non-solvent mass in the feed before spray drying (Equation 1). Due to the difficulty in product collection (some powders stuck to the drying chamber), the encapsulation efficiency (Equation 2)

was defined as the ratio of lysozyme units per mg product (after spray drying) to those per mg non-solvent mass in the feed (before spray drying). Lysozyme units per mg product were estimated using solutions prepared by dissolving 27 mg of a powdered product in 1.5 mL 55% aqueous ethanol. Lysozyme units in the feed were measured before spray drying.

$$\text{Particle yield}\% = 100\% \times \frac{\text{Mass of collected product}}{\text{Non-solvent mass in the feed}} \quad (1)$$

$$\text{Encapsulation efficiency}\% = 100\% \times \frac{\text{Lysozyme units per mg product}}{\text{Lysozyme units per mg non-solvent mass in the feed}} \quad (2)$$

6.3.6. Evaluation of *in vitro* Release Kinetics

In vitro release kinetics was evaluated using 66 mM potassium phosphate buffers adjusted to pH 2-8 by using 1 N potassium hydroxide or 1 N HCl. The 27 mg microcapsules were suspended in 1.5 mL buffers contained in polystyrene microcentrifuge tubes. The suspensions were gently agitated using an end-to-end shaker (Lab Industries Inc., Berkeley, CA) at room temperature. At a predetermined release time point, suspensions were centrifuged at 5,000 × g for 5 min (model MiniSpin Personal, Eppendorf, Westbury, NY), and 1 mL of supernatant was transferred and measured for lysozyme activity. The remainder sample was supplemented with 1.0 mL of the fresh corresponding buffer, resuspended and mixed for continued release studies. The total amount of lysozyme in unit mass of powders was measured by dissolving 27 mg of powders in 15 mL 80% ethanol that dissolved both zein and lysozyme completely.

Calculation of accumulative lysozyme release was as follows:

Let the lysozyme volumetric concentration (U/ml) at the first sampling time, t_1 to be a_1, the accumulative release at time t_1 is:

$$R_{t_1}(\%) = \frac{1.5\,a_1}{U_o} \times 100\% \quad (2)$$

where U_o is the total lysozyme units included in the dispersion before release tests, and the prefix 1.5 before a_1 is the total volume of dispersion.

After removing 1 mL of supernatant, there are 0.5 a_1 units of lysozyme due to the release after time t_1, which carries to the second sampling at time t_2. Similarly, if the volumetric concentration of supernatant from the second sampling is a_2 (U/ml), the total lysozyme released up to this point is a_1 + 1.5 a_2, which gives an accumulative release at time t_2:

$$R_{t_2}(\%) = \frac{a_1 + 1.5\,a_2}{U_o} \times 100\% \quad (3)$$

Therefore, at the i^{th} sampling, the accumulative release is:

$$R_{t_i}(\%) = \frac{\sum_{n=1}^{i-1} a_n + 1.5\,a_i}{U_o} \times 100\% \quad (3)$$

6.3.7. Measurement of Lysozyme Activity

Lysozyme activity was determined according to a method of Sigma-Aldrich (St. Louis, MO) for product HEW lysozyme (catalog number L6876) after slight modification. *Micrococcus lysodeikticus* was used as the test microorganism, suspended in a 66 mM potassium phosphate buffer at pH 6.24 to achieve a cell substrate with a final concentration of ~0.015% (w/v). The mixture of sample and buffer with the test microorganism was monitored the reduction in absorbance at 450 nm for 3 min by using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA). The cuvette cell had a thermal jacket with a recirculating water stream at 25 °C. One unit of lysozyme is defined as a reduction of absorbance by 0.001 per min at the above test conditions. Each sample was tested in triplicate.

6.3.8. Statistical Analysis

Significant differences of particle sizes were analyzed with a least-significant-difference ($P < 0.05$) mean separation using SAS software (v. 9.1, SAS Institute, Cary, NC).

6.4. Results and Discussion

6.4.1. Yield, Encapsulation Efficiency, and Particle Size

The yields, encapsulation efficiencies and particle sizes of synthesized microparticles under different treatments are summarized in Table 6.1. The particle yields were 56-80%, typical for a lab-scale spray dryer due to the difficulty in sample collection (i.e., from the portion sticking to the drying chamber wall). The encapsulation efficiency was 78-100%, indicating the good thermal stability of lysozyme. Particles produced with low zein% and high ethanol% had a smaller average diameter (Trials A1-A4 in Table 6.1). Reducing the zein concentration alone or adding 0.033% of a hydrophobic compound, thymol, also reduced the average particle diameter (Trial A4 vs. B1 and D1); while the addition of 0.05% surfactant Tween 40 did not significantly change the average particle diameter (Trial A4 vs. C1). The specific effects of each variable however were not conclusive because of the limited data points.

Encapsulation efficiencies are higher than the calculated particle yields for all samples (Table 6.1). One reason is that the incomplete sample collection (of the portion sticking on the drying chamber wall) reduces the particle yield but may not affect the proportion of lysozyme and zein before and after spray drying, assuming both zein and lysozyme precipitate similarly during spray drying. As for the particle size (Table 6.1), reducing zein% and increasing the ethanol% in the feed (Trials A1-A4) significantly decreased the microcapsule diameter and polydispersity (smaller standard deviation). Reducing polymer concentration facilitates the

atomization (due to a lower feed viscosity) during spraying, while a higher ethanol concentration facilitates the solvent evaporation during drying. Both factors may have contributed to the observations.

6.4.2. Surface Morphologies and Inner Structures

Representative particles of Trials A1-A4 are shown in Figure 6.1. Most particles showed irregular surfaces which indicated porous structures. Conditions facilitating formation of smaller particles (Trials A1 to A4, A4 vs. B1; Table 6.1), i.e., a lower zein% and a higher ethanol%, generally reduced the roughness of capsule surface. When the additive Tween 40 or thymol was used in the 90% ethanol stock solution, the surface smoothness of particles was improved (Figure 6.2 vs. Figure 6.1 A4).

All particles shown in Figure 6.1 were very fragile and fractured completely when sheared by the razor blade during the preparation step for SEM experiments, exemplified in Figure 6.3 for the 60% and 70% ethanol samples (Figures 6.2A1$_i$ and 6.2B1$_i$). The open inner structures corresponded well to the indication of porous structures based on surface morphology (Figure 6.1). When Tween 40 was added into the stock solution before spray drying, the inner structure of these capsules showed packed nanometer-sized particles with the presence of some cavities (Figure 6.3C1$_i$). In contrast, the sample containing thymol had a finer inner structure without identifiable nanoparticles (Figure 6.3 D1$_i$).

The variables of ethanol% and zein% presumably affected microstructure formation by the following two phenomena. The first one is that evaporation of ethanol (which evaporates faster than water) gradually reduces the solvency of aqueous ethanol in the atomized droplets with respect to zein that is soluble in 55-90% ethanol. This means that zein will become insoluble more quickly for samples with a lower ethanol concentration, which contributes to

quicker solidification of zein (a biopolymer) during spray drying. The second one is related to the effects of polymer concentration on polymer solidification. Similar to crystallization, more nuclei are formed and the growth of nuclei is faster at a higher polymer concentration (Kashchiev and van Rosmalen 2003). Both phenomena lead to faster polymer solidification, which results in a higher degree of microstructure irregularity (Dixon et al. 1993) at a lower ethanol% and higher zein% for Trials A1-A4, also Trials A4 vs. B1.

For the particles produced with additives (Tween or thymol), the enhanced smoothness in particle surface is apparently due to the additional zein (protein)-surfactant and protein-thymol interactions. Understanding and investigation of these interaction mechanisms will be future research topics. Nevertheless, because lysozyme is encapsulated inside capsules, different inner structures of samples produced with various formulations indicate that lysozyme will experience different mass transfer resistances when diffusing outwards to the capsule surface.

6.4.3. *In vitro* Release Kinetics of Lysozyme from Capsules without Additives

In vitro release kinetics of samples prepared from formulations A1-A4 in Table 6.1 are presented in Figure 6.4. Three trends were observed: (1) the release quickly reached equilibrium, which means no sustained release, (2) a smaller amount of lysozyme was released at a higher buffer pH, and (3) a smaller amount of lysozyme released from capsules prepared with the feed adjusted to a higher ethanol concentrationand a lower zein concentration (except for the 90% ethanol treatment).

The first and third trends may have resulted from, respectively, porous structures of capsules and a more porous structure of capsules produced at a higher zein concentration and lower ethanol concentration. The second trend may be explained by the hydrophobic and electrostatic interactions between zein and lysozyme at different pH conditions. At a higher pH

(closer to the isoelectric point of lysozyme, 10.5~11.0; Johnson and Larson 2004), the increased hydrophobicity of lysozyme strengthens hydrophobic interactions between lysozyme and carrier zein, retaining more lysozyme in capsules. Additionally, lysozyme is always positively charged at the pH conditions used, while the carrier zein (pI of 6.8; Cabra et al. 2005) was positively charged at pH 2 and 6 and negatively charged at pH 8. The electrostatic interactions between lysozyme and carrier zein molecules were thus attractive at pH 8 and repulsive at the other two pH conditions. Overall, the interactions between zein and lysozyme were more attractive at a higher pH, which resulted in more lysozyme retention in zein microcapsules.

6.4.4. *In vitro* Release Kinetics of Lysozyme from Capsules with Tween 40 or Thymol

The effects of additives in formulations during microencapsulation were studied for the feed adjusted to 90% ethanol, with the addition of a surfactant (0.05% Tween 40) or a hydrophobic compound (0.033% thymol). *In vitro* release kinetics of HEW lysozyme is plotted in Figure 6.5. The addition of Tween 40 did not change the characteristics of release profiles, i.e., no gradual release, but retained more lysozyme in capsules at all pH conditions (Figure 6.5). The small amount of Tween 40 added in the formulation changed the microstructure of capsules from a porous structure (Figure 6.3A1$_i$) to a continuous inner structure consisting of nanoparticles (Figure 6.3C1$_i$), which may have resulted in the more retention of lysozyme in particles. Tween 40 is a nonionic surfactant and the small amount of Tween 40 possibly is insufficient to modulate molecular interactions between lysozyme and zein.

The addition of thymol resulted in a steady increase of released lysozyme: less noticeable at pH 2 and 6 and more significant at pH 8 (Figure 6.5). A smaller amount of release at a higher pH is similar to other treatments; therefore it is likely that the addition of thymol did not have

significant impact on molecular interactions between zein and lysozyme. Indeed, the amount of released lysozyme at pH 2 and 6 is similar to the sample with added Tween 40 after several hours (Figures 6.5A and 6.5B), and the amount of released lysozyme at pH 8 is similar to the comparable sample without additives (Figure 6.5C). Microstructurally, the added thymol (0.035%v/v) in the formulation formed a more compact structure that provides a stronger mass transfer resistance for lysozyme diffusion than those without additives or with Tween 40.

The impact of additives on release characteristics of lysozyme gives interesting directions for future research. Thermodynamically, molecular interactions between lysozyme and zein determine the equilibrium of lysozyme attracted to the carrier biopolymer. This equilibrium determines how much lysozyme can be eventually released, if given enough time. Kinetically, the "free" lysozyme in microcapsules undergoes typical mass transfer processes: diffusion from internal structures to the particle surface and then from the surface to the incubation buffer. Since lysozyme is soluble in water, at pH 2-8, the mass transfer resistance from the particle surface to the ambient aqueous phase is expected to be minimized. The strategy is then how to control capsules microstructure formation to manipulate internal mass transfer. Both thermodynamic and kinetic bases for improvement of release characteristics may be to modulate interactions between biopolymers (lysozyme and zein; zein and zein) during particle production and during release. Experimentally, this may be achieved by studying various particle production conditions and formulations to include additives of plasticizers, surfactants or other compounds that can affect the thermodynamics and microstructure formation.

6.5. Conclusions

This work illustrated the promise of using spray drying to manufacture delivery systems of antimicrobials with gradual release. Physical properties of capsules, i.e., particle size, surface

morphology and internal structures, were significantly affected by the formulations used in spray drying. The addition of a surfactant Tween 40 and a hydrophobic essential oil thymol assisted the formation of continuous capsule matrices but had different effects on release profiles. Tween 40 did not change the release characteristics of lysozyme, i.e., no sustained release for the capsules without additives, while thymol resulted in gradual release of lysozyme, especially at pH 8. For all samples, a slower release was observed at a higher pH due to stronger attraction between the carrier polymer zein and the encapsulated lysoyzme. The results emphasized the significance of both capsule microstructure and molecular interactions for designing antimicrobial delivery systems. Although the exact physicochemical mechanisms associated with the addition of Tween 40 and thymol are still unknown, this work demonstrated the feasibility to produce food grade delivery systems of antimicrobials by carefully studying formulations and processing conditions during spray drying. Low cost ingredients and scalable processes used are advantageous to produce affordable antimicrobial delivery systems to enhance microbial food safety and reduce food spoilage.

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Appendix

Table 6. 1 Summary of yields, encapsulation efficiencies and particle sizes of treatments.

Trial #	Feed composition			Diameter (μm)*	Yield%**	Encapsulation efficiency%***
	Ethanol%	Zein%	Additive			
A1	60%	6.4	——	4.92 ± 2.84^{B}	79.99	100
A2	70%	4.8	——	7.84 ± 2.72^{A}	57.63	78.64
A3	80%	3.2	——	3.96 ± 2.67^{CD}	64.59	94.08
A4	90%	1.6	——	4.03 ± 1.62^{BCD}	56.64	84.48
B1	90%	0.4	——	3.66 ± 1.59^{D}	64.36	83.13
C1	90%	1.6	0.05% Tween 40	4.67 ± 2.25^{BC}	56.64	89.17
D1	90%	1.6	0.033% thymol	3.67 ± 2.12^{D}	74.52	89.75

* Numbers are averages ± one standard deviation. Numbers with different superscripts are statistically different ($P < 0.05$).

** Yield% = 100% × (mass of collected product)/(non-solvent mass in the feed).

*** Encapsulation efficiency% = 100% × (lysozyme units per mg product)/(lysozyme units per mg non-solvent mass in the feed).

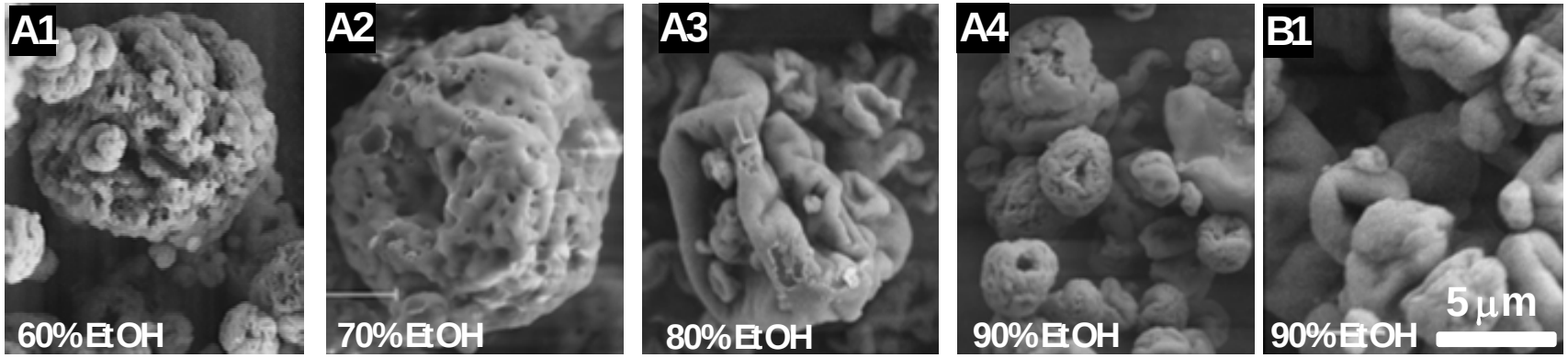

Figure 6. 1. Microparticles of spray-dried samples. Image codes correspond to Trial #s in Table 6.1.

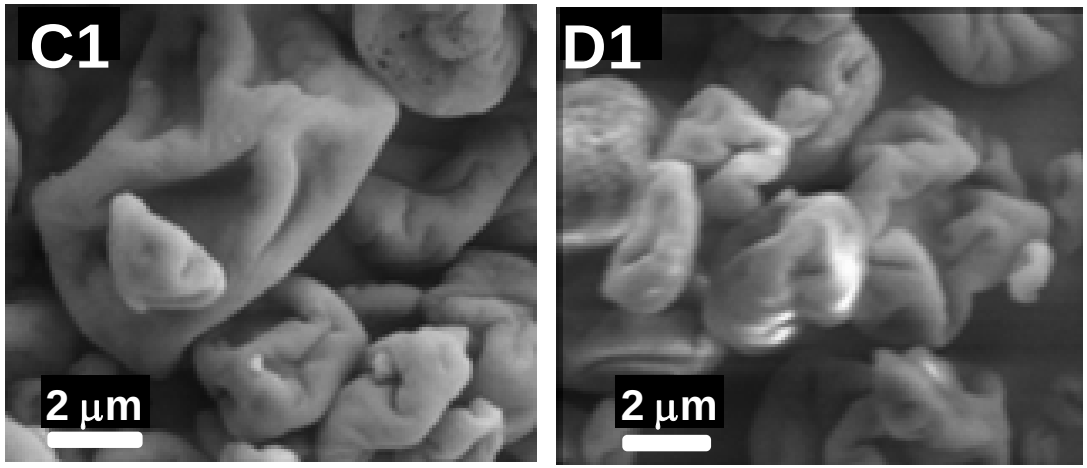

Figure 6. 2. Microparticles of spray-dried samples C1 (with Tween 40) and D1 (with thymol) in Table 6.1.

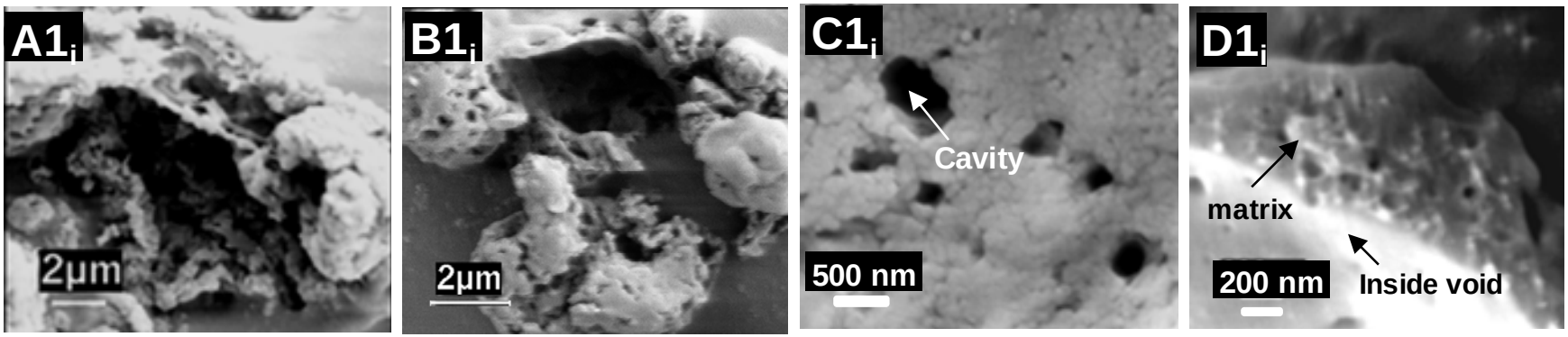

Figure 6. 3. Comparison of microcapsule inner structures of samples A1 (60% ethanol), B1 (70% ethanol), C1 (with 0.5% Tween 40) and D1 (with thymol) in Table 6.1.

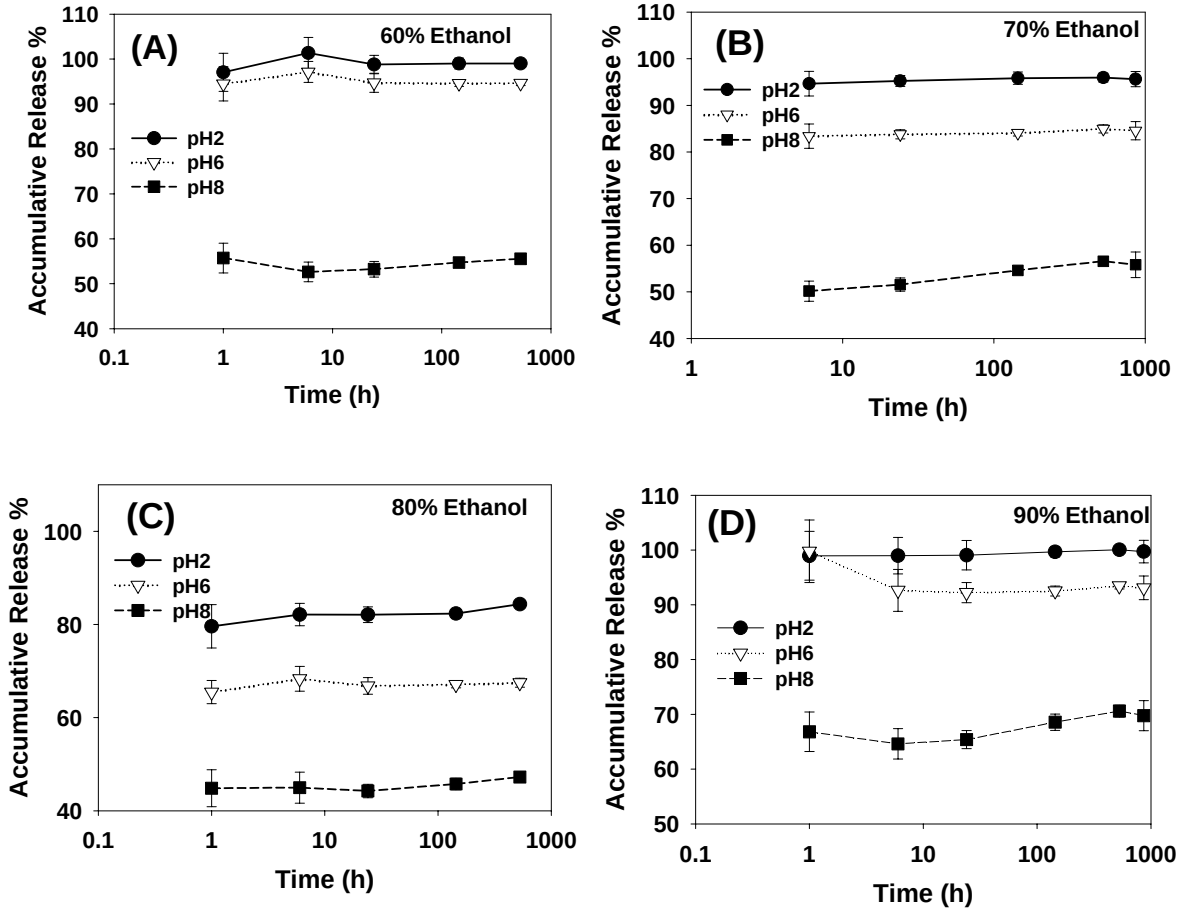

Figure 6. 4. *In vitro* release kinetics of lysozyme from zein microcapsules in potassium phosphate buffers at pH 2, 6, and 8. The capsules were produced using formulations of (A) 6.4% w/v zein dissolved in hen egg white (HEW) extract adjusted to 60% v/v ethanol, (B) 4.8% w/v zein dissolved in HEW extract adjusted to 70% v/v ethanol, and (C) 3.2% w/v zein dissolved in HEW extract adjusted to 80% v/v ethanol and (D) 1.6% w/v zein dissolved in HEW extract adjusted to 90% v/v ethanol. Error bars are 95% confidence intervals from triple tests.

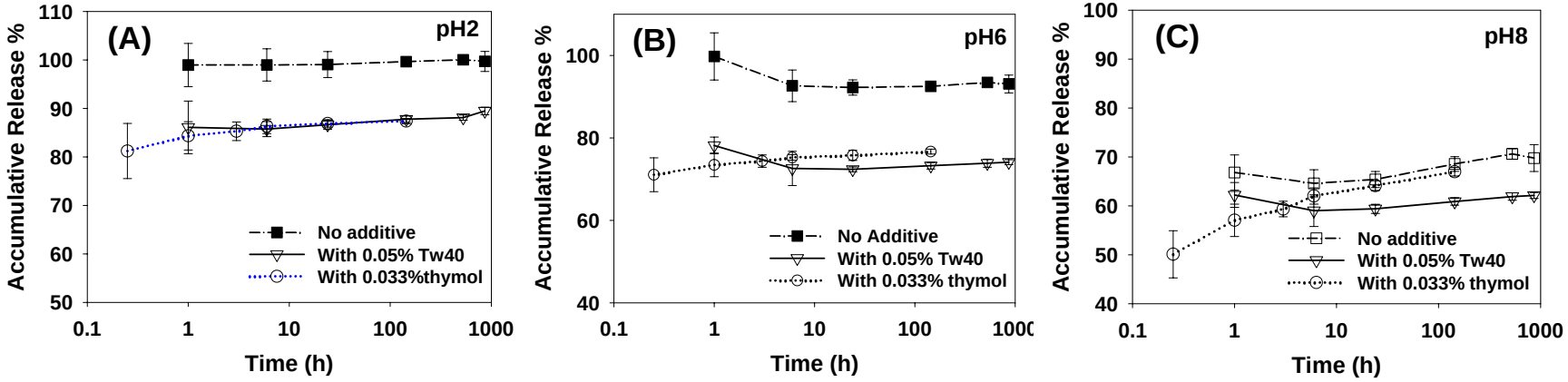

Figure 6. 5. *In vitro* release kinetics of lysozyme from zein microparticles in potassium phosphate buffers at (A) pH 2, (B) pH 6, and (C) pH 8.
Microcapsules were produced from a hen-egg-white extract adjusted to 90% ethanol and subsequently dissolved with 1.6% zein, with or without additional 0.05% Tween40 or 0.033% thymol. Error bars are 95% confidence intervals from triple tests.

Vita

Minfeng Jin was born on June 20, 1979 in Shanghai, China, where her mother and her sister still reside. After graduating from Minhang High School in Shanghai, she entered Huazhong Agriculture University to purse a bachelor's degree in Food Science and Engineering. She completed her B.S. degree in Food Science in June of 2001, with a minor in Marketing received in December of 2000. In July of 2001, Minfeng started her career as a product specialist in a healthcare food company in Shanghai. She led the new product team to develop and launch a supplementary calcium tablet fermented with beneficial bacteria to enhance the absorption ability in GI track, for which the sales was ￥6 million (RMB Yuan) in the first year after launching. After that, she joined a committee to establish a new company which produced beef/fish snack foods in Shanghai. She set up the food chemistry and food microbiology labs, and complemented the Standard Operation Procedures of processing food products and ISO management documents for the company.

Minfeng always wanted to broaden and deepen her knowledge and insight of food industry in the global economy. In August of 2006, she came to Knoxville, TN, to begin her M.S. degree in the Food Science and Technology Department at the University of Tennessee, under the guidance of Dr. Qixin Zhong on the development of microencapsulation techniques for a naturally-occurring antimicrobial, lysozyme with a biopolymer corn zein. After completion of her M.S. study, Minfeng hopes to receive advanced education and explore opportunities in the food industry of the U.S.A.