

**Encapsulation of lactase in dispersible biopolymer particles as a
potential delivery system to control lactose hydrolysis in milk**

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ABSTRACT

Encapsulation of lactase has been considered as an alternate strategy to overcome lactose intolerance and negative sensory properties of lactose-free milk if capsules can be dispersed in milk, retain lactase during storage, and selectively release lactase in the intestines after ingestion. The overall objective of this thesis was to study lactase-loaded biopolymer capsules with characteristics suitable for delivering lactase in milk. Lactase-loaded zein/pectin capsules were prepared by antisolvent precipitation of zein with assistance of ionic cross-linking of low-methoxyl sugar beet pectin (SBP) with Ca^{2+} . Mass ratios of zein:SBP and CaCl_2 :SBP were studied for impacts on encapsulation efficiency, particle size and morphology, zeta potential, and stability and turbidity of dispersions. Encapsulation of lactase was verified using electrophoresis of capsules. The capsule formation mechanisms were additionally studied for surface hydrophobicity (H_0), dispersion stability, and fluorescence spectroscopy as affected by molecular force blockers. Capsules were assessed for release kinetics at 4, 25, and 37 °C at pH 7, lactose hydrolysis during 3-week storage in whole and skim milk, and lactose hydrolysis during *in vitro* digestion. The optimal formulation was observed at a zein:pectin mass ratio of 3:2 and a pectin: CaCl_2 mass ratio of 5:1 corresponding to an encapsulation efficiency of 93.0%, Z-average mean diameter of 652.7 nm, and the spherical capsules. The SDS-PAGE of the capsules showing protein bands corresponding to lactase and zein verified encapsulation of lactase. Zeta potential and H_0 revealed the core-shell structure of the capsules. Electrostatic interactions, hydrophobic interactions, hydrogen bonding, and Ca^{2+} cross-linking between SBP molecules were responsible for nanoparticle formation

based on turbidity and fluorescence intensity of dispersions as affected by sodium dodecyl sulfate, ethylenediaminetetraacetic acid, and urea. Capsules resulted in 33.1% and 40.0% lactose hydrolysis in whole and skim milk, respectively, at milk storage conditions. During *in vitro* digestion, encapsulated lactase resulted in 100 % and 89.4 % lactose hydrolysis in whole and skim milk, respectively, contrasting with negligible lactose hydrolysis by the same units of free lactase. Findings from this thesis suggest the potential of the studied capsules to incorporate lactase in milk, prevent lactose hydrolysis during storage, and hydrolyze lactose in milk during digestion.

Keywords: lactase, biopolymer nanoparticles, formation mechanism, delivery system, controlled hydrolysis of lactose in milk

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Lactase (β -galactosidase) is responsible for hydrolyzing lactose in milk to glucose and galactose. When lactose fails to be hydrolyzed in the upper digestive tract, the excessive lactose rises up the osmotic pressure leading to diffusion of tissue fluid and electrolytes into bowel; when in the colon, colon microflora ferment lactose into short chain fatty acids, hydrogen, carbon dioxide and other gases which increase transit time and pressure in the colon (Lomer, Parkes, & Sanderson, 2008). The phenomena result in symptoms like diarrhea, and the fermentation by colon microflora results in abdominal pain, bloating, and flatus (Kretchmer, 1972). The term, lactose intolerance, is used to describe the digestive conditions when lactose from dairy products is not sufficiently digested in upper gastrointestinal tract due to lack of lactase or lactase activity (American Academy of Allergy, Asthma & Immunology, 2018).

Nearly 65 % of the human population has the reduced lactose digestibility after weaning, and the prevalence of adult lactase deficiency or lactose intolerance in East Asian is over 90% (US Department of Health & Human Services, 2018). Other lactose intolerance populated regions include the Mediterranean, Africa, North and South Americas (Heyman, 2006). Thus, to address this common deficiency of lactase in human body, several treatments have been developed, such as gradual increase of lactose ingestion to induce body adaption, production and consumption of lactose-free dairy products, lactase supplements, and/or lactase-fortified dairy products. However, lactose-free products may have nutrition loss and changes in sensory perceptions (glucose and galactose are about four times sweeter than lactose), and the sensitivity of lactase to high

temperature and acidic gastric conditions limits the efficiency of supplemental lactase (Zhang, Zhang, Chen, & McClements, 2016; Kwak, Ihm, & Ahn, 2001). Therefore, a new strategy of incorporating lactase was studied to first encapsulate lactase in solid-in-oil-in-water (S/O/W) emulsions before including capsules into dairy products (Zhang & Zhong, 2018). Such a method is expected to have minimal effects on product taste as lactase remains encapsulated during storage of milk but promote lactose digestion after consumption. Yet, emulsions are thermodynamically unstable, and loading efficiency remains to be augmented. Lactase was also encapsulated in κ -carrageenan hydrogels with improved thermal and pH stabilities (Zhang, Zhang, Chen, & McClements, 2016). However, κ -carrageenan hydrogel beads showed relatively large pore size and fast enzyme leakage. Hydrogels also have large dimensions and poor mechanical strength limiting their applications in food products (Luo, Teng, Wang, & Wang, 2013). In addition, Kwak et al. (Kwak, Ihm, & Ahn, 2001) fabricated solidified lactase/fatty acid esters emulsions and found restricted lactase release and lactose hydrolysis during 8 or 12 days storage in milk. However, the fatty acid esters had off tastes compared to market milk in the sensory tests.

On the other hand, biopolymer-based nanoparticles, especially, zein nanoparticles-based delivery systems have been used to encapsulate a water-soluble antimicrobial peptide nisin and an antimicrobial protein lysozyme. For instance, nisin or lysozyme encapsulated in zein showed sustained release and improved activity at certain pH, temperature, and formulation (Zhong & Jin, 2009; Xiao, Gömmel, Davidson, & Zhong, 2011). Zein is a proline rich protein derived from maize, and the special polyproline

structure and hydrophobic properties provide zein functionalities for drug/nutrient delivery, film forming, foaming, packaging and many other applications in food, medical, and cosmetic industries (Luo & Wang, 2014; Wang, Yin, Yin, Yang, Shi, Tang, et al., 2013; Trezza, & Vergano, 1994; Salerno, Oliviero, Di Maio, & Iannace, 2007).

According to the reported ability to control the release of enzyme and its Generally Recognized as Safe (GRAS) regulatory status, encapsulating lactase in zein nanoparticles is a promising novel method.

Antisolvent precipitation is one of the most commonly used low energy method to fabricate zein solid nanoparticles. Briefly stated, it is driven by the imbalance of solute-solute and solute-solvent interactions when mixing a bad solvent (antisolvent) of a material with its solution in a good solvent leads to the precipitation of solute molecules (Joye & McClements, 2013). Normally, water and aqueous alcohol are applied to plant and milk proteins due to their miscibility over a broad concentration range. The particle formation is similar to crystallization process (Thorat & Dalvi, 2012). The advantages of this method include predictable particle size and polydispersity by controlling crystallization parameters, hardening agent free, and sustained release (Luo & Wang, 2014). In addition, surface modifications of zein nanoparticles by polysaccharides (e.g., pectin) can not only enhance colloidal dispersion stability and functionality but also mucoadhesive properties (Chang, Wang, Hu, Zhou, Xue, et al., 2017; Liu, Fishman, & Hicks, 2007).

Overall, lactase-loaded zein nanoparticles coated with sugar beet pectin (LZP NPs) were fabricated in this study using the antisolvent precipitation method. An optimization

of formulation is included in the fabrication study to gain a more efficient encapsulation and loading of lactase in the zein/sugar beet pectin delivery vehicle. In order to understand the particle formation and interactions between each ingredient, physicochemical properties of L郑 NPs were characterized with various techniques such as scanning electron microscopy (SEM), differential scanning calorimetry (DSC), and fluoresce spectroscopy. Lastly, for the purpose of assessing the effectiveness of the delivery and the prevention of lactase activity in dairy products during storage and after consumption, the release kinetics of lactase from L郑 NPs after storage different temperatures were evaluated, as well as lactose hydrolysis in milk during 14-day storage and *in vitro* digestion.

Literature review

Overview of lactose intolerance

The role of lactase in lactose digestion

Lactase (β -galactosidase) functions as the enzyme to split milk disaccharide, lactose, into monosaccharides, glucose and galactose. In human body, lactase is located in the jejunum of the small intestine mucosa brush border, specifically, at the apical surface of the microvilli (Lomer, Parkes, & Sanderson, 2008). There are three types of lactase deficiency. First, congenital lactase deficiency is present when the patient cannot synthesize lactase since birth and is a very rare condition (Wilt, Shaukat, Shamliyan, Taylor, MacDonald, Tacklind, et al., 2010). The secondary lactase deficiency is due to the damage of brush border (Heyman, 2006). Persistent diarrhea, cancer chemotherapy, or any other celiac or intestinal diseases result in the damage of small intestine mucosa and can lead to reversible secondary lactase deficiency (Wilt, Shaukat, Shamliyan, Taylor, MacDonald, Tacklind, et al., 2010; Heyman, 2006). The third and primary type of lactase deficiency (or lactase nonpersistence) occurs in approximately 70% of the world's population (Heyman, 2006). In this case, lactase synthesis is normal at birth and during infancy, but decreases significantly in mammals after weaning (Wilt, Shaukat, Shamliyan, Taylor, MacDonald, Tacklind, et al., 2010). Thus, lactose intolerance in adults can be considered as “normal” while the “abnormal” lactose tolerance groups are involved from natural selection or gene mutation overtime by the amount of reduced dairy intake in daily diet (Kretchmer, 1972).

Lactose intolerance induced by lactose malabsorption

The terminology, lactose intolerance, is a clinic syndrome that occurs when the lactose consumption exceeds the hydrolytic ability of body lactase caused by lactase deficiency (Swagerty, Walling, & Klein, 2002; Shaukat, Levitt, Taylor, MacDonald, Shamliyan, Kane, et al., 2010). The terminology, lactose malabsorption is the physiological condition that lactose ingestion and absorption are imbalanced (Wilt, Shaukat, Shamliyan, Taylor, MacDonald, Tacklind, et al., 2010). Unabsorbed lactose physically increases the osmotic pressure in bowel thus draws more fluid out of tissue to the bowel. Biochemically, colon bacteria ferment lactose rapidly and produce short chain fatty acids, hydrogen, and other gases (Lomer, Parkes, & Sanderson, 2008). The two phenomena lead to symptoms such as abdominal pain, distension, explosive diarrhea, and excessive flatus (Swagerty, Walling, & Klein, 2002; Kretchmer, 1972). Besides, many patients suffer from nausea, vomiting, and headaches, and some patients have constipation due to the reduced motility of the intestine (Matthews, Waud, Roberts, & Campbell, 2005). Additionally, the toxic agents generated by colon bacteria such as acetaldehyde, ethanol, methane, and peptide/protein toxins may affect signaling of nerve, immune, or muscle systems (Matthews, Waud, Roberts, & Campbell, 2005).

Prevalence of lactose intolerance

The overall lactose intolerance prevalence is affected by many factors such as lactose ingestion, gastrointestinal illness, race and ethnicity, age, etc. and ranges from 2% to 100% in different regions of the world (Swagerty, Walling, & Klein, 2002; Heyman, 2006). The historical use of dairy products results in a genetic difference of lactose

intolerance. Mediterranean, Asians, Blacks, Latinos are predominate groups of lactase deficiency, while northern Europe is the least common group (Heyman, 2006; PubMed Health, 2015). In the United States, the total number of Americans who are lactose intolerant is up to 40 million (Statics Brain, 2017). At age level, maldigestion of lactose can happen in both infant and adult after 0.5-2 h consumption of lactose containing products (Brown-Esters, Mc Namara, & Savaiano, 2012). While it is uncommon for infants to show lactase deficiency before age 2-3, it is common among older children and adolescents (Heyman, 2006). The older age groups between 60-69 years had significantly higher breath hydrogen than younger groups with age less than 50 years old (Rao, Bello, Warren, & Brown, 1994).

Diagnosis of lactose intolerance

A standard lactose intolerance test usually involves with administering an oral lactose load of 1 g/kg body weight, maximum 50 g in 10-20% solution (Maffei, Metz, Bampoe, Shiner, Herman, & Brook, 1977). Hydrogen breath test is the most agreed lactose intolerance diagnosis method due to its specificity and sensitivity of recognizing pulmonary hydrogen produced by colon (Casellas & Malagelada, 2003). Hydrogen is produced after 60-90 min of ingesting lactose and reaches the peak at around 120 min by an adult with lactose intolerance (Maffei, Metz, Bampoe, Shiner, Herman, & Brook, 1977). Lactose tolerant adults produce none or little hydrogen. A hydrogen concentration greater than 20 ppm against baseline is considered to be lactose intolerant (Swagerty, Walling, & Klein, 2002).

Another lactose intolerance test is the blood glucose test. Normally after ingestion of lactose, blood glucose concentration rises up (Levitt, Wilt, & Shaukat, 2013). When blood sugar concentration increases less than 20 mg/dl after lactose intake, the individual is suggested to be lactose intolerant (Law, Conklin, & Pimentel, 2010). However, in some cases where the subjects' stomach emptying is slow, blood glucose concentration seems to be similar to those who are lactose intolerant (Dahlqvist, Hammond, Crane, Dunphy, & Littman, 1963). Moreover, stool tests, biopsy tests of jejunum or duodenum, and gene tests have been applied to identify lactose intolerance (Mattar, de Campos Mazo, & Carrilho, 2012).

Effects of dietary factors on lactose intolerance

Milk and other dairy ingredients and products are the major sources of dietary lactose (Table 1.1, FSANZ, 2010; Canadian Dairy Commission, 2011). Fat, flavors, probiotics and other food ingredients in the dairy products influence the digestibility of lactose and, therefore, lactose maldigestion symptoms (Brown-Esters, Mc Namara, & Savaiano, 2012). Studies have shown skim milk or chocolate milk induces more lactose intolerance symptoms than whole fat milk with a higher amount of breath hydrogen and a higher mean rise of blood glucose (Dehkordi, Rao, Warren, & Chawan, 1995). The possible reason is that fat delays gastric empty and transition time thus slows down lactose fermentation (Dehkordi, Rao, Warren, & Chawan, 1995). Probiotics or yogurt containing live cultures such as *Lactobacillus acidophilus* and *Bifidobacterium sp.* are suggested to enhance lactose digestion and reduce related symptoms by increasing bacterial lactase activity and slowing gastric empty time (Vesa, Marteau, Zidi, Briet,

Pochart, & Rambaud, 1996; Lomer, Parkes, & Sanderson, 2008). In addition, ingestion of cereals and other food solids and higher food temperature prolong gastric empty time and increase lactose exploration to intestine lactase (Lomer, Parkes, & Sanderson, 2008).

Management of lactose intolerance and critics

Besides steadily increasing the amount of lactose intake to develop colon bacteria adaptation, one of the strategies to prevent lactose intolerance is to consume lactose-free diets. However, lactose-free diet is proved to not meet the daily calcium intake requirement by affecting calcium absorption, which could influence bone mineralization and possibly contribute to osteoporosis (Shaukat, Levitt, Taylor, MacDonald, Shamliyan, Kane, et al., 2010; Newcomer, Hodgson, McGILL, & Thomas, 1978). Another promising lactose intolerance treatment strategy is to provide supplemental lactase or probiotics to facilitate body lactose hydrolysis (Ratzinger, Wang, Wirth, & Gabor, 2010). With the growing educational knowledge and self-diagnosis of lactose intolerance, lactose-free products are becoming more and more popular especially among the developed countries. Overall global lactase demanding has grown rapidly over the past few years. A recent report predicted that lactose-free products in US market will reach to a revenue of 700 million dollars by 2020 which was valued at ~490 million in 2016 (Technavio, 2016). Additionally, lactase supplements are reported to be the second most preferred form of lactase (Persistence Market Research, 2018). Lactase as a kind of enzyme is very sensitive to its surrounding environment due to its weakly stabilized structures. Supplemental lactase can be deactivated easily by acidic pH and pepsin in gastric liquid before it promotes lactose hydrolysis in the small intestine. Commercial supplemental

lactase produced by *Kluyveromyces lactis* yeast strains was reported to have an optimal activity at pH 7 and 25 °C and lost most of the activity at pH 4 or 55 °C (Zhang, Zhang, Chen, McClements, 2016). Thus, the development of technologies such as encapsulation to protect lactase activity and deliver supplemental lactase into the small intestine is required to fulfill the gap between the unstable properties of lactase and lactase market demands (Kwak, Ihm, & Ahn, 2001).

Delivery systems of lactase

Encapsulation of lactase is an efficient method to meet the needs of lactose intolerant costumer if the entrapped lactase is withheld during gastric digestion and be released until reaching the small intestine to facilitate lactose hydrolysis. Hence, the coating material and encapsulating method are essential to maintain capsules intact in gastric condition and release lactase quickly in the small intestine by disintegration or swelling. Besides, the size and stability of the lactase-loaded capsules, cost of encapsulating method and materials, the safety of using organic or synthetic materials are important parameters to justice if a method is suitable for lactase encapsulation. Many studies have been done on lactase encapsulation since the 1980s, including liposomes, emulsions, hydrogels, and polymer based microparticles (Kwak, Mijan, & Ganesan, 2014).

Liposomes

Liposomes are aggregates formed by amphiphilic polar lipids such as phospholipids in spherical shape with one or multiple bilayers (Taylor, Weiss, Davidson, & Bruce, 2005). The interior aqueous phase and inner hydrophobic bilayer allow liposomes to

encapsulate both hydrophilic and hydrophobic compounds such as drugs, enzymes, antimicrobials, and pesticides (Walde, & Ichikawa, 2001; Taylor, Weiss, Davidson, & Bruce, 2005). Rao et al. (1994) fabricated lactase encapsulated liposomes with phosphatidylcholine and cholesterol by two different methods, dehydration-rehydration vesicle (DRV) and reverse-phase evaporation vesicle (REV). The study suggested that REV produced lactase liposomes had higher encapsulation efficiency (EE%) of 27.6% and hydrolyzed 20% of lactose in milk during the 20-day storage test, and liposomes formed by both methods hydrolyzed 50% of lactose during *in vitro* digestion. In a later study, Rodríguez-Nogales and López (2006) conducted an optimization study on encapsulation of lactase in DRV liposomes with the same lipids, reporting the enhanced EE to 28 % by comparing the effects of cholesterol:phosphatidylcholine ratio, type of sonicator, and enzyme:lipid ratio. However, conventional liposomes yield low EE in regards to certain drugs and are unstable in aqueous form during storage (Mugabe, Azghani, & Omri, 2006; Williams, 2003). Freeze-dried lactase liposomes with trehalose treatment were fabricated by Kim et al. (1999). The trehalose treatment prevented fusion of liposomes and linkage of the enzyme, and freeze drying enhanced the stability of lactase in storage. The EE was found to be increased to 87 % even after 60-day storage at 17 °C, and the liposomes stayed intact in simulated gastric fluid but were lysed immediately by bile salt to release lactase.

Emulsions

Hydrophilic lactase has also been encapsulated in emulsions. For example, lactase encapsulated in W/O emulsion was fabricated with medium-chain triacylglycerols (MCT)

and polyglycerol monostearate (PGMS) by Kwak et al. (Kwak, Ihm, & Ahn, 2001). Microcapsules were formed when the lipid phase solidified. In this study, EE was achieved to 94.9 % and 72.8 % by MCT and PGMS, respectively. The sensory test of the milk with and without encapsulated lactase showed no difference in sweetness. However, the off-tastes of MCT and PGMS were high after 8 and 5 days storage, respectively. In another study, Kwak et al. (2002) evaluated the *in vitro* stability of lactase-loaded MCT and PGMS microparticles. The two kinds of microparticles hydrolyzed up to 11 and 13 % lactose in simulated gastric fluid, respectively, and up to 68.8 % and 60.8 % of lactose was hydrolyzed in a simulated intestinal fluid. Furthermore, to improve the disadvantages of converting W/O emulsion to powder form and lipid oxidization, lactase was encapsulated in W/O/W emulsions with MCT as the primary emulsion, and WPI coating as the secondary emulsion (Ahn, Lee, & Kwak, 2013). The yield of encapsulation was high up to 99 %. However, the effectiveness of the W/O/W capsules hydrolyzing lactose was limited to 5.26 % lactose per gram of lactase capsules in a simulated intestinal fluid. In addition, S/O/W emulsions constituting of anhydrous milk fat and sodium caseinate (NaCas) were used to incorporate lactase (Zhang & Zhong, 2018). The obtained EE was 75 %. Less than 15 % of lactose in milk was hydrolyzed by lactase capsules after 3-week storage and approximately 60 % of lactose was digested by encapsulated lactase in the *in vitro* digestion.

Hydrogels

Hydrogels are the most studied lactase encapsulation method. Carrageenan, alginate, silica gels, etc. have been used to encapsulate lactase (Zhang, Zhang, Chen, &

McClements, 2016; Traffano-Schiffo, Castro-Giraldez, Fito, & Santagapita, 2017; Nichele, Signoretto, Ghedini, 2011). Cross-linking agents (e.g., metal cations), chemical hardening agents (e.g., glutaraldehyde), disaccharides (e.g., trehalose), or combination with other polymers (e.g., guar gum) are applied to stabilize hydrogel beads or reduce enzyme leakage through macropores (Sriamornsak, 1999). Injecting the lactase-polymer mixture into hardening solutions is the main hydrogel fabrication method. Others include opposite electrostatic attraction and coacervation (Nichele, Signoretto, & Ghedini, 2011). Hydrogels of κ -carrageenan achieved a lactase EE of about 63 %, and the encapsulated lactase displayed higher activity at a range of pH and temperature than free lactase (Zhang, Zhang, Chen, & McClements, 2016). However, due to the existence of large pores, lactase leaked approximately 25% and 45 % over 8 hours at 25 and 37 °C, respectively. In addition, in order to improve the pH stability of lactase against gastric acid, the same research group produced $\text{Mg}(\text{OH})_2$ buffer loaded carrageenan hydrogels (Zhang, Zhang, & McClements, 2017). This technique effectively maintained the internal pH of the gel beads when exposed to stomach condition. Traffano-Schiffo et al. (2017) incorporated lactase in calcium alginate beads and suggested that the inclusion of trehalose, arabic, and guar gums enhanced the thermal stability against drying, freezing, and thawing of beads.

Polymeric nano- and microparticles

Polymeric nano- and microparticles have been considered as a good method to encapsulate lactase since particles made of biodegradable and biocompatible polymers such as, poly(lactic acid) (PLA) and poly(lactide-co-glycolide) (PLGA) are usually semi-

permeable, which ensures the diffusion of small lactose molecules into the particles but the resistance to the diffusion of outward lactase or inward protease (Sheng, He, & Zou, 2014). Solvent emulsification-evaporation and spray drying are the two commonly methods in the production of polymeric particles (Wischke and Schwendeman, 2008). Conventional PLA or PLGA particles are negatively charged and hence have poor mucoadhesion with also negatively charged mucosa membrane (Musumeci, Ventura, Giannone, Ruozzi, Montenegro, Pignatello, et al., 2006; Jackson, Bush, Washington, & Perkins, 2000; Smart, 2005). Therefore, surface modifications have been applied to PLA and PLGA nano- and micro particles. For example, PLA nanoparticles were coated with water-soluble cationic chitosan or wheat germ agglutinin as delivery vehicles of lactase (Sheng, He, & Zou, 2014). The surface modified nanoparticles presented a good stability against enzymatic degradation and a progressed lactose hydrolysis in simulated intestinal juice. In addition, the half-life of the surface modified nanoparticles in the small intestine was significantly improved than conventional PLA nanoparticles. In another study, lactase was encapsulated in PLA nanoparticles that were then filled in hydroxypropyl methylcellulose phthalate capsules (He, Zhang, & Sheng, 2014). The enteric coating achieved lactase activity retention against artificial gastric fluid, about 80 % of activity release in the intestinal fluid, and almost 100 % of lactose hydrolysis in milk. Moreover, lactase-loaded microcapsules were prepared with a pH-sensitive polymer Eudragit L-100 (Squillante, Morshed, Bagchi, & Mehta, 2003). Due to the solubility properties of this polymer (insoluble at pH below 6), there was no lactase release in acidic media and 80 % of lactase was released in pH 6.8 media. Besides synthetic polymers, biopolymer,

chitosan has been investigated as lactase delivery systems. Even though natural chitosan is only soluble in acidic solutions, chemical modification of chitosan allows it to be a suitable coating material by changing its solubility to be water-soluble (Estevinho, Rocha, Santos, & Alves, 2013). Estevinho et al. (2014; 2015) prepared chitosan microparticles containing lactase by spray drying. However, certain loss of lactase activity after spray drying and 60-day storage at 4 °C was observed.

Disadvantages of current lactase delivery systems

There are several disadvantages of the above delivery systems of lactase. (1) Liposomes and emulsions are thermodynamically unstable systems due to the energy needed to create a new interface area (McClements and Rao, 2011). The stability of liposomes or emulsions may change under changes in environmental pH, temperature, or ionic strength (Zhang & Pawelchak, 2000; Guzey & McClements, 2006). The lipid and water phases always tend to separate over time. (2) The usage of a large portion of lipids in liposome fabrication increases fat content in food, and lipid oxidation can occur during storage. (3) Even though EE of lactase has been improved by drying or use of stabilizers, loading efficiency of lactase in liposomes and emulsions is not optimal. (4) Synthetic polymers are not feasible in the food industry, and there are conflicts about the safety of synthetic polymers PLA and PLGA (Xiao, Gömmel, Davidson, & Zhong, 2011; Sheng, He, & Zou, 2014). (5) Organic solvents (e.g. chloroform), spacers (e.g. polyethyleneimine), coupling agents (e.g. glutaraldehyde), and surfactants are widely used in the preparation of the delivery systems mentioned above. These ingredients are generally undesired in food production. (6) Some of the formation methods such as,

ultrasonication requires high energy input and high cost, which could be difficult to scale up.

Biopolymer particles as delivery systems of enzymes

Most of food biopolymers are generally recognized as safe (GRAS) and abundant. Biopolymer-based particles have been reported to be able to encapsulate, protect, and deliver bioactive compounds. In addition, these particles can be fabricated by relatively low cost, low energy, and easily scalable up methods. Therefore, biopolymer nano- and microparticles have been developed to deliver enzymes and peptides. Among enzymes and peptides, two specific compounds, lysozyme and nisin have drawn attention in food packaging and safety areas due to their excellent antimicrobial activities against gram-positive bacteria (e.g. *Listeria monocytogenes*; Fabra, Sánchez-González, & Chiralt, 2014; Xiao, Gömmel, Davidson, & Zhong, 2011). According to these studies, biopolymer-based delivery systems have shown a reasonable EE and loading capacity of enzymes, a controlled release profile over different pH ranges, and enhanced enzymatic activity in food (Wan, Guo, & Yang, 2015; Krivorotova, Cirkovas, Maciulyte, Staneviciene, Budriene, Serviene, et al., 2016; Shamloofar, Hoseini, Kamali, Motalebi Moghanjoghi, & Poorgholm, 2015). The addition of surfactants, stabilizers, cross-linking agents, coating, or co-encapsulation compounds may enhance the effectiveness of the delivery systems (Amara, Kim, Oulahal, Degraeve, & Gharsallaoui, 2017; Xiao & Zhong, 2011). Specific preparation parameters, preparation methods, formulation, pH, and temperature influence the encapsulation and release properties (Zhong, Jin,

Davidson, & Zivanovic, 2009; Jin & Zhong, 2009; Xiao, Davidson, & Zhong, 2011).
Delivery systems based on proteins and polysaccharides are briefly reviewed below.

Protein-based particles as delivery systems

Zein, a hydrophobic protein from corn, is a widely investigated material for incorporating antimicrobials (Li, Yin, Yin, Tang, Yang, & Wen, 2013; Wan, Guo, & Yang, 2015). Zein nano- and microparticles can be made by spray drying or antisolvent precipitation (Xiao, Davidson, & Zhong, 2011; Zhong & Jin, 2009). Sustained release of antimicrobials and improved activity in food have been shown in many studies. For example, spray-dried nisin-loaded zein capsules achieved sustained release of nisin to more than 80% over 72 hours and inhibited the growth of *L. monocytogenes* by 0.47 log CFU/mL more than free nisin in 2% reduced fat milk in the 48-hour test (Xiao, Davidson, & Zhong, 2011). Furthermore, applying surfactants, surface modification, or co-encapsulation of multiple antimicrobials enhance the effectiveness of the delivery systems. For instance, co-encapsulation of nisin, thymol, and Tween 20 in zein microparticles by spray drying dramatically increased EE at certain formulations, and a controlled release kinetics were observed at pH 6 and pH 8. (Xiao, Gömmel, Davidson, & Zhong, 2011). Besides, lysozyme has been encapsulated in zein by supercritical antisolvent precipitation or spray drying in other studies (Zhong, Jin, Davidson, & Zivanovic, 2009; Zhong & Jin, 2009).

Other proteins have been used as a film or coating material for antimicrobials encapsulation. Pea protein isolate films have shown similar properties as those produced from soy protein isolate, WPI or zein (Fabra, Sánchez-González, & Chiralt, 2014). Pea

protein isolate and cornstarch films with lysozyme have shown sustained diffusion in food. The films retained lysozyme activity at 10 °C, but, failed at room temperature (Fabra, Sánchez-González, & Chiralt, 2014). Whey protein isolate and NaCas were studied for antimicrobial films with controlled release of nisin and reduced the population of *L. innocua* in Muller Hinton broth and contaminated cheese, respectively (Ozer, Uz, Oymaci, & Altinkaya, 2016; Cao-Hoang, Chaine, Grégoire, & Waché, 2010).

Polysaccharide-based particles as delivery systems

Polysaccharides are inexpensive materials that can be made into particles by simply spray-drying, cross-linking or complexation with proteins. Noticeably, a low concentration of polysaccharides ($\leq 1\%$, w/v) is essential in nanoparticle formation (Luo, Pan, & Zhong, 2015; Alishahi, 2014; Krivorotova, Cirkovas, Maciulyte, Staneviciene, Budriene, Serviene, et al., 2016). In one study, chitosan-nisin nanoparticles were cross-linked in the presence of tripolyphosphate (Alishahi, 2014). The average particle size was about 86 nm, and the maximum EE was 78.8 %. The chitosan nanoparticles showed a burst release in the first day then a progressed release at a pH range of 2.5 to 7.5. However, the final amount of released nisin varied due the shift of diffusion to diffusion plus erosion release mechanism with the increase of pH. In another study, pectin nanoparticles loaded with nisin were formed through complexation of oppositely charged materials (Krivorotova, Cirkovas, Maciulyte, Staneviciene, Budriene, Serviene, et al., 2016). The degree of esterification of pectin and pH displayed important roles in particle size and dispersion turbidity. Both chitosan and pectin nanoparticles with nisin showed

activity against gram positive bacteria. Furthermore, spray-dried water-soluble chitosan microparticles loaded with lactase are discussed in the previous section.

Techniques of fabricating biopolymer particles

Nano- and microparticle fabrication methods are divided into two types, top down methods and bottom up methods. Top down methods intend to break large particles or droplets into smaller sizes, including homogenization or shearing, sonication, grinding, milling, and extrusion (Zhong & Jin, 2009; Tang, Huang, & Lim, 2003; Cho, Chang, Kil, Kim, & Jang, H.D., 2009; Yadav, Yadav, & Singh, 2012; Neradovic, Soga, Van Nostrum, and Hennink, 2004). Particle size and shape can be controlled by top down methods, but high energy input is required. Besides, there is a limited size range when applying top down methods due to mechanical limitations or “over processing” effect of micro fluidization (Qian & McClements, 2011; Jafari, Assadpoor, He, & Bhandari, 2008). Bottom up methods assist biopolymer molecules to aggregate or assemble into small particles. Antisolvent precipitation, drying, spontaneous assembly, coacervation, phase inversion emulsification, and templating are common bottom up methods (Pan & Zhong, 2016). Bottom up methods produce particles with well-defined structure and controlled size (Shimomura & Sawadaishi, 2001). Energy input in these methods is low and the produced nanoparticles are less contaminated by erosion or residues from bottom up processing equipment than top down methods (Müller, Jacobs, & Kayser, 2001; Thorat & Dalvi, 2012). The production techniques of biopolymer-based, particularly, zein-based delivery systems are discussed below.

Drying method

Drying methods are easy, fast to produce powdered particles and easy to be scaled up. Spray-drying and freeze-drying are two major drying methods to fabricate particles. Spray-drying produces particles with relatively more uniformed structures than freeze-drying (Anwar & Kunz, 2011). However, the high temperature of the spray-drying procedure may result in a loss of activity in heat sensitive compounds (Desobry, Netto, & Labuza, 1997). In this case, freeze-drying is favored. In terms of the fabrication of biopolymer particles, spray-drying is more widely applied. The solution is atomized to fine droplets in spray drier, and then solvent is quickly evaporated by a stream of hot air, resulting in aggregation of solute molecules (Joye & McClements, 2014). Particle properties are controllable by adjusting inlet/outlet temperature, solution concentration, air flow rate, etc. Zein microparticles have been prepared by spray-drying (Xian, Davidson, & Zhong, 2011). Xiao et al. (2011) studied the effects of spray-drying inlet temperature (75, 95, 105, and 125 °C) on the encapsulation properties of nisin-loaded zein particles. The mass yield was found to be lower at a higher inlet temperature, which is possibly caused by the biopolymers becoming more amorphous at a higher temperature. However, specific activity of nisin was higher in capsules produced at high temperature due to the shorter exposure of nisin to high heat.

Antisolvent precipitation

Nanoparticles can be obtained by decreasing the solvent power of a solute with the addition of a miscible antisolvent (Dalvi and Dave, 2009). For hydrophobic biopolymers (e.g. zein and gliadin), the solvent is usually aqueous alcohol and the antisolvent can be

water or supercritical liquid (e.g. supercritical carbon dioxide; Davidov-Pardo, Joye, & McClements, 2015; Zhong, Jin, Xiao, Tian, & Zhang, 2008). On the contrary, water is the solvent for hydrophilic compounds (e.g. whey protein isolate) and alcohol is the antisolvent (Mansouri, Woo, & Chen, 2013). The formation of the solute aggregates is driven by the imbalance between solute, solvent, and antisolvent interactions. When the solute-solute interaction is favored over a critical antisolvent concentration, aggregates are formed (Joye & McClements, 2013).

Four main steps are involved in the generation of nanoparticles by antisolvent precipitation: supersaturation, nucleation, particle growth through coagulation or condensation, and particle agglomeration that may happen in uncontrolled particle growth (Thorat & Dalvi, 2012; Joye & McClements, 2013). Figure 1.1 illustrates the steps of particle formation. The degree of supersaturation and nucleation rate are important for producing small uniform particles. First of all, supersaturation is the main driving force of precipitation (Bristow, Shekunov, Shekunov, & York, 2001). The degree of supersaturation (S) of a single biopolymer is defined as

$$S = \frac{c}{c_{eq}} \quad (1.1)$$

where c is the current concentration of the biopolymer in liquid and c_{eq} is the equilibrium saturation concentration of this biopolymer in solvent/antisolvent mixture (Beck, Dalvi, & Dave, 2010). When c exceeds c_{eq} , supersaturation is achieved. When c further exceeds a critical supersaturation concentration, nucleation starts (Joye & McClements, 2013).

Secondly, nucleation only happens when its driving energy is higher than the energy barrier (ΔG_b , see equation 1.2; Joye & McClements, 2013).

$$\Delta G_b = \frac{16 \times \pi \times \sigma^3 \times V_s^3}{3 \times k_B^2 \times T^2 \times (\ln S)^2} \quad (1.2)$$

where σ is the interfacial tension at the solid-liquid interface, V_s is the volume of a solute molecule, k_B is the Boltzmann constant, and T is the temperature. It is clearly demonstrated that a lower surface tension and a higher supersaturation degree lead to lower energy barrier and thus favor nucleation.

Thirdly, according to Dalvi and Dave (Dalvi & Dave, 2009), the rate of nucleation (J) is defined as:

$$J = N_0 \nu \times \exp(-\Delta G_b) \quad (1.3)$$

where N_0 is the number of solute molecules per volume, ν is the frequency of molecular transport to the solid-liquid interface, and ν is defined as

$$\nu = \frac{k_B T}{3 \pi \alpha_0^3 \eta} \quad (1.4)$$

where α_0 is the mean diameter of the solute molecules and η is the viscosity (Dalvi & Dave, 2009). From equation 1.4, an increase of viscosity results in the decrease of molecule transfer frequency and thus inhibits nucleation rate.

Therefore, any processing parameter that influences solute supersaturation, nucleation, or particle growth will affect the size and polydispersity of the produced particles. Take an example, the Damkohler number, which is the ratio of mixing time to precipitation time ($Da = \tau_{mix} / \tau_{precipitation}$, $\tau_{precipitation} = \tau_{coagulation} + \tau_{condensation}$), is a useful parameter that can be used to evaluate the effectiveness of a mixing process (Matteucci, Hotze, Johnston, et al., 2006). At $Da < 1$, mixing time is reduced and results in greater supersaturation and more rapid nucleation, and precipitation is relatively slow leading to slower particle growth and thus smaller particles (Matteucci, Hotze, Johnston, et al.,

2006). The type of mixing devices and mixing parameter affect mixing time and Reynolds number (Re), which are related to supersaturation and nucleation rate (Zhao, Wang, Wang, Chen, & Yun, 2007; Bristow, Shekunov, Shekunov, & York, 2001). A decreased crystal size was observed with the increased Re when Re was below 2×10^4 in Bristow's study (2001). Beck et al. (Beck, Dalvi, & Dave, 2010) studied the effects of ultrasound and power input of an ultrasonic driven T mixer on several drugs particle formation. The employment of ultrasonication ensured $Da < 1$. Moreover, it was suggested that not only the degree of supersaturation, the solubility of solute in aqueous phase and the polarity of solvent affected nucleation rate and resultant particle size significantly. Compounds with low solubility in aqueous phase diffused fast in solvent with high polarity. On the contrary, compounds that were more soluble in water diffused slower in high polar solvent. This diffusion rate is related to the particle growth at the solid/liquid interface as seen in equation 1.3: a faster diffusion rate results in higher rate of nucleation and smaller particle size.

In addition, as mentioned above, low surface tension at the solid/liquid interface is favored for nucleation in antisolvent precipitation. For instance, the addition of a low amount of Tween 80 prevented zein particle aggregation at pH 4 (Hu & McClements, 2014). 0.008% Tween 80 resulted in particles smaller than 1000 nm, and 0.04% Tween 80 resulted in particles smaller than 100 nm. Meanwhile, the turbidity of the zein colloidal dispersions was very low when the surfactant concentration was higher than 0.04 %. Moreover, other stabilizers such as 2 % polymer polyvinylpyrrolidone or sodium

caseinate prevented zein nanoparticle aggregation at neutral or alkaline pH (Patel, Hu, Tiwari, & Velikov, 2010).

Nanoparticles produced with antisolvent precipitation, particularly zein nanoparticles have shown sustained release controlling abilities in many studies due to the insolubility of zein in water and relatively compacted particle structure. Hence this method usually is good for encapsulation of hydrophobic bioactive compounds where they are dissolved in alcohol and adsorbed or co-assemble to nanoparticle matrix spontaneously during the particle growth process. Such compounds are curcumin, Vitamin D, peppermint oil, fish oil, etc. (Dai, Sun, Li, Mao, Liu, & Gao, 2017; Luo, Teng, & Wang, 2012; Chen & Zhong, 2015; Zhong, Tian, & Zivanovic, 2009). Moreover, modifying zein nanoparticles with stabilizers like polysaccharides (e.g. pectin), surfactants (e.g. Tween 20), and sodium casein enhanced zein nanoparticles stability at pH close to isoelectric point of zein, surface properties or redispersibility (Xiao & Zhong, 2011; Chen & Zhong, 2015; Li, Yin, Yin, Tang, Yang, & Wen, 2013).

Protein-polysaccharide complexes

Protein and polysaccharides form complexes through two different mechanisms. One is covalent conjugates through the Millard reaction. Millard conjugation is usually achieved by dry-heat treatment (Dickinson and Galazka, 1991). This type of complexes has shown improved stability, emulsifying and foaming properties at certain pH, temperature, and ionic strength (Oliver, Melton, & Stanley, 2006).

The second type of protein polysaccharide complexes are formed through predominately electrostatic attraction between two oppositely charged polyelectrolytes

(e.g. positively charged proteins at pH lower than pI and negatively charged polysaccharides; Turgeon, Schmitt, & Sanchez, 2007). The mechanism of this reaction starts from understanding the consequence of mixing protein and polysaccharides. When two different polymers are mixed, two mechanisms could happen, complex coacervation or thermodynamic incompatibility (Turgeon, Beaulieu, Schmitt, and Sanchez, 2003). When there are thermodynamically favorable interactions (electrostatic attraction, hydrophobic interaction, hydrogen bonds, etc.), protein and polysaccharide form soluble or insoluble complexes. The insoluble complex, so called complex coacervation, is the separated concentrated polymer phase of a colloidal mixture, while another phase is the diluted phase (de Kruif, Weinbreck, & de Vries, 2004). Complexation is entropy or enthalpy driven depending on the magnitude of electrostatic interactions (Patino & Pilosof, 2011; Turgeon, Schmitt, & Sanchez, 2007). The soluble or insoluble complex occurs under different pH and ionic strength conditions. Take ovalbumin-carboxymethylcellulose (OVA/CMC, degree of substitution for CMC was 0.7 and 1.2) coacervation as an example (Xiong, Ren, Tian, Yang, Li, & Li, 2017), three pH values are the critical points in the coacervation, pH_c , pH_{ϕ_1} , and pH_{ϕ_2} . At pH_c (4.5 for CMC_{0.7} and 4.7 for CMC_{1.2}), soluble complexes began to form as the colloidal turbidity increased slightly. As pH decreased to pH_{ϕ_1} (4.39 for CMC_{0.7} and 4.2 for CMC_{1.2}), the turbidity increased rapidly, and the microscopic phase separation occurred. The amount of insoluble complexes increasing until reached an optimum pH, pH_{opt} (3.5 for both CMC). Coacervates started to dissociate as further acidification caused by the protonation of CMC and weakened electrostatic attraction. When pH is lower than pH_{ϕ_2} (1.78 for

CMC_{0.7} and 1.26 for CMC_{1.2}), the dissociation/ protonation of CMC was completed. At pH above protein isoelectric point, unfavorable driving forces, steric and electrostatic repulsion, lead to thermodynamic incompatibility between protein and polysaccharides, where the two polymers tend to separate from each other (Patino & Pilosof, 2011). This phenomenon can be expressed by a phase diagram based on Flory-Huggins or depletion interaction theories, where above the binodal curve the mixture system remains homogenous and below the binodal curve, the system may separate into two phases (Doublier, Garnier, Renard, and Sanchez, 2000; Turgeon, Beaulieu, Schmitt, and Sanchez, 2003). In addition to pH, ionic strength plays an important role in formation of the complexes since a higher ionic strength lowers the surface charge density (de Kruif, Weinbreck, & de Vries, 2004).

The functionality studies of protein-polysaccharide complexes are mostly focused on rheological, emulsifying, and gelation properties. In the rheology study of OVA-chitosan coacervation (Xiong, Ren, Jin, Tian, Wang, Shah, et al., 2016), the samples were found to be more elastic than viscous, and a higher salt concentration and OVA/CS ratio resulted in lower elasticity. In terms of the stabilization of emulsions and foams, OVA/carrageenan complexes were found to be able to stabilize foams at pH below pI and residue foam was observed for up to 30 hours (Miquelim, Lannes, & Mezzenga, 2010). Whey protein/xanthan-locust bean emulsions produced stable emulsions and inhibited lipid oxidation at pH 3 and pH 7 (Owens, Griffin, Khouryieh, & Williams, 2018). In regards to gelation properties, protein-polysaccharide hydrogels usually have better characteristics than gels formed by individual protein or polysaccharide including higher

gel strength, good capability of water entrapment, low concentration and low heat or no heat required during gel formation (Le, Rioux, & Turgeon, 2017; Bertrand & Turgeon, 2007). The excellent functionality of protein-polysaccharide complexes leads to its application in encapsulation and preservation of food compounds bioavailability, particularly hydrophobic compounds such as orange oil and fish oil (Jun-xia, Hai-yan, & Jian, 2011; Eratte, Wang, Dowling, Barrow, & Adhikari, 2014).

Structures of common food biopolymers used to fabricate particles

Structures of proteins

Typically, proteins have three types of conformations (Figure 1.2) based on their primary (amino acids sequence), secondary (α -helix and β -sheet), and tertiary structures: globular, partially unfolded or rod-like, and random coil (unfolded) proteins. Under mild denaturation conditions, globular proteins can become partially unfolded (Mezzenga & Fischer, 2013). Globular proteins are closely packed with hydrophobic regions buried inside and hydrophilic regions exposed outside to minimize Gibbs free energy (Anfinsen, 1973). Most of the food proteins are globular such as bovine whey proteins β -lactoglobulin (β -lg), α -lactalbumin (α -lac), and serum albumin; egg white proteins ovalbumin and lysozyme (Nicolai, Britten, & Schmitt, 2011; Stevens, 1991). Random coil proteins can be considered as a polyelectrolyte. This kind of structure exists in linear proteins that are more disordered and flexible, e.g. caseins (Dickinson, 2001). Some proteins have intermediate partially unfolded structures and can change when the environment changes (Mezzenga & Fischer, 2013). A classic example of protein intermediate structure is gelatin. Collagen consists of three peptide chains that form a

triple helical structure (Prystupa, & Donald, 1996). Gelatin is the hydrolysate of collagen and is suggested to have a coil structure over 40 °C but reforms helical structures when cooled down (Prystupa, & Donald, 1996). Above a critical concentration (0.4-1.0%), gelatin partially reverses to triple helices in the junction zone during gelling (Ross-Murphy, 1992). In aqueous solutions, proteins can be present as monomers, dimers, or multimers. When environment changes, e.g. pH, temperature, salt concentration, or with the presence of other biopolymers, proteins can undergo 3-dimensional folding/unfolding and aggregation, 2-dimensional adsorption at interfaces and 1-dimensional fibril formation (Ridgley, Claunch, & Barone, 2012; Mezzenga & Fischer, 2013). Furthermore, protein amino acid sequence, structures, molecular weight, biological properties (e.g. catalysis, affinity), and physicochemical properties (e.g. pI value, thermal stability, amphipathy) affect its gelation, emulsifying, self-assembly, and other functionalities. A few popularly studied animal and plant derived proteins are discussed as follows.

Whey proteins and caseins are two main classes of milk proteins. Whey proteins are globular proteins mainly composed of β -lg, α -lac, and serum albumin. Both β -lg and α -lac contain disulfide bonds; however, the latter doesn't have free thiol group (Nicolai, Britten, & Schmitt, 2011). When heated at a temperature above 65-70 °C, the configuration entropy exceeds in folded whey proteins (mainly β -lg and α -lac) and drives proteins to unfold (Mezzenga & Fischer, 2013). Partially unfolded proteins are more mobile and flexible with internal hydrophobic and thiol groups exposed, causing them to aggregate (Croguennec, T O'Kennedy, & Mehra, 2004). The aggregated whey proteins can form gels when its concentration is higher than a critical gelling concentration (C_g),

and C_g decreases when electrostatic repulsion is weakened by decreasing charge density or increasing ionic strength (Hudson, Daubert, & Foegeding, 2000). Moreover, temperature, pH, salt concentration and salt types affect not only size but also morphology of aggregated particles. For example, when heating temperature or heating rate increases, particle size decreases (Bromley, Krebs, & Donald, 2006). In addition, β -lg aggregates obtained at pH 2.0, 5.8, and 7.0 showed structures shaped in rods, spheres, and worms (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008). Heat treated whey proteins are widely studied to produce emulsions, gels/microgels, as well as to encapsulate compounds (Sarkar, Murray, Holmes, Ettelaie, Abdalla, & Yang, 2016; Hudson, Daubert, & Foegeding, 2000; Zhang & Zhong, 2012).

The composition and structure of caseins have been studied since the early 80s (McMahon & Brown, 1984). Casein micelles and ingredients contain α_{s1} -, β -, κ -, α_{s2} - and γ -caseins (Horne, 2002). κ -Casein is well agreed by scholars for its role in the stabilization of casein micelles (Horne, 2002). At a pH away from pI, glycomacropeptide segment of κ -casein behaves as a charged tail attaches to the casein micelles surface; as pH close to pI, glycomacropeptide structure collapses and lost its ability to balance casein micelle sterically (Horne, 1998; Holt, 1992). Meanwhile, many studies illustrated the important role of calcium phosphate cluster cross-linking sub-micelles of α_s and β -caseins, with details described by Holt (1992). de Kruif et al. (de Kruif, Huppertz, Urban, & Petukhov, 2012) built a similar model as Holt's describing casein micelles with an internal homogeneous collection of small (~ 2 nm) calcium phosphate nanoclusters. The distance between each nanocluster is 18.6 nm, and the casein tails are associated through

weak interactions. Furthermore, the association and dissociation of casein micelles can be changed by thermal, acidic or dissociation agent (sodium dodecyl sulfite, urea, ethylenediaminetetraacetic acid, e.g.) treatments (Anema & Klostermeyer, 1997; Ward, Goddard, Augustin, & Mckinnon, 1997; Lefebvre-Cases, Gastaldi, & de la Fuente, 1998). Monomeric caseins have a flexible random coil structure distinctly different from whey proteins. This allows caseins, particularly β -casein, adsorb on interfaces, with hydrophobic trains and small loops attaching to the oil phase and hydrophilic tails sticking into the water phase (Dickinson, 2001). β -Casein has no cysteine residues but is abundant of proline residues which give β -casein little ordered secondary structures and a large portion of hydrophobic patches (Dickinson, 1999). In addition, β -casein has no intramolecular covalent bond, however, the negatively charged 5 phosphoserine residues play a key role in stabilizing emulsions by electrostatic repulsion and steric restriction but also could destabilize emulsions in the presence of calcium ions owing to its strong binding to calcium (Dickinson, 2001; Dickinson, 2010). α_{s1} -Casein has similar properties as β -casein but contains more negative net charges and 8 phosphoserine residues. Thus, the adsorbed layer of α_{s1} -casein at the interface is presented in the loop configuration (Dickinson, 2010). Caseins are well known for their self-assembly properties and casein micelles are the natural self-assembled protein aggregates. NaCas is a modified casein with excellent solubility in water and therefore has been studied as an encapsulation material (e.g., protein-polysaccharide complex), self-assembly/co-assembly material (e.g. co-assembly with proanthocyanidin), surface modifier (e.g. improving wettability of zein nanoparticles), emulsifier, and foaming agent (Luo, Pan, & Zhong, 2015; Liu, Li, Yang,

Xiong, & Sun, 2017; Wang, Yin, Yin, Yang, Shi, Tang, et al., 2013; Sánchez & Patino, 2005).

Plant derived proteins, in general, are biodegradable, biocompatible, and more economic and environmentally friendly than those derived from animals (Nesterenko, Alric, Silvestre, & Durrieu, 2013; Tapal & Tiku, 2012). Many plant proteins have not only nutritional values but also special properties such as special solubility and/or gelation properties (Young & Pellett, 1994; Nesterenko, Alric, Silvestre, & Durrieu, 2013). Thus, plant proteins extracted from soybean, corn, pea, or wheat have been studied as materials for nano- and microencapsulation.

Maize protein zein and wheat gluten protein gliadin are water-insoluble protein materials. There are four types of zeins (α -, β -, γ -, and δ) and three types of gliadins (α -, γ -, and ω -) according to their variations in amino acid compositions, primary and secondary structures, molecular weight, and solubility (Zhu, Kale, & Cheryan, 2007; Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012). Firstly, both zein and gliadin are rich in hydrophobic proline and zein is also rich in polar glutamine residues (Shukla & Cheryan, 2001; Rombouts, Lamberts, Celus, Lagrain, Brijs, & Delcour, 2009). Polyproline is responsible for the conformation of α -helix with three proline residues per turn (Kogan, Dalcol, Gorostiza, López-Iglesias, Pons, Sanz, et al., 2001). Zein's 3D structure is based on the repeating of α -helices antiparallelly connected by glutamine-rich 'turns' through hydrogen bonds (Figure 1.3. Argos, Pedersen, & Marks, 1982; Matsushima, Danno, Takezawa, & Izumi, 1997). In a good solvent, α -helices of zein transform into antiparallel β -sheet strands. The strands then side by side pack into a

ribbon. Driven by hydrophobic interaction, the ribbon rolls into a ring until the center of the ring is closed. Then new β -sheets start to collapse onto the nuclei, followed by growth to particles (Figure 1.4. Wang & Padua, 2012). In terms of α - and γ - gliadins, their non-repetitive C-terminal domains are rich in α -helices, while repetitive N- terminal domains are rich in β -reverse turns giving them an overall globular conformation stabilized by disulfide bonds (Sato, Matsumiya, Higashino, Funaki, Kitao, Oba, 2015). However, in ω -gliadin, low levels of ordered α -helix and β - sheet structures were suggested, giving ω -gliadin a coil structure (Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012). Secondly, the unique solubility of zein and gliadin allows them to form nanoparticles by an easily controlled, low energy antisolvent precipitation process (Patel & Velikov, 2014). Typically, the method is to induce zein/gliadin precipitation by adding a miscible bad solvent (e.g. water) into the protein solution (with 70- 90% aqueous ethanol, methanol, or propanol, or vice versa; Patel & Velikov, 2014). Zhong and Jin (2009) fabricated zein nanoparticles by shearing zein ethanol solution in deionized water with a high-speed homogenizer. The produced zein nanoparticles had a dimension of 100-200 nm and could be controlled by adjusting the shear rate, ethanol concentration, and zein concentration. Joye et al. (2015) produced gliadin nanoparticles by pouring gliadin extract into water with continuous stirring. The particle size varied from approximately 100-250 nm affected by process parameters like mixing conditions (e.g. shear speed), addition rate and order, solvent/antisolvent ratio, etc. The advantages of using water-insoluble proteins to produce nanoparticles or delivery vehicles are (1) particle size can be controlled easily by controlling process parameters; (2) no surfactants or hardening

agents are required to preserve the particle integrity; (3) due to limited dissolving and slow digestion rate of zein/gliadin, the produced nanoparticles have excellent ability to control sustainable release of encapsulated compound (Patel & Velikov, 2014). The mechanisms and other details about antisolvent precipitation are discussed in the previous section. Last, but not the least, according to current reports, the more hydrophobic, partially aggregated and insoluble of the proteins are, the more it is favored to enhance the stability of emulsions and foams (Binks & Horozov, 2005; Dickinson, 2010). Zein and gliadin are ideal candidates for Pickering stabilizer since they are less soluble in aqueous phase. One study successfully produced colloidal zein stabilized o/w emulsions at a pH range of either above or below its pI value with contact angles close to 90° (de Folter, van Ruijven, & Velikov, 2012). Another study obtained foams stabilized by gliadin nanoparticles with highest foam half-life of ~3200 min at pH 5.8 (Peng, Jin, Tang, Lu, Wang, Li, et al., 2017).

The information about soybean protein structure and functionality has been reported since 1970's (Wolf, 1970). Soybean proteins consist of mainly two proteins, 7 S and 11 S globulins. The two proteins are different in molecular characteristics, structures, function properties, and mechanisms during physicochemical reactions (Utsumi & Kinsella, 1985). Soybean proteins contain quaternary structures of acidic and alkaline subunits (Badley, Atkinson, Hauser, Oldani, Green, & Stubbs, 1975). The subunits associate or dissociate as the disulfide cross-linked bonds change under changing of pH, temperature, and the presence of other association/dissociation agents (e.g. calcium and urea; Wolf, 1970). This behavior allows soy protein to aggregate and gel by heating and

cooling, pH, and metal ionic treatments (Petrucelli & Anon, 1995). In addition, it has been reported soybean proteins possess interfacial and emulsifying properties (Molina, Papadopoulou, & Ledward, 2001). Nano- and microparticles are usually prepared by cold gelation, conservation, spray-drying, and desolvation of soy protein isolate (SPI; Wan, Guo, & Yang, 2015; Liu & Tang, 2013; Tang & Li, 2013). For cold gelation, proteins are unfolded and polymerized by pre-heating, and gels are formed by subsequent cooling down and the addition of multivalent cations to provide bridging forces between polypeptide chains (Zhang, Liang, Tian, Chen, & Subirade, 2012). SPI nano- and microparticles are good for encapsulation of both hydrophobic and hydrophilic bioactive compounds, development of delivery systems, and preparation of Pickering emulsions (Wan, Guo, & Yang, 2015; Liu & Tang, 2013).

Other plant-derived proteins such as pea protein isolate, rice protein, and barley proteins are also studied as materials in nano- and microparticle production at their either native or structurally modified form (Klemmer, Korber, Low, & Nickerson, 2011; Wang, Wang, Chen, & Zhong, 2016; Wang, Tian, & Chen, 2011). These materials are not further discussed here.

Structures of polysaccharides

Polysaccharides are mostly built up by pyranose ring monomers (e.g. D-glucose; Morris, 1986). The variation in monomer structure, molecular weight, etc. results in distinct properties between each species. Polysaccharides can be divided into anionic, cationic, and nonionic according to their ionized groups such as carboxylates, sulfates, and amines and the pK_a value of those groups (Jones & McClements, 2010). Alginate,

pectin, xanthan, carboxymethyl cellulose (CMC), and carrageenan are anionic; chitosan is cationic; while dextran is nonionic (Joye & McClements, 2014; de Kruif, Weinbreck, & de Vries, 2004). The conformation of polysaccharides relies on the linkage pattern and chemical groups (Jones & McClements, 2010). Glycosidic linkage rotation restriction makes linear chain favored (Jones & McClements, 2010). The abundant hydroxyl groups lead to hydrogen bond formation between polysaccharide chains. Then these hydrogen bonds form helical structures in some polysaccharides (e.g., cellulose; Dumitriu, 2004). Specifically, cellulose, chitin, and mannan are extended flat ribbons; alginate and pectin chains are more likely to be buckled ribbons due to the isomeric conversion of pyranose rings, and both form inter-chain junctions with Ca^{2+} as mentioned before in the bridging forces; agar and carrageenan, however, are composed of a coaxial double helix by repeating anhydride bridges (Morris, 1986). The functional properties of polysaccharides are their gelation abilities, emulsifying abilities depending on the amount of structural modification or the presence of protein on the molecular structure (e.g. sugar beet pectin), good bioadhesive abilities given by hydrophilic groups, and their complexation with proteins (Racoviță, Vasiliu, Popa, & Luca, 2009; Drusch, 2007; Liu, Jiao, Wang, Zhou, & Zhang, 2008; de Kruif, Weinbreck, & de Vries, 2004).

Pectin is commercially used in food products for its gelling, thickening, and stabilizing properties (Akhtar, Dickinson, Mazoyer, & Langendorff, 2002). Pectin is the cell wall extracts from citrus, apple and sugar beet (Round, Rigby, MacDougall, & Morris, 2010). Typically, pectin consists of a semi-flexible linear chain of D-glucose connected by α -(1, 4)-glycosidic (Racoviță, Vasiliu, Popa, & Luca, 2009). The degree of

esterification (DE) is important to classify pectin. Low methoxyl pectin with < 50% DE forms gel by calcium-induced galacturonic acid chain cross-linking (Racoviță, Vasiliu, Popa, & Luca, 2009), and high methoxyl pectin with > 50% DE gels differently when pH is below 3.6 and in the presence of > 50% sucrose (Oakenfull & Scott, 1984). The protein associated with sugar beet pectin during industry production plays a key role in pectin emulsifying ability (Siew & Williams, 2008). It was reported that the segments of citrus and beet pectin adsorbed onto oil-water interface contained more proteins than those in the aqueous phase (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003). The same research group (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003) also pointed out hydrophobic acetyl groups improve the emulsion stability by preventing calcium flocculation. Moreover, molecular weight, accessibility of protein and ferulic acid groups, degree of esterification, and degree of acylation work together in pectin emulsification instead of simple relationships (Williams, Sayers, Viebke, Senan, Mazoyer, & Boulenguer, 2005). Pectins with higher molecular weight, higher protein content, and higher ferulic acid content are easier to absorb at oil-in-water interface (Siew & Williams, 2008). A hypothetical cartoon of pectin-stabilized emulsion is illustrated in Figure 1.5 (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003).

Gum arabic (GA) is also a surface-active polysaccharide and has been used in beverages for flavor (e.g. citrus oil) emulsification and encapsulation. It is a heteropolymer of six carbohydrate moieties consisting of β -(1, 3) linkage (Islam, Phillips, Sljivo, Snowden, & Williams, 1997; Osman, Menzies, Williams, Phillips, & Baldwin, 1993). GA also contains a small amount (~2 %) of protein covalently bound to the

polysaccharide chains (McNamee, O'Riorda, & O'Sullivan, 1998). The amphiphilic protein contributes to surface activity of GA and the polysaccharide chain provides electrostatic and steric repulsion for emulsion stabilization (Snowden, Phillips, & Williams, 1987). The structure of GA is mostly agreed with Fincher's "wattle blossom" model, which describes GA molecules with several polysaccharide chains linked to a common protein coil (Fincher, Stone, & Clarke, 1983). This structure is more compact than linear chain structure of pectin, and GA thus has more surface coverage than pectin when absorbing at O/W interface (Nakauma, Funami, Noda, Ishihara, Al-Assaf, Nishinari, & Phillips, 2008). Dickinson et al. (1991) studied the molecular weight effects on GA emulsifying properties and suggested high molecular weight fraction (10% of natural gum) had better emulsion stabilizing ability than low molecular weight fraction. Overall, GA is a suitable material for emulsification and encapsulation (McNamee, O'Riorda, & O'Sullivan, 1998).

Another abundantly studied and applied polysaccharide for delivery systems is alginate. Alginate is linearly composed of (1, 4)-linked β -D-mannuronate (M-block) and α -L-guluronate (G-block) blocks with alternate sequences (Lee & Mooney, 2012). When two G-blocks are arranged side by side, a diamond shaped hole is formed, and it is an ideal shape for calcium binding (the "egg box" theory; George & Abraham, 2006). Native alginate does not gel under thermal treatment (Lee & Mooney, 2012). However, alginate gels can be prepared by ionotropic gelation through cross-linking -COOH groups with multivalent cations (Racoviță, Vasiliu, Popa, & Luca, 2009). Divalent cations Ca^{2+} , Sr^{2+} , Ba^{2+} and trivalent cation Al^{3+} induce alginate gelation yet monovalent

ions and Mg^{2+} do not (Racoviță, Vasiliu, Popa, & Luca, 2009). The type of the ions and block type of alginate (M- or G-) are an essential factor affecting gel stability and quality such as gel strength, permeability, and water uptake (Mørch, Donati, Strand, & Skjåk-Bræk, 2006).

Chitosan is the derivative of deacetylated chitin (Kumar, 2000). It is a linear copolymer of β -(1, 4)-linked D-glucosamine and N-acetyl-D-glucosamine (George & Abraham, 2006). Due to the presence of amine groups, chitosan is the only natural cationic polysaccharide in neutral or basic conditions (Rinaudo, , 2006; Agnihotri, Mallikarjuna, & Aminabhavi, 2004). Chitosan's solubility and the ability of ionotropic gelation are caused by the protonation of $-\text{NH}_2$ in acidic solution (Agnihotri, Mallikarjuna, & Aminabhavi, 2004). The protonation provides electrostatic repulsion that enhances the polysaccharide solubility as well as the positive charge of chitosan chains that interact with anions such as sodium tripolyphosphate (Rinaudo, 2008; Fan, Yan, Xu, & Ni, 2012). The solubility and biocompatibility of chitosan allow its wide application in drug delivery (George & Abraham, 2006). Some of the examples are: antibiotic gastric delivery by freeze-dried chitosan-poly (ethylene oxide) hydrogels, insulin gastrointestinal delivery by alginate/chitosan nanoparticles, and indomethacin/sulphamethoxazole colon delivery by chitosan modified amidated pectin hydrogels. (Patel & Amiji, 1996; Sarmento, Ribeiro, Veiga, Sampaio, Neufeld, & Ferreira, 2007; Munjeri, Collett, & Fell, 1997).

Furthermore, cellulose and its derivatives such as CMC, dextran, starch, and many other native or modified polysaccharides are found to be structurally, economically,

functionally, and biologically friendly for designing delivery systems (Kosaraju, 2005; Liu, Jiao, Wang, Zhou, & Zhang, 2008). These polysaccharides are also not reviewed.

Structures of biopolymer particles

According to the United States National Nanotechnology Initiative (2015), nanostructured materials have a dimension of about 1 to 100 nm and they display different properties from those of bulk materials and single atoms or molecules. In food science, nano- and microparticles have their unique influences on food bulk matrix appearance, viscosity, release kinetics, biological mucoadhesion, etc., and these properties usually change as the particle size changes gradually (Joye & McClements, 2014). The nanoparticles in this thesis are defined as larger than single biopolymer molecule but smaller than 1 μm .

The shape of a biopolymer nanoparticle is usually spherical. It can also be a rod, a fiber, a tube, or laminates when different methods are used (Mai and Eisenberg, 2012). For example, drug-loaded zein nanofibers were prepared by electrospinning and zein-curcumin composite spherical particles were obtained by antisolvent precipitation (Huang, Zou, Li, Jing, Xia, & Liu, 2013; Patel, Hu, Tiwari, & Velikov, 2010). Further, the internal structure of a nanoparticle is crucial to determine the particle functionalities like permeability and release profile. Jones and McClements (Jones & McClements, 2010) categorized three kinds of biopolymer particle internal structures as homogeneous, dispersion, and core-shell. For homogeneous particles, the composition is a single biopolymer (e.g. whey protein microgel particles) or two miscible biopolymers (e.g. mixed gels of pectin and gelatin, Schmitt, Moitzi, Bovay, Rouvet, Bovetto, Donato, et al.,

2010; Tolstoguzov, 1998). Dispersion structured nanoparticles normally have one or more discrete phase dispersed in other continuous phase, e.g. hybrid chitosan nanoparticles loaded in phospholipid (Grenha, Remuñán-López, Carvalho, & Seijo, 2008). The most commonly produced nanoparticle structure is the core-shell structure with one core surrounded by a coating shell. This shell can be one or more layers, e.g. zein nanoparticles coated by alginate or zein nanoparticles coated by NaCas and chitosan double layers (Hu & McClements, 2015; Zhang, Niu, Luo, Ge, Yang, Yu, et al., 2014).

Forces involved in the dispersion stability of biopolymer particles

The stability of a colloidal system depends on the balance between weak attraction and repulsion forces. The main attraction forces are hydrophobic interactions, hydrogen bonds, the electrostatic forces between two oppositely charged entities, and bridging forces. The main repulsion forces are the electrostatic forces between particles of a same type of charge and steric forces. Understanding and manipulating these interactions by changing environmental pH, ionic strength, presence or absence of surfactants or metals are essential for nanostructure design.

DLVO forces

The basic starting theory for colloidal particle stability is the classic Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, that is, the total potential interaction between two globular colloidal particles (F_{DLVO}) is the sum of their electrostatic repulsion force (F_e) and van der Waals attracting force (F_d , see equation 1.5, Mezzenga & Fischer, 2013; Israelachvili, 2011).

$$F_{DLVO} = F_d + F_e \quad (1.5)$$

$$F_d = -\frac{AR}{12D} \quad (1.6)$$

$$F_e = \frac{2\pi\sigma^2 R}{\kappa_B^2 \epsilon_0 \epsilon_r} e^{-\kappa D} \quad (1.7)$$

where A is the Hamaker constant, D is the distance between two particles, R is particle radius, σ is the charge density of the particles, ϵ_0 and ϵ_r are constants, and κ^{-1} the Debye length.

Since van der Waals force is only strong enough within a very short distance (Equation 1.6), while the electrostatic force decreases exponentially (Equation 1.7), there is usually a repulsion peak (F_{max} , energy barrier) in a distance range of approximately 1-5 nm for highly charged surfaces (Israelachvili, 2011). This energy barrier is often higher than thermal energy ($k_B T$, where T is the absolute temperature), and the particles remain apart from each other and stay kinetically stable (Ikeda & Zhong, 2012). When the surface charge density is low, energy barrier can't overcome $k_B T$, particles aggregate slowly overtime. When the energy barrier falls below 0, particles aggregate rapidly. The Debye length is affected by total ionic strength, and the energy barrier of aggregation increases with the decrease of ionic strength (Mezzenga & Fischer, 2013).

Hydrophobic interactions

Israelachvili and Pashley (1984), found additional strong attractive forces between two hydrophobic surfaces in the range of 4-10 nm that caused energy barrier move to a longer separation distance. This attractive force is the hydrophobic force (F_h) and can be described as equation 1.8 within 0-10 nm (Israelachvili & Pashley, 1984).

$$\frac{F_h}{R} = -Ce^{-D/D_0} \quad (1.8)$$

where C and D_0 are constants representing the strength of the interaction and decay length, respectively, and R is the radius of curvature of the mica surface to normalize F_h . Globular proteins have their hydrophilic moiety exposed to water and hydrophobic moiety folded inside the globule. When protein unfolds in solvent or under heat, hydrophobic domains are exposed to water and then hydrophobic forces must be considered. Thus equation 1.5 can be adjusted to

$$F_{DLVO} = F_d + F_e + F_h \quad (1.9)$$

From equation 1.8, it is clear that hydrophobic interaction is similar to electrostatic repulsion that decays exponentially with the increase of distance, and it is highly dependent on colloidal particle size and distance. Thus, the competition between hydrophobic attraction and electrostatic repulsion cannot be neglected between particles with large portion of hydrophobic patches since the two forces can be within the same magnitude (Mezzenga & Fischer, 2013). In addition, hydrophobic force is affected by many factors such as double layer charge potential, surface contact angle, and structure rearrangement. Hence, the calculation and modeling in many colloidal systems are much more complex (Ducker, Xu, & Israelachvili, 1994; Yoon, Flinn, & Rabinovich, 1997; Mezzenga & Fischer, 2013).

Hydrogen bonds

Hydrogen bond is a type of strong (stronger than van der Waals but weaker than covalent bonds) orientational dipole-dipole interaction that atom H^+ forms bonds with electronegative atoms such as O^- , N^- , and F^- (Israelachvili, 2011). Hydrogen bonds play

an important role in biopolymer intra- and inter-molecular structure formation (Jones & McClements, 2010), for instances, protein secondary helix and β -sheet structures, casein micelle nanocluster self-association behavior, protein gelation, and film forming chain to chain binding, interactions between bioactive compounds and proteins, and biopolymer mucoadhesion (Derewenda, Lee, & Derewenda, 1995; de Kruif, Huppertz, Urban, & Petukhov, 2012; Nicolai, Britten, & Schmitt, 2011; Patel, Hu, Tiwari, & Velikov, 2010; Dai, Sun, Li, Mao, Liu, & Gao, 2017; Smart, 2005).

Bridging forces

Another significant interaction force between biopolymer particles is the bridging effect. There are mainly two types of bridging mechanisms. The first type, so called bridging flocculation, is when biopolymer concentration is low, the adsorption of biopolymer on particle surfaces is limited, thus one biopolymer chain may adsorb on two surfaces at a time causing particle aggregation (Jenkins & Snowden, 1996).

The second type is ionic bridging of biopolymers. The most applied agent is divalent cations (e.g. Ca^{2+}) to cross-link polyuronates as previously discussed for alginate and low methoxyl pectin. Alginate and pectin are two classic polyuronates consisting of guluronate and galacturonate residues, respectively (Fang, Al-Assaf, Phillips, Nishinari, Funami, & Williams, 2008). According to Grant et al. (1973), the binding mechanism between Ca^{2+} and alginate can be described as an “egg box” model where Ca^{2+} is surrounded by 2 pairs of guluronate helixes. Low methoxyl pectin has a similar but more complicated interaction with divalent ions due to its randomly distributed binding sites (Fang, Al-Assaf, Phillips, Nishinari, Funami, & Williams, 2008). On one hand, cross-

linking agents produce bridges between biopolymers to form network structures; on the other hand, cross-linking improves biopolymer structures against surrounding environment changes (e.g., pH and temperature), as well as mechanical stress, and thus is widely applied in nano- and microencapsulation (Janes, Calvo, & Alonso, 2001; Draget & Taylor, 2011).

Excluded volume interactions

Excluded volume interaction or steric repulsion is caused by restricted biopolymer segment regions that exclude other segments to fit in, and thus the real polymer chain is extended or swollen (Ikeda & Zhong, 2012). The biopolymer chain radius of gyration (R_g) and its steric interaction is influenced by the interaction between solvent and biopolymers (Minton, 2005). This can be expressed by excluded volume parameter (v) and Flory-Huggins parameter (χ_{12} ; Ikeda & Zhong, 2012; Mai & Eisenberg, 2012).

$$\chi_{12} = \left(\frac{z}{k_B T}\right) \left[\varepsilon_{12} - \frac{1}{2}(\varepsilon_{11} + \varepsilon_{22}) \right] \quad (1.10)$$

$$v = v_c \left(\frac{1}{2} - \chi_{12} \right) \quad (1.11)$$

where z is the number of nearest neighbors per repeat unit in the polymer, ε_{12} , ε_{11} , and ε_{22} are the interaction energies of solvent-solute, solvent-solvent, and solute-solute respectively, and v_c is a constant. When solvent quality is good, $\chi_{12} < 1/2$, $v > 0$, the interactions between solvent and biopolymer are more favorable, and the chain is extended; when solvent quality is bad, $\chi_{12} > 1/2$, $v < 0$, the interactions between biopolymer itself are more favorable, so the chain is more compact (Ikeda & Zhong, 2012; Mai & Eisenberg, 2012). In addition, from equation 1.10, it is obvious that thermal

energy or temperature is a factor that influences solvent quality. A higher temperature results in a stronger excluded volume interaction. Excluded volume is essential for biopolymer interactions and stability such as thermal incompatibility of proteins and polysaccharides resulting in phase separation (larger excluded volume can lead to lower compatibility) and steric repulsion stabilization (Jenkins & Snowden, 1996; Tolstoguzov, 1991).

Chemical cross-linking

Chemical cross-linking mechanisms in proteins and polysaccharides are covalent bonding involved with protein amino residues, carboxyl, and hydroxyl groups (Peters & Richards, 1977). Heating and denaturation lead to WPI protein intra-molecular cleavage and inter-molecular rearrangement of disulfide bonds (Zhang & Zhong, 2012). Enzyme transglutaminase (TGase) can transfer an acyl group from glutamine to a ϵ -lysine and form ϵ -(γ -glutamyl)-lysine bridge (Færgemand, Murray, & Dickinson, 1997). This reaction is widely used in milk protein cross-linking due to enzyme's general label-friendly characteristic. In addition, aldehydes, most commonly glutaraldehyde, are utilized to cross-link protein amino groups and polysaccharide hydroxyl groups, e.g. gliadin, gelatin, chitosan, and alginate (Joye, Nelis, & McClements, 2015; Berger, Reist, Mayer, Felt, Peppas, & Gurny, 2004; George & Abraham, 2007).

Characterization of biopolymer particles

Particle size, morphology, and polydispersity

Particle size and morphology affect optical properties of colloidal systems (Pan & Zhong, 2016). The appearance of nanoparticle dispersions is important for the production

of beverages. More turbid dispersions might be preferred in dairy drinks, while more transparent dispersions might be preferred in juices and some fortified drinks. Take emulsions as an example. Conventional emulsions have relatively large droplets with a dimension similar to light wavelength ($r \approx \lambda$); and therefore, the scattering of light is strong and emulsions appear to be turbid or opaque (McClements & Rao, 2011). On the other hand, nano- and microemulsion droplets are much smaller ($r \ll \lambda$), and they scatter light relatively weakly, thereby optically appearing to be clear or slightly turbid. Next, systems composed of smaller particles are more stable to gravitational separation (Joye, Davidov-Pardo, & McClements, 2014). The less polydispersed particles are, the more stable the systems are against Ostwald ripening of emulsions (Dickinson, 2010). Then, inherent high surface area allows nanoparticles to easily adhere to mucosa, transport to cell, and penetrate through Peyer's patches and the enterocytes (Hecq, Amighi, & Goole, 2016). The morphology studies conducted by scanning electron microscopy (SEM), transmission electron microscopy (TEM), or other techniques, provide information about the external or internal structure of nanoparticles (Pan & Zhong, 2016). These methods are also ways to confirm or investigate the mechanism of nanoparticle formation.

Surface properties

The surface charge properties of nanoparticles is usually measured for zeta-potential, which is not identical to the surface potential (ψ_0 ; Walstra, 2003). Zeta-potential can be used to estimate the electrostatic force and implies the stability of a system. Theoretically, particle aggregation is unlikely to occur when zeta-potential is higher than $|20|$ mV (Gonzalez-Mira, Egea, Garcia, & Souto, 2010). In addition, surface

hydrophobicity (H_0) is an important parameter in estimating the hydrophobic interactions in particle formation (Pan & Zhong, 2016). Both two surface properties are also valuable parameters in verifying the successful assembly of individual materials and estimating the overall configuration of nanoparticles. For example, the zeta-potential of zein-curcumin particles decreased with the increased amount of curcumin on particle surface (Patel, Hu, Tiwari, & Velikov, 2010); and a lower H_0 corresponded to a greater surface coverage of NaCas on zein nanoparticles (Chen & Zhong, 2014).

Thermal properties

Thermal behaviors, such as glass transition, crystallization, melting, or pasting of starch, as well as heating capacity of a material or enthalpy change parameters, are important to analyze the thermal stability of individual and mixed materials, the internal structures of nanoparticles, and the miscibility between two materials (Pan & Zhong, 2016; Traffano-Schiffo, Aguirre Calvo, Castro-Giraldez, Fito, & Santagapita, 2017). For instance, a right shifted endothermic peak of a compound indicates improved thermal stability (Liu, Wang, Sun, McClements, & Gao, 2016). According to the presence of glass transition point, crystallization point or the trend of the heat flow curve (e.g. smooth), the state of a compound can be recognized (e.g. glassy, crystallized, or amorphous; Traffano-Schiffo, Aguirre Calvo, Castro-Giraldez, Fito, & Santagapita, 2017; Hu, Huang, Gao, Huang, Xiao, & McClements, 2015). Additionally, the change of glass transition temperature (T_g) between individual biopolymers and their mixtures indicates that they are miscible (Pan & Zhong, 2016). The absence of a melting or crystallization

peak after encapsulation can be used to determine if the compound is well dispersed into the encapsulating material (Pan, Zhong, & Baek, 2013).

Release mechanisms of biopolymer particles as delivery systems

To evaluate the release profile, bioavailability and bioaccessibility of bioactive ingredient, simulation models of gastrointestinal (GI) tract digestion conditions have been developed. Some of the features in different parts of simulated GI tract are summarized in Table 1.2 (Minekus, Alming, Alvito, Ballance, Bohn, Bourlieu, et al., 2014; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005; Joye, Davidov-Pardo, & McClements, 2014; Liu, Fishman, Kende, & Ruthel, 2006; Wyatt, Horn, Gee, & Johnson, 1988; Gibson, Willems, Reading, & Collins, 1996; Asvarujanon, Ishizuka & Hara, 2005; Sugano, Yamada, Yoshida, Hashimoto, Matsuo, & Kimoto, 1988). The release mechanism of a nanoparticle depends on its composition and structure. Proteins can be digested by proteases and acidic environment of gastric fluid, lipids can be digested by gastric lipase in stomach and pancreatic lipase with assistance of bile in small intestine with assistance of bile; while polysaccharides may be hydrolyzed in colon (Joye, Davidov-Pardo, & McClements, 2014; Singh, Ye, & Horne, 2009). The encapsulated bioactive compounds are expected to be absorbed and ideally released at different locations within GI tract. Many nutrients like vitamins and peptide/proteins (insulin, e.g.) are targeted to small intestine, while other compounds (apigenin, e.g.) and drugs (indomethacin, e.g.) with anticancer or inflammation effects can be targeted to treat colon disease (Wang, Wang, Chen, & Zhong, 2016; Lohcharoenkal, Wang, Chen, & Rojanasakul, 2014). Encapsulated compounds could be released through four types of

mechanisms (Figure 1.6; Jones & McClements, 2010). (1) The bioactive components diffuse into aqueous environment driven by concentration gradient. Diffusion is more likely to happen when the biopolymer wall mesh size is large. (2) Compound diffusion induced by swelling or shrinking of the biopolymer matrix. Such biopolymers could be polysaccharides or WPI. (3) Biopolymer erosion caused by chemical or enzymatic degradation or dissociation of non-covalent bonds can induce the release of encapsulated compounds. (4) Burst release can be triggered by physical disruption by shear or dissociation of the biopolymer matrix. In addition, studying release kinetics of a delivery system helps to evaluate compound delivering effectiveness, testify if the encapsulation meets design requirements, and imply the release mechanisms of the delivery vehicle. Some colloidal systems showed release kinetics following a specific order and can be presented in mathematic models. For instance, β -carotene in emulsion droplets coated with rice proteins followed zero order release kinetics in simulated intestinal digestion where the slope indicated the release rate (Wang, Wang, Chen, & Zhong, 2016. Sarkar et al. (2016) revealed the structure changes and release mechanisms of WPI microgel stabilized Pickering emulsions by combining methodologies of measuring droplet size, size distribution, zeta-potential, collecting confocal laser scanning micrographs, determining protein molecular weight distribution by protein electrophoresis, and release kinetics of free fatty acids. These studies suggested the important role of remaining WPI microgel particle fragments in stabilizing O/W emulsions during simulated gastric digestion and significant influence of bile salts and free fatty acids on displaying those fragments hence droplet coalescence in simulated intestinal digestion. The overall *in vitro* and even *in vivo*

digestion studies on evaluating delivery systems are rather complicated and are not further discussed in this section.

Future perspectives

Biopolymer nano- and microparticles are promising structures to improve and control food products sensory related properties such as texture and appearance, develop nutraceutical target delivery systems with high bioactivity and bioavailability , and prolong food shelf life and solve food safety issues. Future researches are expected to focus on (1) the understanding of quantitative structure-function relationships for the accurate design of nanoparticles; (2) the discovery of novel food grade biopolymers; (3) the development of novel nanoparticle fabrication methods that are easy to scale up; and (4) the understanding of toxicity and health issue related to nanoparticle ingestion and digestion (Ikeda & Zhong, 2012; Thorat & Dalvi, 2011; Hoet, Brüske-Hohlfeld, & Salata, 2004).

Hypothesis

Many studies have proven that biopolymer particles including zein nanoparticles are suitable for the encapsulation of bioactive hydrophobic and hydrophilic compounds with characteristics for delivery systems with desired functionalities. Previous studies showed that spray-dried zein particles can be applicable to incorporate hydrophilic compounds (e.g. nisin) with sustained release (Xiao, Gömmel, Davidson, & Zhong, 2011; Xiao & Zhong, 2011). Surface modification of zein nanoparticles by coating with polysaccharides can improve colloidal system stability and dispersibility (Chen & Zhong,

2014). Hence, the hypothesis of this study is that zein nanoparticles coated with sugar beet pectin (SBP) can be used to encapsulate lactase, and the produced nanoparticles can retain lactase activity during storage and release lactase to the target site as a potential delivery system to control lactose hydrolysis in milk.

Objectives

The overall goal of this project is to produce lactase-loaded zein/sugar beet pectin nanoparticles (LZP NPs) with a controlled release during storage of milk and simulated digestion of lactose in milk. The specific objectives are to fabricate lactase-encapsulated nanoparticles using antisolvent precipitation, characterize particle physicochemical properties, investigate possible mechanisms of the nanoparticle formation, estimate release kinetics of lactase from the fabricated particles, and characterize the hydrolysis of lactose in milk during storage and simulated digestion

CHAPTER II

MATERIALS AND METHODS

Materials

Encapsulating materials. Commercial lactase product Maxilact® LGX 5000F in liquid preparation was kindly provided by DSM Food Specialties (Delft, Netherlands). Zein powder was purchased from Sigma-Aldrich Corp. (St. Luis, MO, USA). Sugar beet pectin (SBP) powder GENU® BETA pectin was kindly provided by CP Kelco U.S. Inc. (Atlanta, GA, USA). According to the vender, the SBP product had ~1% protein, 20.9 % degree of acetylation, ~ 50% degree of esterification, and a molecular weight of 67 kDa.

Enzymatic assay reagents. O-nitrophenyl- β -D-galactopyranoside (ONPG) lactase assay reagent and 2-nitrophenol (ONP) were obtained from Thermo Fisher Scientific (Waltham, MA, USA) and Sigma-Aldrich Corp., respectively.

Protein assay reagents. Mini-PORTEAN® precast TGX gels (4-20%), 10 $\times$ Tris/Glycine/SDS Buffer, Bio-Safe™ Coomassie G-250 Stain, 2 $\times$ Laemmli Sample Buffer, and Precision Plus Protein™ dual color standards used in protein electrophoresis were bought from Bio-RAD Laboratories, Inc. (Hercules, CA, USA). 2-Mercaptoethanol (β -ME, electrophoresis grade) was bought from Fisher Scientific (Pittsburgh, PA, USA). Pierce™ Coomassie (Bradford) Protein Assay Kit was purchased from Thermo Fisher Scientific.

Materials used in release and *in vitro* digestion studies. Grade A whole milk (Vitamin D fortified) and fat-free milk (skim milk) were obtained from Publix grocery store (Knoxville, TN, USA). Glucose assay reagent (HK), pepsin from porcine gastric mucosa, lipase from porcine pancreas, and pancreatin from porcine pancreas were purchased from Sigma-Aldrich Corp. Pectinase from *Aspergillus niger* (3797 U/g; one

unit was defined as the amount of enzyme required to release one μmol of galacturonic acid from polygalacturonic acid per minute at pH 4.0 at 25 °C according to the product description) was obtained from MP Biomedicals (Solon, OH, USA).

Other chemicals were obtained either from Fisher Scientific or Sigma-Aldrich Corp.

Methods

Characterization of lactase product

Enzymatic activity assay

Enzymatic assay followed the method described by Park, Santi, & Pastore (1979), with modifications. 200 μL of suitably diluted enzyme solution in PBS at pH 7.0 (a dilution factor corresponding to absorbance at 410 nm of 0.2-2.0 was considered as suitable) was added into 1 mL of 1% (w/v) ONPG substrate solution pre-equilibrated to 37 °C, followed by incubation at 37 °C in a shaking water bath for 5 min. 1.8 mL of 10% Na_2CO_3 was added subsequently to stop the reaction. The final test solution was measured for absorbance at 410 nm with a UV-vis spectrophotometer (model Evolution 201, Thermo Scientific Co., Waltham, MA, USA). A reagent blank and an enzyme blank were used to correct the absorbance. The reagent blank was prepared with 200 μL PBS and 1 mL of the ONPG substrate solution. The enzyme blank was prepared with 200 μL of an enzyme solution and 1 mL of PBS. Both blanks were subjected to same treatments as above. The standard curve was built with different concentrations of ONP against absorbance. One Unit of enzyme activity was defined as the amount of enzyme that was required to hydrolyze one μmol of ONPG per minute under the conditions described above ($\mu\text{mol}/\text{min}$). Enzyme activity of an unknown sample was determined as follows.

$$\text{Activity (U/mL)} = \frac{\frac{\text{Corrected Abs}_{410 \text{ nm}}}{0.0705} \times \text{Volume of test solution} \times \text{Dilution factor}}{\text{Reaction time} \times \text{Total volume of actase sample added}} \quad (2.1)$$

where 0.0705 is the slope of the standard curve in $\text{mL}/\mu\text{mol}$.

Effects of pH and ethanol on enzymatic activity

To understand possible denaturation of lactase during nanoparticle preparation and other assays, the effects of pH and ethanol on lactase activity were tested. In the pH test, 1 mL of the liquid commercial lactase after 100-fold dilution was added into 9 mL PBS, followed by adjusting pH to 2.0-10.0 with 1 M and 0.5 M HCl or NaOH and mixed at room temperature (RT, ~21 °C) for 15 min on a stirring plate at 600 rpm. Subsequently, 200 µL of the lactase solution was added into the 1% ONPG substrate solution previously adjusted to the same pH and the activity was determined. In the ethanol test, 1 mL of the commercial lactase was dispersed into 50 mL 35% ethanol, stirred in an ice bath for 4 hours, and equilibrated to RT, followed by activity measurement. Three independent replicates were measured in pH and ethanol tests.

Protein concentration determination

Protein concentration was measured with PierceTM Coomassie (Bradford) Protein Assay Kit. Commercial liquid lactase was diluted for 100 times before reacting with Coomassie reagent. 30 µL of diluted lactase sample was added into 1.5 mL Coomassie Reagent after incubating at RT for 10 min, absorbance at 595 nm was measured for the sample and corrected with reagent blank consisting of 30 µL PBS and 1.5 mL Coomassie reagent. Standard curve was built by plotting absorbance at 595 nm of each bovine serum albumin standard versus the concentration (ug/mL).

Fabrication and formulation optimization of LZIP NPs

Fabrication of LZIP NPs

SPB stock solutions as prepared by dissolving 1% (w/v) SPB powder in 10 mM PBS (pH 7.0) at 80 °C followed by stirring at RT for 2 h. The stock solution of zein was prepared at 5% w/v in 70% (v/v) aqueous ethanol at RT. The stock solutions were placed in a refrigerator overnight to ensure fully hydration and were centrifuged at 11,952 *g* for 30 min (Sorvall RC-5B plus, Sorvall, Newtown, CT, USA) to remove insoluble impurities the next day. The final pH of SBP solution was adjusted to pH 7.0 with 1 M NaOH. 1% (w/v) CaCl₂ stock solution was also prepared. The stock solutions were diluted to the desired concentrations for formulation optimization (Table 2.1).

Liquid commercial lactase preparation (1 mL) was mixed with 25 mL SBP solution followed by stirring at 600 rpm for 5 min. Then 10 mL zein solution was poured into the lactase-SBP mixture. After stirring at 600 rpm for 30 min to facilitate nucleation and particle growth, 2 mL CaCl₂ solution was added to the mixture followed by stirring for another 5 min to cross-link SBP. The final pH of the mixture was 6.8-7.0. All the beakers were placed in an ice bath while stirring to prevent enzymatic activity loss by the heat generated from magnetic stir plate. Then the mixture was centrifuged at 1,912 *g* for 30 min to remove insoluble and large particles. For the purpose of removing free molecules and collecting lactase capsules, the supernatant collected from the last step was centrifuged at 23,426 *g* for 30 min and precipitates were collected as encapsulated lactase. Three times of sedimentation-redispersion cycles using PBS were used to wash off ethanol and free molecules with DI water, and the ethanol residue is considered to be

less than 10⁻⁴% (w/w; Binks & Horozov, 2005). The final precipitates were re-dispersed in 20 mL PBS. Subsequently, freshly prepared samples were stored in a 4 °C refrigerator and used within 48 h. Separate samples were freeze-dried (model VirTis Advantage Plus EL-85, SP Scientific, Gardiner, NY, USA) for SEM and DSC measurements.

Formulation optimization

The effect of zein:SBP and CaCl₂:SBP mass ratios were studied with a constant liquid lactase volume of 1 mL. The zein:SBP mass ratios were tested for 4:1, 2:1, 3:2, 1:1. From preliminary experiments, an SBP concentration higher than 0.4% resulted in relatively high turbidity and large particle size (> 1 μm), hence a fixed SBP concentration of 0.4% was selected for all tests. The effect of CaCl₂:SBP mass ratios of 1:5, 1:10, 1:20, and 0:1 was studied with four zein:SBP mass ratio sets. The optimal formulation was selected based on encapsulation properties, as well as particle size, zeta potential, turbidity, and the appearance of dispersions after two-week storage at 4 °C. The optimal formulation was used in other experiments. Three independent replicates were tested for this experiment.

Characterization of LZP NPs

Encapsulation efficiency (EE, %)

The supernatant collected above by centrifugation at 23,426 g for 30 min was used to determine the amount of free lactase was with the ONPG method. The EE was then calculated using equation 2.2. Three independent replicates were measured.

$$EE(\%) = \frac{\text{Total amount of lactase added (U)} - \text{Free lactase (U)}}{\text{Total amount of lactase added (U)}} \times 100 \quad (2.2)$$

Particle size, polydispersity index (PDI), and zeta potential

Freshly prepared dispersions without free molecules as described in the nanoparticle preparation section, were measured for Z-average diameter, PDI, and zeta potential using a zetasizer instrument (model ZEN3600, Malvern Instruments Ltd., Malvern, Worcestershire, UK). To prevent multiple-particle scattering, dispersions were diluted until particles reached to the smallest values with DI water and the pH of the diluted dispersions was adjusted to pH 3.0-8.0 with 1 M or 0.5 M HCl or NaOH. Three independent replicates were measured.

Turbidity

Turbidity was measured according to Chen's method (Chen and Zhong, 2015). Freshly prepared samples were measured for absorbance at 600 nm with the above UV-vis spectrophotometer with DI water as a blank. Three independent replicates were measured.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

In order to confirm the successful encapsulating of lactase in LZP NPs, protein electrophoresis was conducted. SBP was not included in the SDS-PAGE analysis due to its limited amount a protein and high viscosity at high concentration caused difficulties with detecting protein fractions in SBP from preliminary experiments. To enable detecting of lactase, 5 mL lactase of the liquid preparation was used to prepare LZP NPs using the method mentioned above. The washed precipitates were re-dispersed in 2 mL DI water. A separate sample was prepared for 1 mL LZP NPs re-dispersion mixed with 0.2 mL of 1% (w/v) EDTA solution and reacted at RT for 15 min to eliminate possible Ca^{2+} cross-

linking preventing particle disintegration. A zein-only dispersion was prepared by pouring 10 mL of 1.5% zein into 32 mL DI water, followed by stirring at 600 rpm for 30 min and removing ethanol and collecting particles as above.

LZP NPs, EDTA-treated LZP NPs, zein colloidal dispersions, and commercial lactase were diluted with a loading buffer for 2, 2, 2, and 10 times, respectively. The loading buffer was prepared by mixing β -ME and Laemmli sample buffer at 1:19 volume ratio. The samples were briefly mixed with a vortex mixer (model S8233-1, American Scientific, Columbus, OH, USA) and then boiled for 5 min. The boiled samples were centrifuged at 8944 *g* (model Sorvall Legend Micro 21 R, Thermo Fisher Scientific, Langenselbold, Germany) for 5 min and 10 μ L of the supernatant was added to the gel well, as well as the protein standard marker. The electrophoresis was carried out under a constant current at 200 V and 400 mA for 30 min using a Mini-PROTEAN precast gel in 10 times diluted Tris buffer. To visualize proteins, the gel was stained with Coomassie Brilliant Blue R250 buffer for 10 min and then rinsed with 50% methanol for 10 min. The gel was soaked in DI water and placed in refrigerator overnight before photographing. The image contrast was adjusted for a clearer resolution. Protein molecular weight was determined by measuring relative mobility (R_m =protein migration distance from top of the gel/distance between top of the gel and tracking dye) of the samples and calculated using the slope of the standard curve (Nielsen, 1998). The standard curve was built by plotting log molecular weight of the standard marker versus R_m . The SDS-PAGE images were printed, and the distance was measured manually with a ruler. The relative amount of lactase and zein in LZP NPs was calculated by comparing

densitometric peak area of each protein band (Gupta, Khan, & Macritchie, 1993). The major peaks and peak areas of the gel plots of L郑 NPs, individual lactase, and zein were determined using software ImageJ 1.52a (National Institutes of Health, USA). Two independent replicates were run for SDS-PAGE and one suitable image was chosen to present in this thesis.

Morphology

Scanning electron microscopy (SEM) images of freeze-dried commercial lactase, freeze-dried zein/SBP (郑) NPs and L郑 NPs powders were collected for morphology study. 郑 NPs were fabricated by pouring 10 mL of 1.5% zein into 32 mL of mixture previously prepared with 25 mL of 0.4% SBP solution and 2 mL of 1% CaCl₂ solution. Free molecules and ethanol were removed by sedimentation-redispersion cycles with centrifuging force of 23,426 *g*. The collected NPs were re-dispersed in 20 mL DI water and freeze-dried. The samples were immobilized on double-sided carbon tape that was attached to a sample stub before imaging using a SEM microscope (model LEO 1525, SEM/FIB Zeiss Auriga, Oberkochen, Germany). Two independent replicates were used in SEM image collecting and suitable clear images were analyzed.

Surface hydrophobicity (H_0)

8-anilino-1-naphthalene sulfonic acid (ANS) is a probe that binds to hydrophobic patches of protein surface (Cardamone & Puri, 1992). Zein colloidal dispersions, SBP, lactase, 郑 NPs, and L郑 NPs were subjected to H_0 determination. Zein colloidal dispersions and 郑 NPs were prepared using the same method in protein electrophoresis and SEM image section. To determine protein concentration in dispersions, zein

dispersions, ZP NPs, and LZP NPs were dissociated by adjusting pH to 11.0 for the Bradford assay. The protein dispersion samples at pH 7.0 were then diluted to a protein concentration gradient of 0.0001, 0.001, and 0.01% (w/v). For SBP-only solution, the protein concentration was low, and the 1% SBP stock solution was diluted to 0.0001, 0.001, and 0.01% with PBS. Samples were adjusted for pH from 3.0 to 8.0 using 1.0 or 0.5 M NaOH or HCl. The ANS solutions were prepared at 8 mM in 10 mM PBS and adjusted to pH 3.0 to 8.0 as well. 10 μ L ANS working solution was added into 2mL of a sample at the same pH, followed by vortex mixing and reaction for 30 min at RT avoiding light to induce ANS interaction with the protein hydrophobic patches. After the reaction, the samples were collected for fluorescence emission spectra from 400 to 600 nm with an excitation wavelength of 370 nm using a fluorospectrophotometer (model RF-1501, Shimadzu Corp., Tokyo, Japan). The emission slit was set to 10 nm. The H_0 was calculated as the slope of the fluorescence intensity versus protein concentration. Two independent replicates were measured.

Thermal properties

Differential scanning calorimetry (DSC) was conducted to study thermal properties of particles. Freeze-dried zein NPs, lactase, and LZP NPs samples were prepared as above. To better characterize properties, the 0.4% w/v SBP solution in NP preparation was also freeze-dried. The DSC instrument was a model Q2000 system (TA Instrument, New Castle, DE, USA). 1-2 mg of each sample was added in aluminum low mass pans (tZero, Midvale, UT, USA) and heated from -50 to 200 $^{\circ}$ C at 10 $^{\circ}$ C/min. The flow rate of

dry nitrogen was 20 mL/min. An empty sealed aluminum pan was used as a reference. Two independent replicates were tested.

Molecular forces involved in LZP NP formation

Dissociation tests were conducted following a literature method with some modifications (Zhang, Liang, Tian, Chen, & Subirade, 2012). 1 mL of 0.2, 0.4, 0.6, 0.8, and 1% (w/v) of SDS, Urea, or EDTA was reacted with 1 mL of freshly produced LZP NP dispersions to study the possible contribution of hydrophobic, hydrogen-bond, and ionic bridging forces, respectively, on nanoparticle formation. The mixture of 1 mL PBS and 1 mL LZP NP dispersion was used as the control. The mixtures were reacted for 30 min before determining turbidity and fluorescence emission spectra of the dispersions.

Following the method of Corradini et al. (Corradini, Demol, Boeve, Ludescher, & Joye, 2017), the protein conformational changes were investigated by analyzing fluorescence emission spectrum of two intrinsic probes, tryptophan and tyrosine. The emission spectrum of samples dissociated with SDS, urea, and EDTA were collected using the above spectrofluorometer. The excitation wavelength for Try and Tyr was 280 nm, and the emission wavelength was from 250 to 420 nm with the slit width of 20nm. Two independent duplicates were tested.

Evaluation of LZP NPs as a delivery system

Release kinetics of lactase from LZP NPs in PBS

5 mL freshly prepared LZP NP dispersions and the same amount of free lactase calculated from EE were added into 15 mL PBS at pH 7.0, respectively. For samples with

free lactase, the volume was made up to 15 mL with PBS. Three sets of the samples were placed in a cooler at 4 °C or a water incubator shaker at 25 and 37 °C with a shaking or stirring speed of 150 rpm, respectively. 1 mL sample was collected at 0, 5, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, and 312 h followed by centrifuging at 23,426 *g* for 3 min and then the activity of the supernatant was measured using the ONPG method. After 14 days, 4 ml dispersions from each temperature treatment were mixed with 1 mL of 0.05% (corresponding to total amount of 200 U) pectinase solution followed by incubation in 37 °C incubator shaker (150 rpm) for 10, 20, 30, 60, and 120 min. 1 mL mixture was collected at each corresponding time point and the released enzymatic activity was determined after centrifugation as above. Independent duplicate samples were tested.

100 % activity was the total amount of lactase in 1 mL L郑 or free lactase sample.

The activity retention or release was calculated by the following equation

$$\text{Activity retention (or release)\%} = \frac{\text{Amount of lactase retained (or released) in 1 ml mixture at the time point}}{\text{Total amount of lactase in 1 ml mixture}} \times 100 \quad (2.3)$$

Lactose hydrolysis in milk during storage

The lactase induced lactose hydrolysis tests were conducted according to Zhang et al.'s method with modifications (Zhang & Zhong, 2018). Encapsulated lactase or free commercial lactase was added to 20 mL whole milk or skim milk at a level of 50 U/mL. The volume amount of plain ZP NP dispersions and PBS corresponding to L郑 NPs and free lactase, respectively, were added in milk as controls. Additional PBS was added in the samples to gain a total volume of 21 mL. The mixtures were vortex mixed and placed in refrigerator at 4 °C for 5 min and were considered as 0 week. 5 mL of each sample was

collected after 0, 1, 2, and 3 weeks storage in a refrigerator at 4 °C. Then the samples were heated at 100 °C for 5min to deactivate the enzyme. After cooling down in an ice bath, the samples were adjusted to pH 4.6 to precipitate casein. Supernatants were collected after centrifuging at 17,530 *g* for 10 min. The amount of glucose in the supernatants was measured with glucose assay reagent (HK) using the method described in the product instruction. The final glucose amount of the samples with LZP NPs and free lactase were corrected with the samples containing ZP NPs and PBS, respectively. The determined glucose amount was then converted to the corresponding amount of hydrolyzed lactose. 100% lactose was the total lactose content in milk, which was measured by hydrolyzing all the lactose in 5 mL whole or skim milk to glucose with 5 mL commercial lactase at 37 °C. To ensure complete lactose hydrolysis, glucose concentration in the samples above was measured every 30 min until it stopped rising (2 h-reaction was sufficient in this experiment). The glucose content in lactose hydrolyzed milk was measured using the same method mentioned above. The total produced glucose was corrected with initial glucose content in milk that was then converted to lactose content. The storage test was conducted for two independent replicates.

Lactose hydrolysis in milk during in vitro digestion

To evaluate the ability of LZP NPs to hydrolyze milk lactose *in vitro*, whole or skim milk containing lactase capsules or free lactase was added/adjusted to simulated gastric fluid, simulated small intestinal fluid, and simulated colonic fluid one by one. First, 15 mL simulated gastric fluid consisting 0.1% pepsin in 10 mM PBS solution at pH 2.0 was prepared before adding 2 mL milk. Then the reaction was started when

encapsulated or free lactase at a level of 50 U/mL was added. The total mixture volume was made up to 18 mL with pH 2.0 PBS and the final pH was about 3.0. The same volume of plain ZP NPs and PBS as LZP NPs and free lactase, respectively, were added to milk and conducted *in vitro* digestion along with test samples as controls. The samples were incubated in a shaking incubator (Model CLASSIC C-76, New Brunswick Scientific Co., INC., Edison, NJ, USA) at 37 °C for 2 h with a stirring speed of 150 rpm, 1 mL samples were collected, and the enzyme was immediately deactivated by heating in 100 °C water bath for 5 min. The glucose produced was measured using the method described in the storage test section above. After simulated gastric digestion, the remaining mixtures were then shifted to simulated small intestinal conditions by adjusting pH to 7.0 and dissolving with 0.3% pancreatic lipase and 1.0% pancreatin (w/v). Subsequently, the mixtures were incubated at 37 °C for 2 h and then 1 mL of each sample was collected at the end-time point to determine glucose content. 5 mL simulated colonic fluid with 1% (w/v) pectinase (total amount of 200 U) at pH 5.0 was subsequently added in the mixture resulting in a final mixture pH of approximately 6.5 and incubated for 8 h. 1 mL samples were collected at 2, 4, 6, and 8 h, respectively. The glucose content in the samples was measured. The amount of produced glucose in milk with LZP NPs and free lactase was corrected with the amount of glucose in milk with ZP NPs and PBS, respectively. In *in vitro* test, 0 % of lactose was considered to be digested at 0 h. Two independent replicates were tested.

The percentage of hydrolyzed lactose was calculated using the equation below.

$$\text{Hydrolyzed lactose (\%)} = \frac{(\text{Amount of glucose in test sample (g)} - \text{Amount of glucose in control (g)}) \times 342.3}{\text{Total amount of lactose in milk (g)} \times 180} \times 100 \% \quad (2.4)$$

where 180 and 342.3 are the molar mass of glucose and lactose, respectively.

Statistical analysis

All experiments were conducted in two or three independent replicates and data was reported as mean $\pm$ standard deviation (SD). One-way ANOVA tests were applied to compare the similarity within groups using SSPS software (IBM® SPSS® Statistics 24.0 for Windows, IBM Corp., Armonk, NY, USA). The confidential level was set to be 95%.

CHAPTER III

RESULTS AND DISCUSSION

Fabrication and formulation optimization of LZP NPs

Characteristics of commercial lactase product

The selection of Maxilact® LGX5000F lactase product was referred to a previous study that reported its relatively high purity and high thermal stability (Zhang, 2016). However, some changes were observed in this product due to different production batches and the use of its liquid form in this study. Briefly, Maxilact® LGX5000F lactase was derived from yeast strain *Kluyveromyces lactis*. *K. lactis* lactase is the most commonly used industry lactase in dairy production due to its natural habitat in milk (Kim, Ji, & Oh, 2004). It was reported that lactase from *K. lactis* had optimal activity at pH 6.6-7.0 and very low activity at its isoelectric point around 5.5 (Zhou & Chen, 2001). The pH stability of commercial lactase used in this thesis is shown in Figure 3.1, where lactase had an optimal activity at pH 7.0 and lost its activity completely at pH 5.0 and below or pH 10.0.

Other properties of the commercial lactase are summarized in Table 3.1. In the simulated NP preparation conditions, lactase activity in 35% ethanol was 26,800 U mL⁻¹. This number was not significantly different from lactase activity measured in pH 7.0 PBS ($P > 0.05$). Therefore, 35% ethanol had little effect on lactase activity within a reasonable time (at least 4 hours).

To estimate the purity of the commercial lactase, its protein concentration and molecular weight (MW) distribution were determined. The protein concentration of the aqueous lactase product was determined as 3.5%, which was in agree with the Safety Data Sheet provided by the manufacturer (1-10%). Figure 3.2 shows lactase molecular

weight distribution based on SDS-PAGE. At least 6 clear bands were observed: the major bands with MW of 81.1 and 106.7 kDa and bands with lower MW distributed at 14.2, 18.2, 34.9, and 54.3 kDa, respectively. The reported molecular weights of purified *K. lactis* monomers were 95.3 and 125.4 kDa (Tello-Solís, Jiménez-Guzmán, Sarabia-Leos, Gómez-Ruíz, Cruz-Guerrero, Rodríguez-Serrano, et al., 2005). Another study reported one lactase protein subunit at 135 kDa (Dickson, Dickson, & Markin, 1979). The slight differences between literature reports and current results may be contributed by glycosylation degree of the enzyme or measurement conditions (Tello-Solís, Jiménez-Guzmán, Sarabia-Leos, Gómez-Ruíz, Cruz-Guerrero, Rodríguez-Serrano, et al., 2005). The smaller protein proportions could be impure proteins which agreed with the report of Tello-Solís et al. (2005).

Formulation optimization for fabrication of LZP NPs

The purposes of formulation optimization were to study the role of each material in nanoparticle formation, obtain the best encapsulation conditions for possible scaling up, and gain the scientific phenomenon under a series of experiments as significant as possible. Encapsulation efficiency (EE), particle size, particle polydispersity index (PDI), zeta potential, and dispersion turbidity are important parameters to evaluate the resultant nanoparticle dispersions. High encapsulation efficiency, small particles, low PDI, high magnitude of zeta potential, and low turbidity are favored for the present study. Characteristics of dispersions as affected by zein:SBP and CaCl₂: SBP mass ratios at neutral pH are summarized in Table 3.2.

EE as affected by formulation

Both zein:SBP and CaCl_2 :SBP mass ratios worked together on influencing EE. At the absence of calcium ion, EE decreased as zein level decreased ($P < 0.05$) indicating the important role of zein in L郑 NPs fabrication. The interactions between zein and lactase was stronger than the interactions between SBP and lactase due to the larger hydrophobic portion of zein while a very hydrophilic property of SBP (Hu, Huang, Gao, Huang, Xiao, & McClements, 2015). When ionic bridging force between SBP molecules was induced by adding calcium, EE of four zein:SBP mass ratios increased significantly ($P < 0.05$). For groups with 2:1 and 3:2 zein:SBP mass ratios, the treatment with a CaCl_2 :SBP ratio of 1:5 resulted in the highest EE of 90.3% and 93. %, respectively. The increased EE indicates the enhanced surface structure of pectin on the NPs, which is in a good agreement with literature (Luo, Teng, & Wang, 2012). Furthermore, the amount of zein still highly influenced EE when calcium was added. At the lowest zein concentration (zein:SBP=1:1), EE was low comparing to other groups at all calcium addition level. At the highest zein concentration (zein:SBP=4:1), EE increased to 92.1% and 91.2% with 1:20 and 1:10 CaCl_2 : SBP mass ratios, respectively; however, EE decreased to 89.4% with 1:5 CaCl_2 : SBP mass ratio. Excess cross-linking by calcium can reduce the efficiency of SBP molecules forming compact structures on ZP NPs (Fraeye, Duvetter, Doungha, Van Loey, & Hendrickx, 2010).

Particle size, PDI, zeta-potential, and turbidity as affected by formulation

CaCl_2 : SBP mass ratio overall had more significant influences on particle size, PDI, zeta-potential, and turbidity than zein:SBP mass ratio. The addition of calcium increased

particle size dramatically for approximately 1.4- to 1.6-fold in groups with 1:1, 3:2, and 2:1 zein:SBP mass ratios. Some not showing a trend. The more calcium added, the larger the particles in these three groups indicating more absorption of pectin on LZP NPs. For the group with 4:1 zein:SBP mass ratio, Z-average diameter at 1:5 CaCl_2 : SBP mass ratio was similar the particles produced without CaCl_2 ($P > 0.05$). The latter opposite result was consistent with the decreased EE of the group with a zein:SBP mass ratio of 4:1 possibly due to the formation of plain ZP NPs with excessive zein. A decreased PDI of the samples with 4:1 and 1:5 zein: SBP and CaCl_2 : SBP mass ratios, respectively, comparing to those without calcium addition, further confirmed this hypothesis. In other test groups, PDI was generally reduced by the addition of more calcium ion. PDI is related to stability of colloidal dispersion against depletion flocculation, and a more uniformed size distribution leads to a more stable system (Tuinier, 2003; Tuinier, Rieger, & De Kruif, 2003). Therefore, calcium induced bridging forces seems to contribute to the stabilization of LZP NP dispersions. This hypothesis is discussed more in the next section. In terms of zeta-potential, there was no significant difference amount treatments ($P > 0.05$). Moreover, since particle dimension is one of the factors (other factors include particle concentration, wavelength of light, length of optical patch, etc.) affecting light scattering and therefore turbidity (Kleizen, De Putter, Van der Beek, & Huynink, 1995), the turbidity of LZP NP dispersions generally showed a similar trend as particle size.

Optimal formulation for fabrication of LZP NPs

The highest EE was obtained in two samples with no statistical difference: zein:SBP and CaCl_2 :SBP mass ratios of 4:1 and 1:20, respectively ; and zein:SBP and

CaCl₂:SBP mass ratios of 3:2 and 1:5, respectively. Except insignificantly difference in PDI of the two samples, the latter showed significantly smaller particles and lower turbidity. Hence, the formulation with zein:SBP mass ratio of 3:2 and CaCl₂:SBP mass ratio of 1:5 was selected for further experiments.

To further optimize lactase loading, 1 and 5 mL lactase liquid sample preparation was mixed with 10 mL of 1.5% zein solution, 25 mL of 0.4% SBP solution, and 2 mL of 1% CaCl₂ solution, and the EE of 5 mL lactase treatment (96.8%) was higher than 1 mL lactase treatment. Dispersions prepared with 5 mL lactase were then used in following characterizations.

Physicochemical characteristics of LZP NPs

Confirmation of lactase encapsulation using SDS-PAGE

The SDS-PAGE electrophoresis of LZP NPs, EDTA-treated LZP NPs, lactase, and zein is shown in Figure 3.2. The MW of lactase is discussed previously. For zein, two major bands were observed at 23.7 and 18.4 kDa respectively. This result agrees with the reported MW of α -zein corresponding to bands at 21-25 kDa and 19 kDa (Shull, Watterson, & Kirleis, 1991). Bands corresponding to both lactase and zein major bands were found in LZP NPs, which confirms the coexistence of lactase and zein in LZP NPs. In the denaturing electrophoresis, proteins disulfide bonds and regions associated through hydrophobic interactions are separated into subunits by β -ME and SDS, respectively (Nielsen, 1998). Other weak interactions are disrupted by heating above protein denaturation temperature. No band shift or new band was observed in the SDS-PAGE indicating the interactions between each compound in the LZP NPs were weak forces other than covalent bonding (Rawel, Rohn, Kruse, & Kroll, 2002). EDTA chelates calcium thus breaks ionic bridging forces between SBP molecules and ensure particle dissociation in the analysis (Zhang, Liang, Tian, Chen, & Subirade, 2012). There was no band shifting between NPs with and without EDTA, and the fusion between each major bands in native LZP NPs became slightly clearer in the EDTA-treated sample. Furthermore, the relative amount of lactase and zein in LZP NPs was evaluated by densitometry. Bands in LZP NPs corresponding to lactase preparation (106.6, 81.1, 54.3, and 34.9 kDa) and zein (23.7 and 18.4 kDa) were quantified and the relative ratio of lactase:zein in LZP NPs was 2.37, which is higher than the original lactase (0.18g

protein):zein (0.15g) ratio. This can partially result from the observation that impurities in commercial lactase (low MW fractions) were not present in NPs (Figure 3.2). Overall, the SDS-PAGE analysis verified the fact of lactase being encapsulated in the NPs.

Morphology of samples

SEM images demonstrated in Figure 3.3 reveal particle surface morphology and nanostructure. Freeze-dried commercial lactase sample had spheroidal or elliptical particles and interparticle matters unresolved by SEM contrasting particular structures of NPs (Figure 3.5). Plain ZP NPs exhibited both spherical and irregular shapes with relatively uniform particle size of approximately 460 nm. The observation of unclear boundary is in good agreement with zein colloidal particles coated with gum arabic (Dai, Sun, Wei, Mao, & Gao, 2018). The freeze-dried L郑 NPs were mostly spherical and some appear agglomerated. SEM results prove the success of nanoparticle fabrication.

Particle size and surface properties of L郑 NPs

Z-average diameter and surface properties (zeta-potential and H_0) of L郑 NPs, ZP NPs, and their components were measured at pH 3.0-8.0 to understand particle structures. Figure 3.4 shows the Z-average diameters of L郑 NPs and ZP NPs. The size of ZP NPs varied from 268.7 to 309.0 nm at pH 5.0 to pH 8.0, and the largest size was observed at pH 6.0 which is close to zein's isoelectric point (~6.2 according to Figure 3.5). Very large dimensions of ZP NPs were found at pH 3.0 and 4.0 with diameters of 4,975 and 1,610 nm, respectively (not shown in Figure 3.4). The dramatically increased particle size may be caused by the reduced solubility of pectin since its pK_a ranges from pH 3.0-4.0

(Chen & Zhong, 2015; Jones, Lesmes, Dubin, & McClements, 2010). The size of L郑 NPs at pH 3.0 and 4.0 was all smaller than 600 nm ($P < 0.05$). This may be due to the additional positive charge provided by lactase at pH 3.0 and 4.0 leading to a stronger binding of SBP. In addition, Z-average diameter of L郑 NPs reduced from 528.0 to 328.6 nm as pH increased from pH 3.0 to 6.0 contributed by increased solubility of SBP. At pH 7.0, surface charge of the three components become all negative and the electrostatic attraction between molecules is limited. Therefore, L郑 NPs had the largest Z-average diameter of 571.4 nm at pH 7.0. Interestingly, when pH was further increased to 8.0, the particles had the smallest diameter of 312.9 nm. Since the electrostatic repulsion between lactase, zein, and SBP is even stronger at pH 8.0 and zein is more soluble at alkaline pH, L郑 NPs could be partially dissociated at pH 8.0 (Pan & Zhong, 2016). Moreover, the diameters of L郑 NPs at pH 5.0, 6.0, and 7.0 were higher than ZP NPs ($P < 0.05$) suggesting the success of incorporating lactase into ZP NPs.

As shown in Figure 3.5, the charge of zein and lactase changed from positive to negative along with the increase of pH, and their zero zeta-potential was observed at about pH 5.0 and pH 6.2, respectively. The results are consistent with the literature (O'connell & Walsh, 2007; Patel, Bouwens, & Velikov, 2010). The zeta-potential of pectin was negative at pH 3.0-8.0 and had higher magnitude at a higher pH. These results are consistent with the literature (Dashevsky, 1998; Patel, Bouwens, & Velikov, 2010; Li, Fang, Al-Assaf, Phillips, & Jiang, 2012). Additionally, ZP NPs and L郑 NPs had a similar zeta-potential profile as SBP, which suggest the formation of nanoparticles with shell of SBP (Hu, Huang, Gao, Huang, Xiao, & McClements, 2015). Zeta-potential of

LZP NPs at pH 7.0 was -16.6 mV. Usually, colloids with zeta-potential magnitude of 20 mV is likely unstable against aggregation (Gonzalez-Mira, Egea, Garcia, & Souto, 2010.). Steric repulsion resulting from SBP on NP surface is significant to the stability of LZP NPs.

Besides zeta potential, H_0 is an important parameter to evaluate particle surface properties. H_0 reflects particle functionality and behaviors such as protein folding/unfolding and protein adsorption rate in emulsification (Delahaije, Gruppen, Giuseppin, & Wierenga, 2014). Higher H_0 means more available hydrophobic groups on a particle surface to contact with the polar aqueous phase (Xiong, Wang, Zhang, Wan, Shah, Pei, et al., 2016). Extrinsic fluorescence probe ANS is used to detect these surface available hydrophobic groups (Bhattacharya, Jain, Bhasne, Kumari, & Mukhopadhyay, 2011). For individual zein NPs, H_0 decreased as pH increased (Figure 3.6). The resultant high H_0 at pH lower than 7 are due to better solubility of zein at acidic pH. Zein NPs partially dissolved and disintegrated exposing the inner protein structures to the polar aqueous environment at lower pH 3.0-6.0 (Chen & Zhong, 2016). Hence, more available hydrophobic patches were able to bind to ANS and led to a high emission intensity. Lactase showed very high hydrophobicity at pH 4.0 and 5.0 and then decreased dramatically at pH 6.0 to 8.0. Like globular proteins, lactase monomer is a compact protein with hydrophobic regions folded inside (Pereira-Rodríguez, Fernández-Leiro, González-Siso, Cerdán, Becerra, & Sanz-Aparicio, 2012). At pH close to lactase pI, lactase denatures and partially unfolds with hydrophobic regions exposed to aqueous environment, and therefore, H_0 increased (Mezzenga & Fischer, 2013). Furthermore,

since SBP is very hydrophilic, and the H_0 was thus very low at the entire pH range of 3.0-8.0. Both ZP and LZP NPs showed similar decreasing trend when pH increased. H_0 of ZP NPs is very high at pH 3.0 and 4.0 showing similar trend as its particle diameter. This may be caused by the separation of SBP and revealed hydrophobic patches of zein molecules. The magnitude of H_0 of ZP NPs was significantly higher than zein colloidal particles since zein molecules in ZP NPs could bind to the protein moiety of SBP directly and still be available to bind ANS unlike highly aggregated zein molecules in zein NPs limiting the exposure of sites to ANS. LZP NPs showed H_0 between lactase, ZP NPs, and SBP at pH 3.0-5.0. The results gave insights that SBP covered on the NPs and the hydrophobic groups of zein and lactase were blocked by pectin coating to restrict the binding with ANS. As electrostatic force changed to be repulsive at pH 6.0-8.0, H_0 became higher than ZP NPs or lactase implying that binding forces could shift from electrostatic interaction to hydrophobic interaction.

Lastly, Figure 3.7 shows the appearance of individual and mixture samples at pH 3.0 to 8.0 before and after overnight incubation at 4 °C. Zein colloidal dispersions are known to have poor stability to aggregation at pH close to its pI value (~pH 6.2) due to limited electrostatic repulsion force between the particles (Patel, Bouwens, & Velikov, 2010). Coating zein NPs with polysaccharides to provide sufficient repulsion forces has shown improved dispersibility and stability of zein colloidal dispersions (Hu and McClements, 2015; Hu, Huang, Gao, Huang, Xiao, & McClements, 2015; Chen & Zhong, 2016). Consistent with these studies, zein NP dispersions (Figure 3.7(b)) precipitated at pH 6 after overnight storage while ZP NP dispersions were stable at pH

6.0. However, as discussed above, ZP NPs were unstable at pH close to pectin or carboxyl pK_a value. Precipitates were observed at pH 3.0 and 4.0 after storage. In terms of lactase, as expected at lactase pI value pH 5.0, precipitates appeared and lactase dispersion was very turbid at pH 4.0 and 6.0. Interestingly, there was no precipitation in LZP NP dispersions at pH 3.0-8.0. The turbidity of LZP NPs at pH 7.0 was higher than at other pH values which agreed with the particle size; however, this sample remained stable for up to two weeks. In summary, the appearance of LZP NPs supported the results of particle size, zeta-potential, and H_0 and suggested the interactions between lactase, zein, and SBP improved the stability of lactase and zein.

Thermal property of LZP NPs

DSC is a good method to assess nanoparticle internal structures by comparing the thermodynamic properties of individual ingredients and/or their mixtures (Podlogar, Rogač, M.B. & Gašperlin, 2005). The absence or shift of a material's glass transition (T_g), melting, or crystallization peaks usually indicates the good miscibility of two materials or the encapsulation of a bioactive compound (Pan & Zhong, 2016; Dai, Sun, Li, Mao, Liu, & Gao, 2017). Figure 3.8 shows the DSC thermograms of freeze-dried LZP NPs, zein, SBP, and lactase powders from -50 to 200 °C. Zein and the protein content in SBP started to melt at around 60 and 50 °C with endothermic peaks at 93.34 and 103.79 °C, respectively. Zein also showed a glass transition temperature of 161.22 °C, which is consistent with around 160 °C in another study (Patel, Bouwens, & Velikov, 2010). Commercial lactase sample had three melting points at 123.23, 151.56, and 187.20 °C. The multiple endothermic peaks in lactase may be a result of different lactase subunits

and impure proteins (Figure 3.2). The heat flow of LZP NPs showed one endothermic peak at 130.03 °C, while other peaks of the individual compounds were disappeared indicating that lactase was well dispersed in the zein/pectin matrices. In addition, this peak was at a temperature higher than those in zein, SBP, and the first peak of lactase samples implied the improved thermal stability of the nanoparticles. The absence of T_g in zein indicates a possible good miscibility between zein, protein portion of SBP, and lactase.

Interaction forces and possible mechanisms of LZP NPs formation and stabilization

Dispersion turbidity as affected by dissociation agents

To understand the interaction forces between LZP NPs, dissociation agents SDS and urea were applied to disrupt hydrophobic interactions and hydrogen bonds, respectively (Lefebvre-Cases, Gastaldi, Vidal, Marchessau, Lagaude, Cuq, et al., 1998), and EDTA acted as a metal chelating agent to identify the role of calcium in forming NPs (Zhang, Liang, Tian, Chen, & Subirade, 2012). The influences of the three chemicals on LZP NPs dispersion turbidity are shown in Figure 3.9. The turbidity decreased sharply with the increase of SDS concentration indicating hydrophobic attraction is one of the main forces to form the NPs. The addition of urea initially reduced the turbidity at 0.2% and 0.4% concentrations, followed by increases at 0.6-1.0% urea. This indicates there was a limited amount of hydrogen bond between LZP NPs (Liu, Li, Yang, Xiong, & Sun, 2017). Moreover, 0.2 % EDTA dramatically reduced the turbidity of LZP NP dispersion demonstrating that even a small amount of Ca^{2+} was essential to serve as a bridging agent to form NPs and maintain the stability of NPs.

Fluorescence spectra of dispersions as affected by dissociation agents

Effects of SDS, urea, and EDTA on fluorescence spectra of LZP NP dispersions are presented in Figure 3.10. Aromatic amino acids Tyrosine (Tyr, polar), Tryptophan (Trp, hydrophobic), and phenylalanine (Phe, hydrophobic) are intrinsic probes of proteins commonly detected by a spectrofluorometer (Lakowicz, 1999). The excitation wavelength and peak emission intensity for Tyr, Trp, and Phe residues are $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 275/304 nm , 280/350~358 nm , 257/283 nm, respectively (VanScyoc, Sorensen, Rusinova, Laws, Ross, & Shea, 2002). Usually, Trp acts as an acceptor of Resonance energy transfer and interactions from Phe and Tyr, therefore, in proteins contain Trp, Phe and Tyr are undetectable (Corradini, Demol, Boeve, Ludescher, & Joye, 2017). Additionally, two types of non-radiative processes may induce protein fluorescence intensity reduction: internal conversion of the energy of excited ^1La state into the vibrational manifold of the indole ring and quenching caused by the interaction between Trp residue and other surrounding chemical groups; such reactions could be molecular rearrangement, energy transfer, collision quenching or ground state complexation (Corradini, Demol, Boeve, Ludescher, & Joye, 2017; Zhang & Zhong, 2012). The detected peaks around 290 nm in the three spectra were possibly due to the disrupted particle structure with hydrophobic regimes exposed in solvent which causes the insufficient transfer of Tyr (Lakowicz, 1999). In SDS-treated the samples, the λ_{max} of Trp red shifted to a greater extent with the increasing SDS concentration, suggesting the increased polarity around Trp residues (Zhang & Zhong, 2012). The lowered emission intensity is caused by the binding of hydrophobic patches by SDS, which resulted in less available Trp in the aqueous system.

The fluorescence emission intensity decreased dramatically, and Tyr and Trp peaks slightly red shifted from 288 to 298 nm and 332 to 336 nm, respectively, with only 0.2% urea treatment. No significant changes were observed from 0.2-1.0% urea addition. On the one hand, changes in Tyr emission peak and intensity suggest hydrogen bonds affected LZIP NPs conformation. On the other hand, the decreased emission intensity was caused by amino acid residue quenching by exposed groups such as $-NH_3$ after the disruption of hydrogen bonds; the red shifts of Tyr and Trp indicate the exposure to the polar environment of the two residues (Lakowicz, 1999). For EDTA-treated samples, there was no shift of λ_{max} indicating ionic bridging had no influence on protein structures, and a lower intensity was also observed at a higher EDTA level. It is likely that Tyr and Trp were quenched by EDTA since there are four carboxyl groups complexing one EDTA molecule (Kim, Thiessen, Bolton, Chen, Fu, Gindulyte, et al., 2016).

Possible mechanisms of LZIP NPs formation

According to physiochemical properties of particles presented previously, a possible LZIP NPs formation mechanism is suggested in Figure 3.11. At neutral pH, zein molecules precipitate and form nuclei rapidly when zein solution is poured into the lactase and SBP mixture solution. During agitation, nuclei grow when zein molecules diffuse to the solid/liquid interface; spontaneously, lactase and the protein patches of SBP bind to zein driven by electrostatic interactions, hydrogen bonding, and hydrophobic forces. Then cross-linking between SBP molecules was induced by the addition of Ca^{2+} while lactase loaded zein nanoparticles were entrapped in SBP shell. Electrostatic and

steric repulsion forces provided by pectin coating enable the stability of the colloidal dispersion.

Evaluation of LZP NPs as a delivery system

Release kinetics of LZP NPs

The release kinetics of lactase from LZP NPs was studied at three temperatures, 4, 25, and 37 °C, corresponding to refrigeration, room, and human body temperature, and pH 7.0 was chosen owing to the high activity of lactase at neutral pH and neutral acidity of milk. To understand release kinetics, the stability of free lactase as affected by temperature was first characterized (Figure 3.12 A). During the 2-week storage, free commercial lactase had 0.0%, , ~14.6% and ~96.0% activity loss at 4, 25, and 37 °C, respectively. In a study (Jurado, Camacho, Luzón & Vicaria, 2004, the relative activity of *K. lactis* lactase reduced overtime at 25, 30, and 40 °C, and the reducing rate was higher at higher temperature, which is similar to the present study. The insignificant change of lactase activity at 4 °C is significant for application in refrigerated milk.

A sustained release of activity from LZP NPs is shown in Figure 3.12 (B). 0.222%, 0.0129%, and 0.00306% activity were released in 14 days at 4, 25, and 37 °C, respectively. This result is consistent with other literature reports on the release properties of zein NPs (Xiao & Zhong, 2011; Zhong, Jin, Davidson, & Zivanovic, 2009; Zhong & Jin, 2009). The slow and progressive release kinetic is contributed by the insolubility and slow digestibility of zein along with many other factors such as pH and temperature (Patel & Velikov, 2014; Xiao, Davidson, & Zhong, 2011). Theoretically, the release rate should be higher at a higher temperature when the Brownian motion and diffusion rate of molecules are faster at a higher thermal energy (Ikeda & Zhong, 2012). Nevertheless, opposite observations were found in Figure 3.12 B and very low lactase release could

partially result from an even faster denaturation rate of the released lactase at 25 and 37 °C (Figure 3.12 A). In addition, the cross-linking between SBP molecules reduces solubility of SBP and release rate (Sungthongjeen, Sriamornsak, Pitaksuteepong, Somsiri, & Puttipipatkachorn, 2004).

The addition of pectinase at the end of 14-day incubation boosted lactase release after 20-30 min. This result is analogous to the release profile lysozyme from pectin-films (Bayarri, Oulahal, Degraeve, & Gharsallaoui, 2014). The observation indicated that pectinase hydrolyzing SBP on LZP NPs increases diffusion of encapsulated lactase. 38.7%, 17.3%, and 1.89% lactase activity were released within 120 min at 4, 25, and 37 °C , respectively. As explained above, a higher temperature can influence the detection of activity of lactase even it is released.

Lactose hydrolysis in milk

Figure 3.13 illustrates lactose hydrolysis in whole fat milk and skimmed milk during 3-week storage at 4 °C. 50 U/mL of encapsulated or free lactase were added in milk for the storage tests. In both whole and skim milk, 100% lactose was hydrolyzed by free lactase after storage at 4 °C for 1 week. On the contrary, only 1.65% and 1.57% lactose was hydrolyzed by LZP NPs after the first week in whole milk and skim milk, respectively. The hydrolyzed lactose increased to 21.4% and 28.6% in whole milk and skim milk by the encapsulated lactase, respectively, at the second week. Therefore, encapsulated lactase was mostly not in contact with lactose in the continuous phase, which agrees with < 10% lactase release after 2 weeks (Figure 3.12). At week three as more lactase was released, 33.1% and 40.0% lactose was hydrolyzed in whole and skim

milk, respectively. In Zhang's study (2018), less than 15% of lactose in milk was hydrolyzed by lactase-encapsulated S/W/O emulsions after 3-week storage. However, the amount of lactase activity added in milk was much lower (20 U in 15 mL milk) than in this thesis. In another study conducted by Rao et al. (1994), approximately 65% and 20% lactose in milk was hydrolyzed by two kinds of lactase loaded liposomes in 20 days. However, the amount of activity addition was not mentioned in this study. Overall, the release and stability of lactase in LZP NPs provide characteristics for delivery in milk.

Lactose hydrolysis during in vitro digestion in milk

The performance of encapsulated and free lactase in whole milk or skim milk under simulated digestion conditions is shown in Figure 3.14. Free lactase only promoted neglectable lactose hydrolysis in both whole and skim milk during 12-hour simulated digestion, due to irreversible loss of lactase activity at gastric acidity (pH 3.0; Figure 3.1). LZP NPs also showed the limited lactose hydrolysis in simulated gastric digestion but were active during simulated intestinal and colonic digestion. After simulated small intestinal digestion for 2 h, 45.6% and 44.5% of total lactose was converted in whole milk and skim milk treated by LZP NPs, respectively. At 6 h in simulated colonic digestion, the hydrolysis of lactose by lactase capsules increased to 99.0% and 88.2% lactose in whole and skim milk, respectively. The hydrolyzed lactose reached to 100% and 89.4% at 8 h in whole and skim milk, respectively, and stopped further reaction with lactose. The results indicated the possible release mechanisms of LZP NPs could be the slow erosion of zein/pectin shell by pepsin, pancreatic protease, and pectinase in stomach, small intestine, and colon, respectively, resulting in sustained release of lactase.

Thus, even if a small portion of lactase (2h) is released and denatured in gastric conditions, further released lactase is able to hydrolyze lactose in simulated intestinal conditions. The fastened release of lactase between 4 h and 6 h was in good agreement with the boosted activity release from LZIP NPs triggered by pectinase (Figure 3.12 B). In the simulated digestion test, LZIP NPs had shown similar lactose hydrolysis percentage as studies conducted by Zhang and Zhong (2018) and Kwak et al. (2002), with about 50% and 58% lactose hydrolysis respectively, in the simulated gastrointestinal digestion tests. However, the prolonged colonic digestion allowed more complete lactose hydrolysis. This result suggested LZIP NPs could potentially alleviate the lactose intolerance symptoms caused by colonic bacteria fermentation of the excessive lactose (Lomer, Parkes, & Sanderson, 2008).

Further, the diffusion of lactase and contact between lactase and lactose in still milk is affected by the higher viscosity of whole fat milk (Zhang & Zhong, 2018; Mahoney & Adamchuk, 1980). Hence, lower lactose hydrolysis was found in still whole milk. Oppositely, more lactose was converted by lactase in whole fat milk during *in vitro* digestion test in the present study applying agitation, which is also in a good agreement with literature reporting that the slow digestion of milk fat improves lactose maldigestion by prolonging lactase function time with lactose when agitation was applied and diffusion of lactase was insignificantly affected by viscosity (Dehkordi, Rao, Warren, & Chawan, 1995; Brown-Esters, Mc Namara, & Savaiano, 2012).

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

This study demonstrated the application of biopolymer-based nanoparticles in lactase encapsulation. Encapsulation properties were influenced by zein:SBP and CaCl_2 : SBP mass ratios. With respective zein:SBP and CaCl_2 : SBP mass ratios of 3:2 and 5:1, a stable dispersion with EE of 93.0% and particles of 652.7 nm was chosen as the optimal formulation at the studied conditions. According to SDS-PAGE, lactase was incorporated in the L郑 NP capsules and the relative amount of lactase:zein was 2.37. Electrostatic interactions, hydrophobic interactions, hydrogen bonding, and calcium bridging were essential to particle formation and maintain L郑 colloidal dispersion stability, suggesting a zein/lactase core and an SBP shell structure that was held by Ca^{2+} . Lactase in L郑 NPs showed a sustained release profile at different incubation temperatures, with 38.7%, 17.3%, and 1.88% of activity released after two-week storage at 4, 25, and 37 °C, respectively, followed by 120 min treatment by pectinase. The L郑 NPs limited lactose from contacting with lactose during milk storage to yield < 40% lactose hydrolysis after 3 weeks but resulted in 100% and 89.4% of lactose hydrolysis in whole milk and skim milk, respectively, at simulated digestion conditions. These characteristics are desired to develop delivery systems of lactase in milk for targeted lactose hydrolysis after ingestion of milk.

Several questions remain to be solved. In regards to lactase product used in this research, the loss of its activity at acidic conditions is to be improved for applications in acidic dairy products such as yogurts. Secondly, considering zein or pectin special taste and the purpose of this study, sensory tests should be done to compare customer reactions towards regular dairy products and those containing encapsulated or free lactase. Lastly,

this study evaluated hydrolysis under simulated digestion conditions only. Future *in vivo* tests are recommended to ensure the effectiveness of encapsulated lactase alleviating symptoms caused by lactose maldigestion.

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Chapter I

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APPENDIX

Appendix A: Chapter I tables and figures

Table 1.1. Major sources of dietary lactose (Food standards for Australia and New Zealand, 2010; Milk ingredients Canada, 2017).

Dairy products	Lactose content (% w/w)
Whey powder	70-75
Whey protein concentrate (35-80 %)	3.5-46.5
Milk, 1% reduced fat	6.1
Milk, skimmed	5-5.1
Milk, whole fat	4.6-4.9
Yogurt, regular fat	3.7-6.7
Yogurt, low fat (< 0.5 %)	3-5.3
Cream cheese	2-6.4
Cottage cheese	2-3.5
Cheddar cheese	0.1-3.8
Cream	1-4.3
Sour cream	2.5
Ice cream	1.9-3.6

Table 1.2. Features of gastrointestinal *in vitro* digestion model at different sites (Minekus, Alming, Alvito, Ballance, Bohn, Bourlieu, et al., 2014; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005; Joye, Davidov-Pardo, & McClements, 2014; Liu, Fishman, Kende, & Ruthel, 2006; Wyatt, Horn, Gee, & Johnson, 1988; Gibson, Willems, Reading, & Collins, 1996; Asvarujanon, Ishizuka & Hara, 2005; Sugano, Yamada, Yoshida, Hashimoto, Matsuo, & Kimoto, 1988).

Digestion sites	pH	Digestion time	Simulated fluids	Shear strength/ environment	Main organic contents	Digestive food ingredients
Mouth	6.8-7.0	2-5 min	Electrolytes/saliva salts	High shear	α -Amylase, lysozyme, mucin	Amylose, water soluble starch
Stomach	2.0-3.0	2 h	Electrolytes	Moderate shear	Pepsin, mucin	Protein, lipids
Small intestine	6.5-7.0	2-4 h	Electrolytes	Low shear	Pancreatin (lipase, protease, amylase), trypsin, bile,	Protein, lipids, amylopectin
Large intestine	5.0	8 h	Electrolytes	Very low shear, anaerobic environment	Microflora	Non-digestible carbohydrates (e.g. poly-, oligo-, and disaccharides), non-digestible proteins (e.g. insoluble soybean protein)

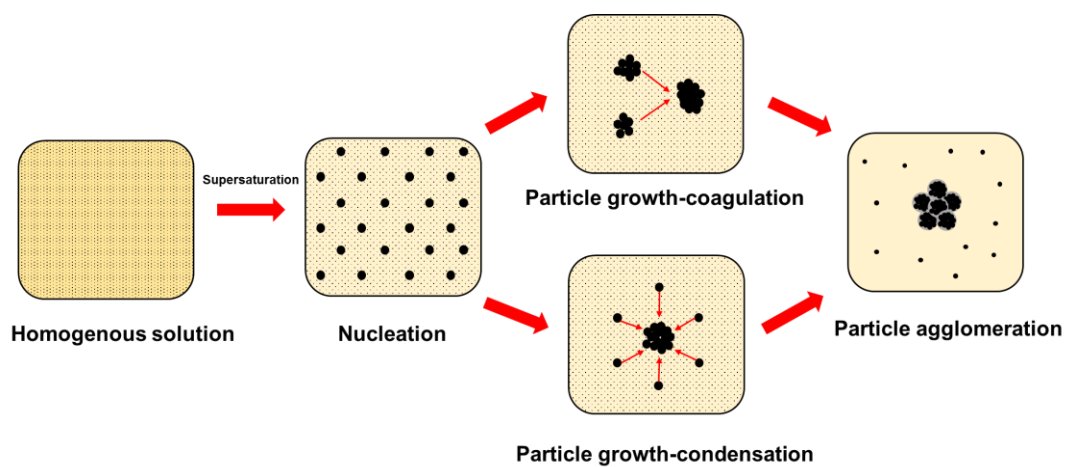


Figure 1.1. Scheme of nanoparticle formation by antisolvent precipitation (redrawn according to Thorat & Dalvi, 2012).

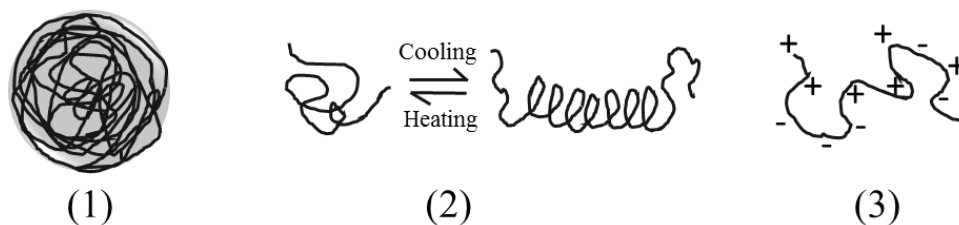


Figure 1.2. Scheme of three types of protein structures. (1) Globular protein; (2) partially unfolded or rod-like protein, when heated the folded structure can be partially unfolded and it is reversible when cooled down; and (3) random coil protein with both positive and negative charges (Mezzenga & Fischer, 2013).

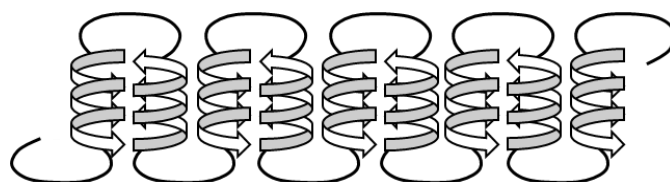


Figure 1.3. α -Zein structure proposed by Matsushima, Danno, Takezawa, & Izumi (1997; figure is redrawn based on the reference).

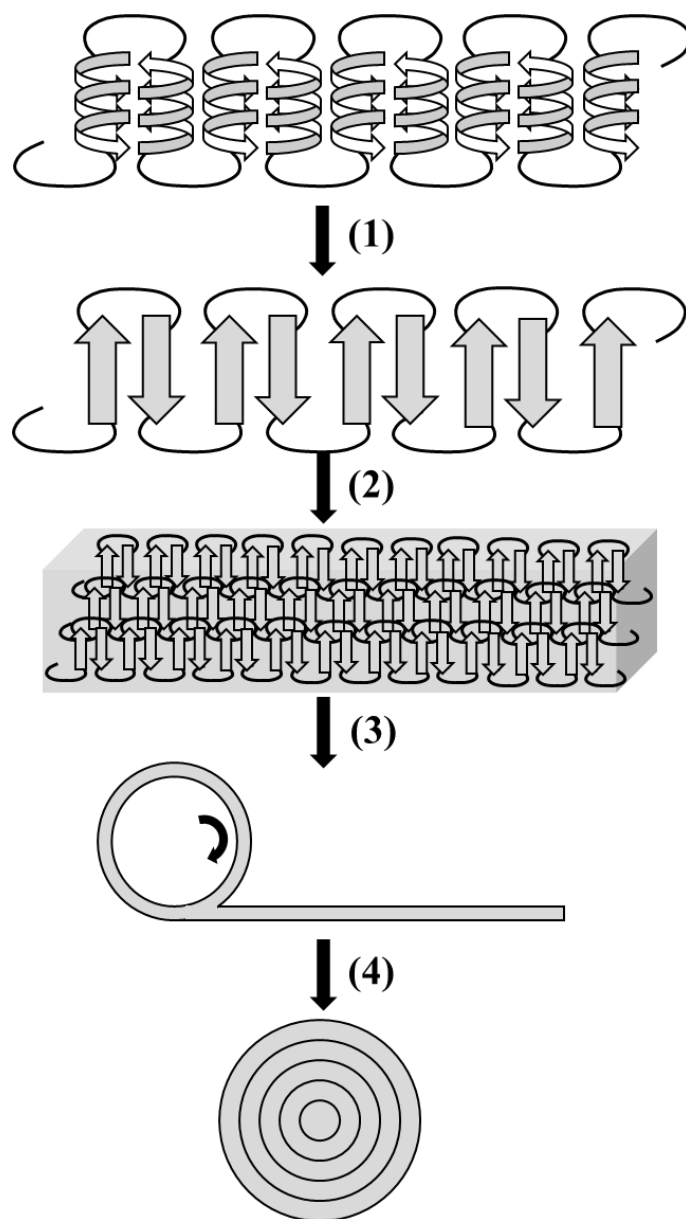


Figure 1.4. Possible mechanism of self-assembly of zein molecules to form particles (Redrawn according to Wang & Padua, 2012). Step (1): original α -helices transform into antiparallel β -sheet strands; step (2): the strands pack into ribbons; step (3): the ribbon roll into a ring driven by hydrophobic interaction until the center cycle is closed; step (4): new β -sheet adsorbs onto the nuclei and the particle grows.

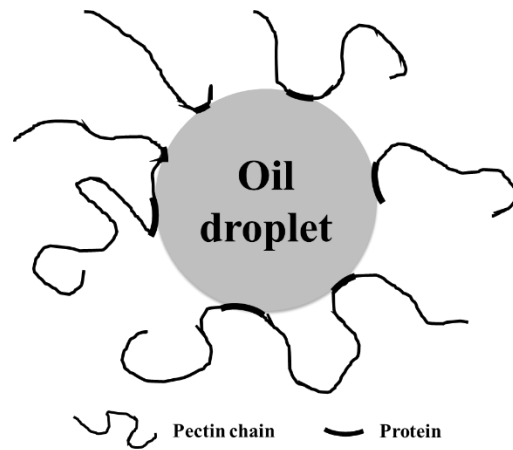


Figure 1.5. Scheme of hypothetical pectin-stabilized oil droplet (Redrawn according to Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003).

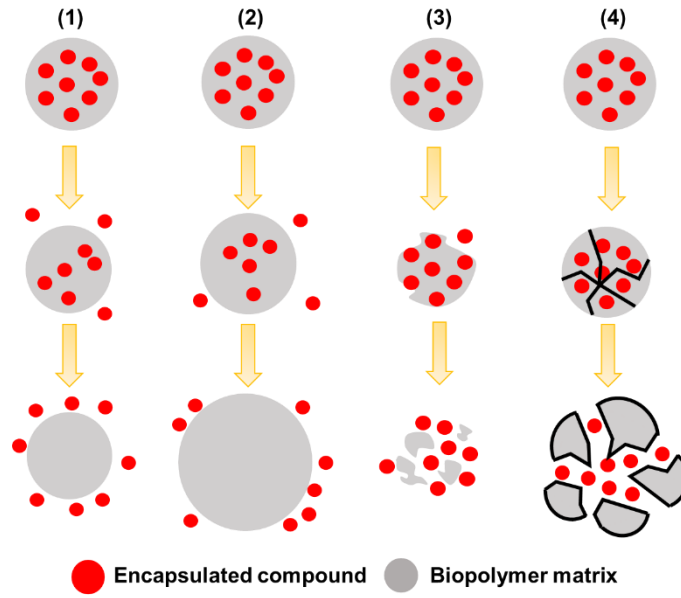


Figure 1.6. Scheme of mechanisms of compounds being released from capsules

(Redrawn according to Jones & McClements, 2010). (1) Diffusion; (2) diffusion caused by particle swelling; (3) erosion; (4) particle disintegration.

Appendix B: Chapter II tables and figures

Table 2.3. Formulations for preparation of LZP NPs.

Sample		Concentration of 10 mL zein (%, w/v)	Concentration of 25 mL SBP (%, w/v)	Concentration of 2 mL CaCl ₂ (%, w/v)	Commercial lactase (mL)
Zein:SBP mass ratio	CaCl ₂ :SBP mass ratio				
4:1	1:5	4.0	0.4	1	1
	1:10			0.5	
	1:20			0.25	
	0:1			0	
2:1	1:5	2.0	0.4	1	1
	1:10			0.5	
	1:20			0.25	
	0:1			0	
3:2	1:5	1.5	0.4	1	1
	1:10			0.5	
	1:20			0.25	
	0:1			0	
1:1	1:5	1.0	0.4	1	1
	1:10			0.5	
	1:20			0.25	
	0:1			0	

Appendix C: Chapter III tables and figures

Table 3.4. Characteristics of commercial lactase product.

Properties	Value*
Enzymatic activity in PBS (pH 7.0, U mL ⁻¹)	27,200±910 ^a
Enzymatic activity in 35% ethanol (U mL ⁻¹)	26,800±294 ^a
Protein concentration (% w/v)	3.5±1.1
Molecular weight (kDa)	Mainly distributed at 81.1±3.0~106.6±2.7 kDa with smaller fractions distributed from 14.1±1.0 to 54.3±0.5 kDa.

* Numbers are mean ± SD (n=3). Values with the same superscript letter represent they are not significantly different from each other (P > 0.05).

Table 3.5. Encapsulation properties and characteristics of LZP NPs prepared with different zein:SBP and CaCl₂:SBP mass ratios*.

Zein:SBP	CaCl ₂ :SBP	EE (%)	Z-average diameter (nm)	PDI	Zeta potential (mV)	Turbidity (Abs _{600 nm})
4:1	1:5	89.4±0.2 ^{cd}	595.8±3.4 ^f	0.374±0.057 ^{cde}	-18.47±0.98 ^{ab}	0.0585±0.0010 ^m
	1:10	91.3±0.1 ^b	860.5±5.3 ^a	0.338±0.050 ^{de}	-18.13±0.99 ^{ab}	0.2621±0.0017 ^a
	1:20	92.1±0.4 ^{ab}	747.9±20.6 ^b	0.340±0.065 ^{de}	-17.97±0.67 ^{ab}	0.2509±0.0010 ^b
	0:1	88.9±0.1 ^d	595.5±18.2 ^f	0.489±0.040 ^b	-18.53±0.48 ^{ab}	0.0791±0.0053 ^d
2:1	1:5	90.3±0.4 ^f	695.5±8.4 ^c	0.427±0.026 ^{bcd}	-19.59±0.85 ^b	0.0871±0.0039 ^j
	1:10	86.3±0.9 ^c	625.8±9.0 ^{def}	0.302±0.016 ^e	-18.83±1.18 ^{ab}	0.1353±0.0010 ^e
	1:20	90.2±0.1 ^{cd}	541.7±18.6 ^g	0.344±0.049 ^{de}	-18.19±1.22 ^{ab}	0.1468±0.0011 ^d
	0:1	86.3±0.1 ^f	365.9±15.5 ⁱ	0.620±0.041 ^a	-17.33±0.77 ^a	0.0326±0.0006 ⁿ
3:2	1:5	93.0±0.1 ^a	652.7±21.9 ^d	0.412±0.091 ^{bcd}	-18.55±1.08 ^{ab}	0.0660±0.0014 ^l
	1:10	88.9±0.1 ^d	634.1±8.2 ^{de}	0.370±0.084 ^{cde}	-19.56±1.47 ^b	0.1132±0.0006 ^g
	1:20	88.8±1.0 ^d	501.1±11.4 ^h	0.459±0.020 ^{bc}	-19.28±0.97 ^{ab}	0.0928±0.0043 ⁱ
	0:1	83.2±0.0 ^g	366.7±12.1 ⁱ	0.593±0.042 ^a	-18.41±0.62 ^{ab}	0.0324±0.0014 ⁿ
1:1	1:5	87.1±2.1 ^{ef}	732.8±11.0 ^b	0.462±0.012 ^{bc}	-18.47±0.70 ^{ab}	0.1183±0.0008 ^f
	1:10	87.6±0.4 ^e	617.8±29.8 ^{ef}	0.369±0.048 ^{cde}	-18.57±1.27 ^{ab}	0.1039±0.0005 ^h
	1:20	87.1±0.2 ^{ef}	542.6±17.4 ^g	0.438±0.066 ^{bcd}	-19.11±1.87 ^{ab}	0.1683±0.0011 ^c
	0:1	79.8±0.9 ^g	336.8±35.6 ⁱ	0.475±0.106 ^{bc}	-18.11±0.68 ^{ab}	0.0355±0.0021 ⁿ

* Numbers are mean ± standard deviation (n=3). Values in same column with different superscript letters represent they are significantly different (P < 0.05).

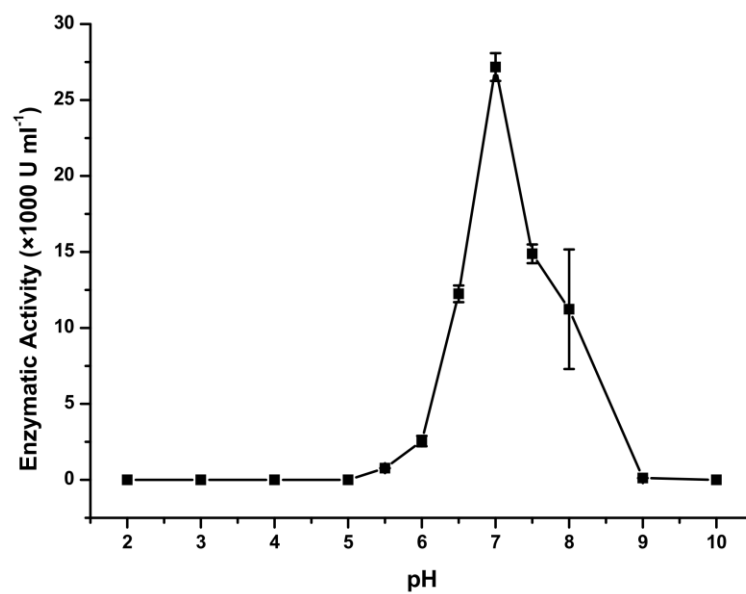


Figure 3.7. Lactase activity at pH 2.0-10.0. Error bars are standard deviations (n=3).

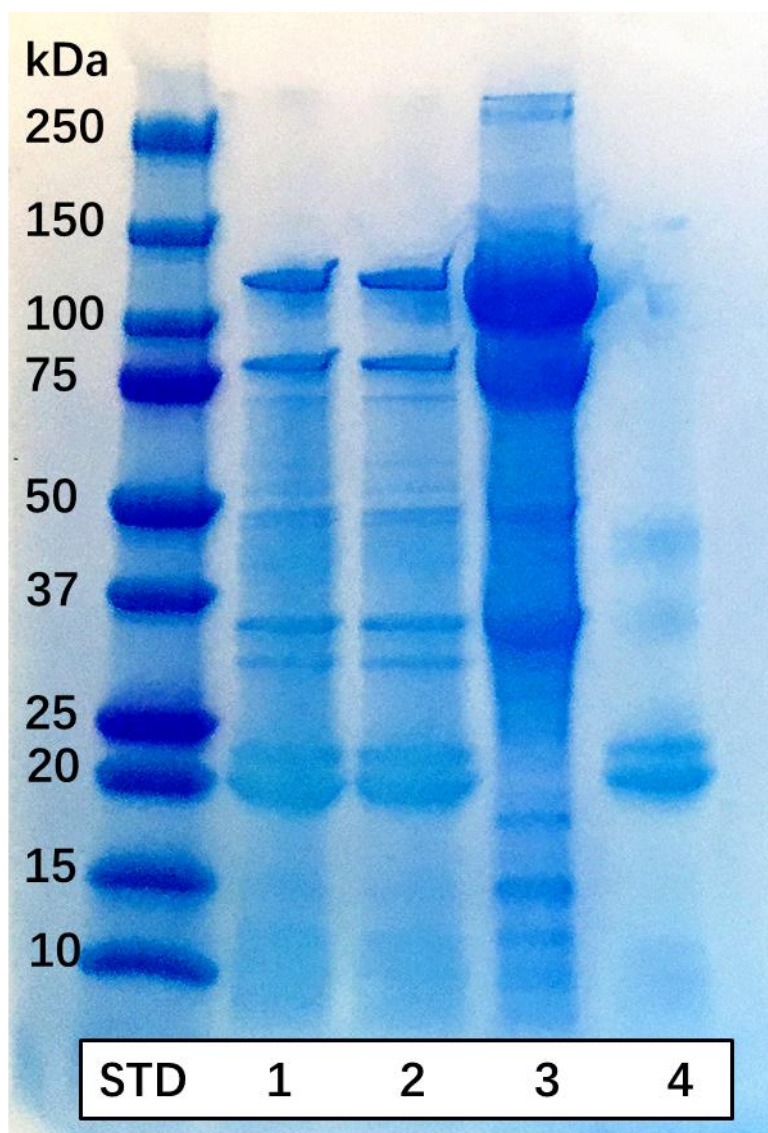


Figure 3.8. SDS-PAGE analysis of freshly prepared LZP NPs (1), EDTA treated LZP NPs (2), native commercial lactase (3), and zein (4). STD is the standard protein marker.

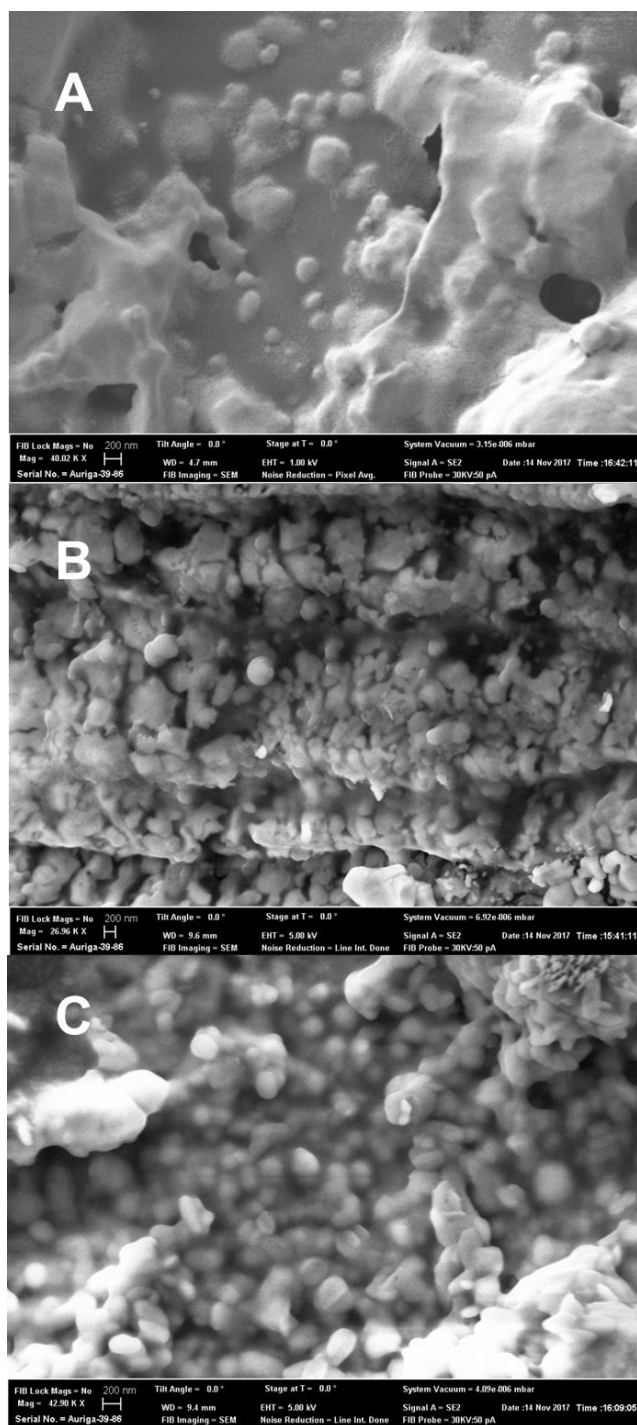


Figure 3.9. Morphologies of freeze-dried commercial lactase (A), ZP NPs (B), and LNP NPs (C). The scale bars represent 200 nm.

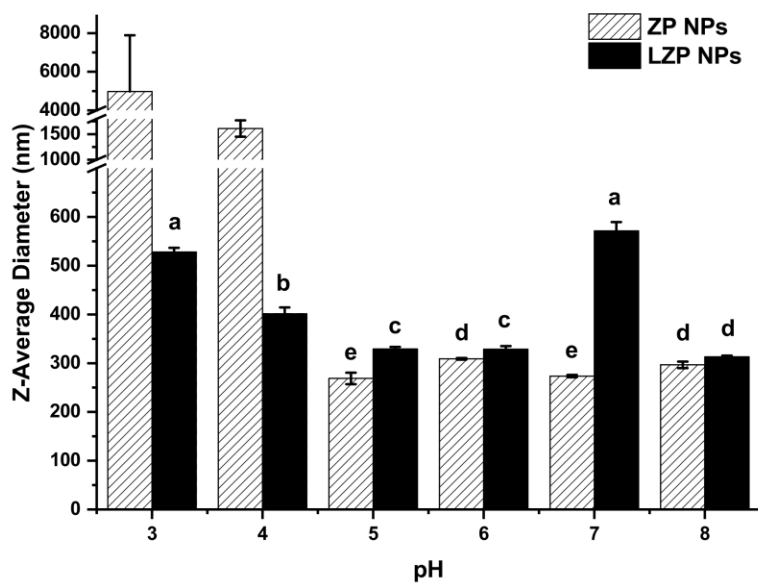


Figure 3.10. Particle size of ZP NPs and L郑 NPs as affected by pH. Bars labeled with different letters are significantly different among those smaller than 1,000 nm ($P < 0.05$). Error bars are SD (n=3).

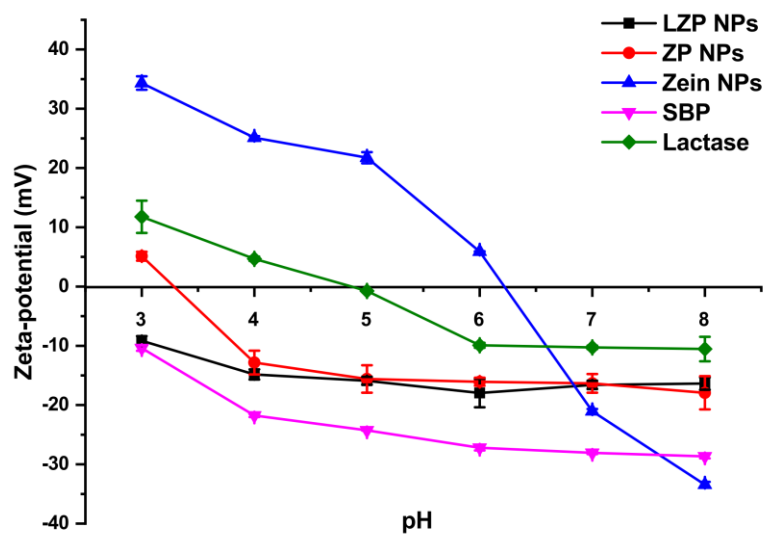


Figure 3.11. Zeta-potential of LZIP NPs, ZP NPs, zein NPs, SBP, and lactase at pH 3.0-8.0. Error bars are SD (n=3).

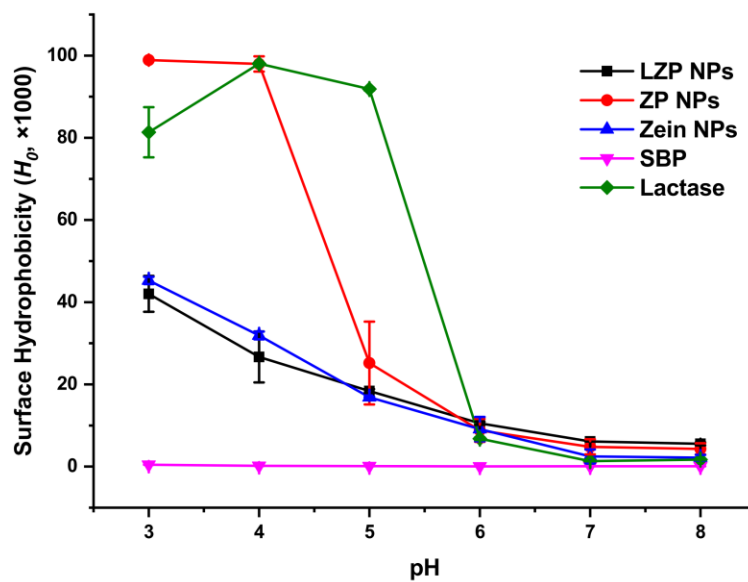


Figure 3.12. Surface hydrophobicity (H_0) of LZIP NPs, ZP NPs, zein, SBP, and lactase at pH 3.0-8.0. Error bars are SD (n=2).

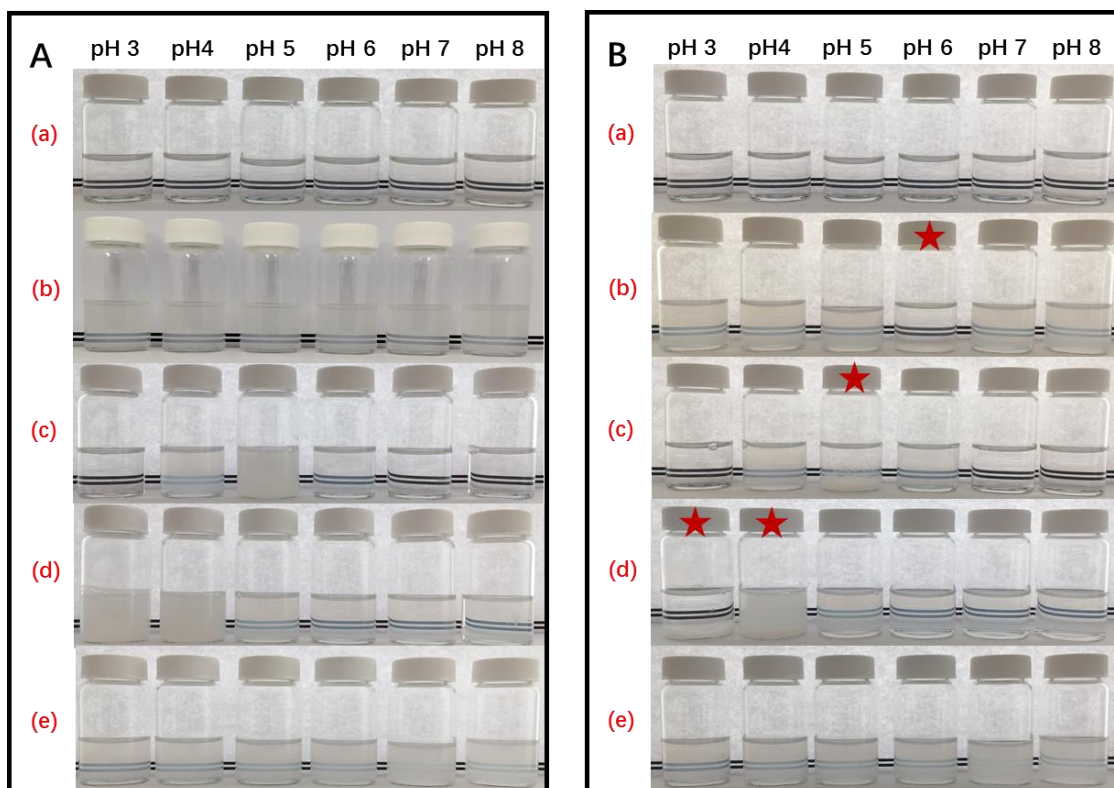


Figure 3.13. Appearance of fresh dispersion (A) with SBP (a), zein NPs (b), lactase (c), ZP NPs (d), and LZP NPs (e), compared with those after overnight storage at 4 °C (B). Dispersions marked with red stars precipitated.

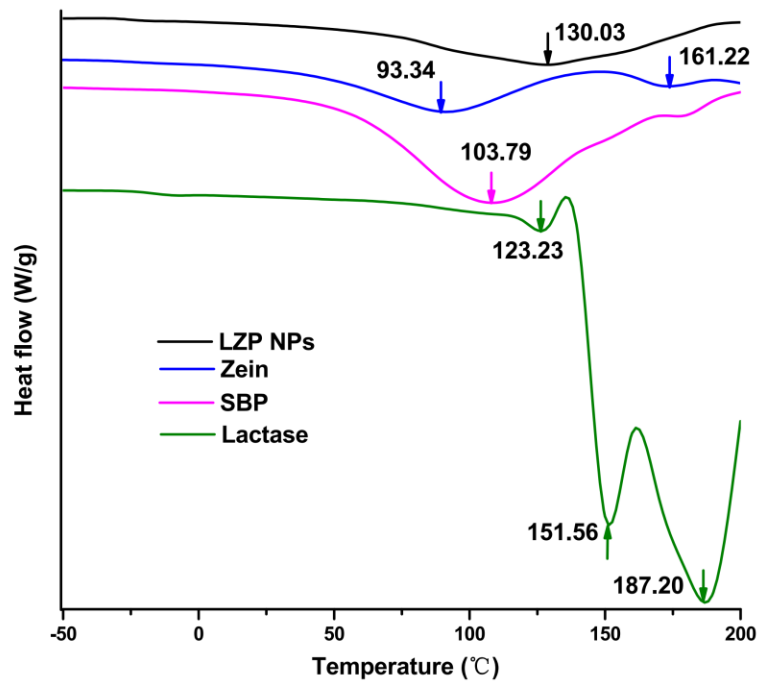


Figure 3.14. DSC thermograms of freeze-dried L郑 NPs, zein, SBP, and commercial lactase powders.

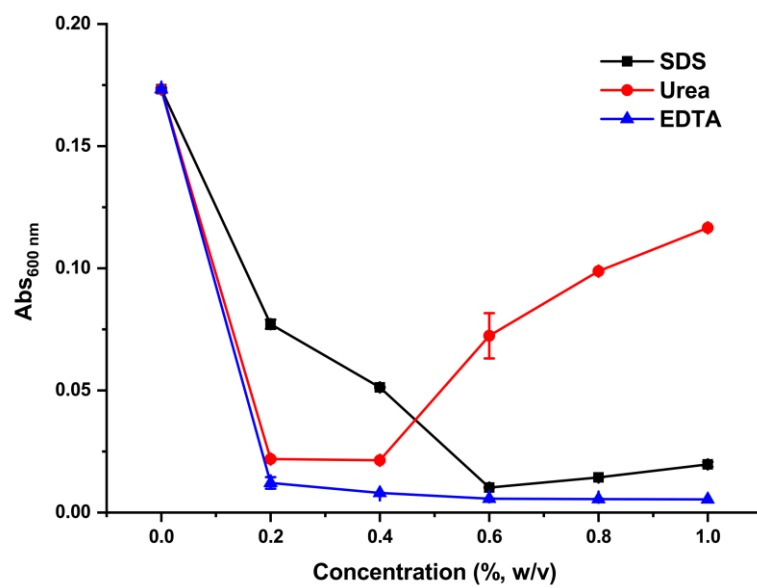


Figure 3.15. Effects of dissociation agent concentration on the turbidity of LZP NP dispersions at neutral pH.

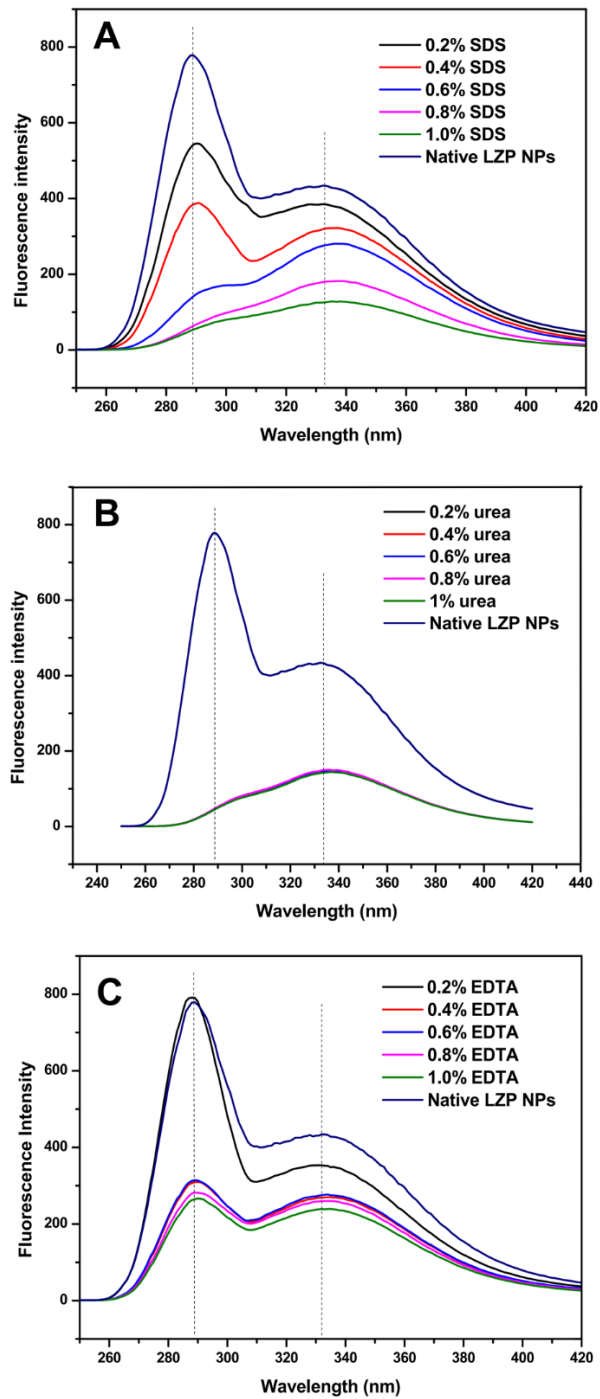


Figure 3.16. Fluorescence spectra of LNP NP dispersions treated by 0.2-1.0% sodium dodecyl sulfate (SDS; A), urea (B), and ethylenediaminetetraacetic acid (EDTA; C).

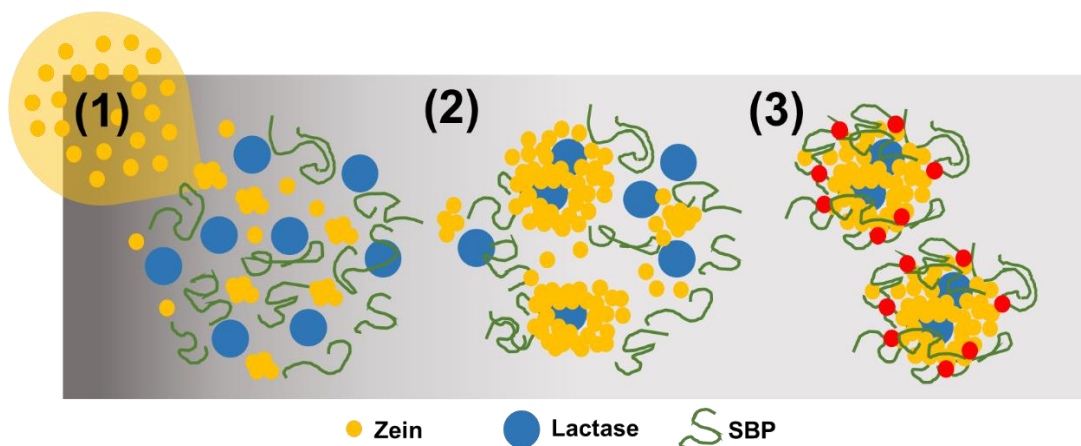
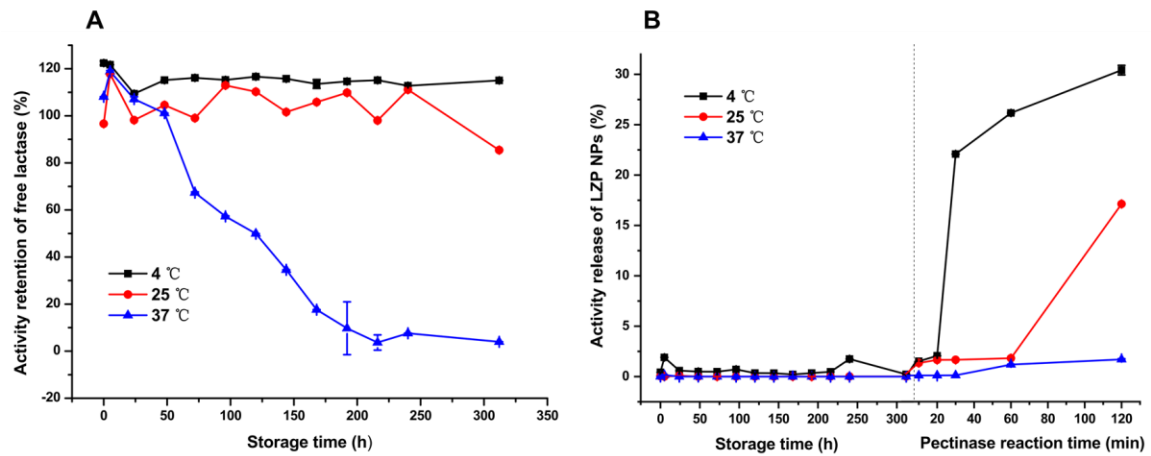


Figure 3.17. Possible mechanisms of LZP NPs formation. (1) The precipitation and nucleation of zein particles as solvent power is decreased; (2) simultaneous binding of lactase and SBP to zein driven by hydrophobic interactions to grow into co-precipitated particles; (3) formation of zein/lactase core and SBP shell that is further cross-linked. The lighter gray in the background color represents the lower ethanol concentration. The darker the gray is, the higher the ethanol concentration.



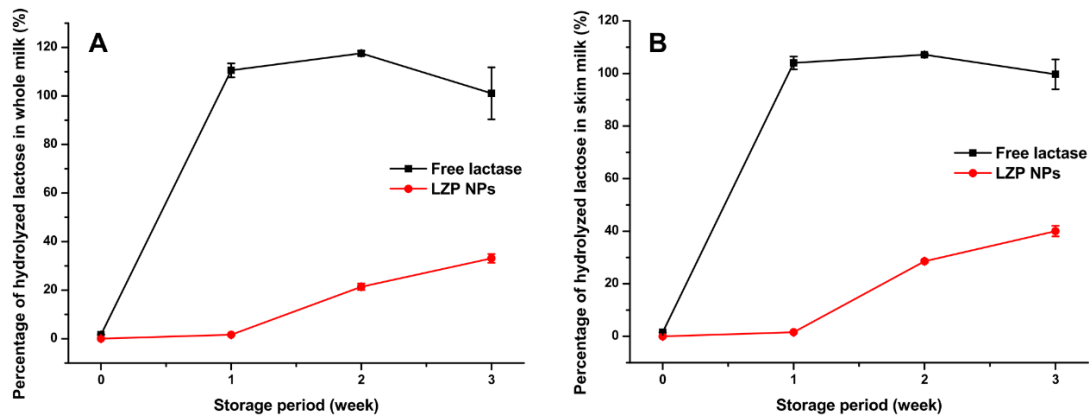


Figure 3.19. Lactose hydrolysis by free lactase and LYP NPs during 3-week storage of whole milk (A) and skim milk (B) at 4 °C. Error bars are SD (n=2).

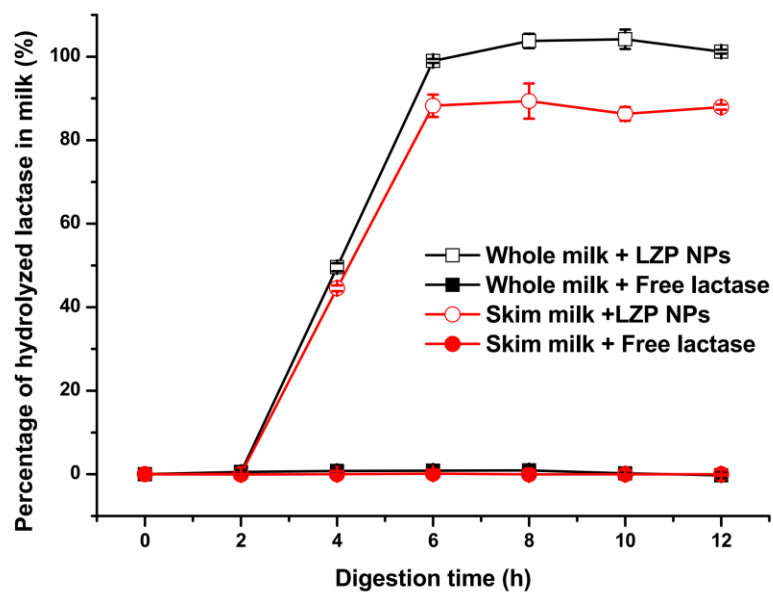


Figure 3.20 Lactose hydrolysis by free lactase and LYP NPs during *in vitro* digestion: gastric conditions with pepsin at pH 2.0 in 0-2 h, intestinal conditions with pancreatic lipase and pancreatin at pH 7.0 in 2h, and colonic conditions with pectinase at pH 5.0 in 8 h. Error bars are SD (n=2)

VITA

Lianger Dong was born in Lingshi, China on April 10th, 1993. She moved to Zhengzhou with her family when she was 12 years old. After 6 years middle and high school education, she was admitted to Jiangnan University (Wuxi, China, 2011) to study animal nutrition and feed science in the School of Food Science and Technology. She enjoyed her college life very much where she became a member of Zhishan Honor School and had the chance to travel to Davis, California, the United States for a one-month culture communication program in 2014. With both of her parents being food scientists, she had developed interests in food science and participated many scientific research activities during undergraduate and her gap year. In summer 2016, she started her graduate school at the University of Tennessee under supervision by Dr. Qixin Zhong. In two years of her M.S. program, Lianger experienced many exciting, fun, or frustrating moments and she really appreciates all the people she met in her life. After getting her Master's degree, she is planning to travel more and pursue a Ph.D. degree.