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**DEVELOPMENT OF ZEIN-BASED ICE NUCLEATION FILM FOR  
FROZEN FOOD APPLICATION**

By

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and approved by

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# ABSTRACT OF THE DISSERTATION

## Development of Zein-Based Ice Nucleator Films for Frozen Food

### Applications

By KE SHI

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Dr. Tung-Ching Lee

This research aimed at developing zein-based ice nucleation films for the applications of frozen foods. Acetic acid was chosen as the solvent to cast zein films with flawless surface. Tributyl citrate (TBC) was mixed into zein films to various proportions and 10% TBC was most effective on improving mechanical properties resulting in 2.3-fold more flexibility and 5.8-fold more toughness compared to unplasticized zein films. But incorporation of 20% TBC significantly decreased the water absorption by 19% compared to 10% TBC. Thus, zein films with 20% TBC with good flexibility and water resistance was used in following experiments.

The activity of ECINs was stable at pH between 4.0 and 9.0 and ionic strength between 0.01 M and 0.10 M. In the development of INFs, the optimum adsorption of ECINs on zein films surface occurred through layer-by-layer method at pH 7.0 and ionic strength of 0.05 M on UV/ozone-treated zein. A novel method was developed to quantify

the activity of INFs, which revealed that the highest activity of zein-based INFs reached 175 units/mm<sup>2</sup>.

The zein-based ice nucleation films (INFs) were used to wrap frozen bread dough during five freeze/thaw cycles. The high-activity INF was as effective as blending ECINs in improving the yeast survival by 40%, and consequently increased the specific volume of bread loaf by 25%. Furthermore, high-activity INFs prevented the dehydration of frozen dough and thus significantly reduced the crumb hardness by 36% and increased the crumb springiness by 1.25 times. On energy saving, zein-based INFs were less potential than blended ECINs. INFs only reduced the time of freezing by 6% compared to the 20% by blended ECINs. However, the total amount of ECINs used on wrapping a piece of dough was only about 1/200 of the amount through blending. In addition, ECINs immobilized on the zein films showed desirable stability to sustain at least fifteen repetitive uses on freezing water.

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## INTRODUCTION

### **I. Zein: a biodegradable material for food packaging**

#### **Characteristics of zein**

##### *Origin and Extraction*

Zein was first found and named by John Gorham in 1821. Early work indentified it as a major storage protein found in the endosperm of corn, comprising 45 ~ 50% of the proteins in corn. Among the four major types of proteins defined by their solubility in certain solvents, zein belongs to the group of prolamine, which are soluble in alcohols or mixture of water and alcohols (Shukla and Cheryan, 2001). Since its isolation in 1821, the extraction of zein has received considerable investigation (Lawton, 2002). Currently, the commercial production includes steps of extracting zein by alcohol and then removing the impurities either by addition of hexane (Swallen, 1941), or through fractional precipitation by chilling (Carter and Reck, 1970). Recently, high-grade zein became commercially available from two manufactures, Freeman Industries in the USA and Showa Sangyo Corp in Japan. Among their products, the yellow Freeman F4000 zein (88-96% protein, <2% ash) and the white Showa  $\alpha$ -zein (96.3% protein, 3.4% water) are widely used for scientific researches.

##### *Composition*

Biologically, zein is a family of proteins with various molecular weights, charges and solubility, named as  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  (Esen, 1987; Woo and others 2001). The  $\alpha$ -zein, the most abundant and well studied group of zein, is defined as those proteins soluble at 95% ethanol solution. On reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis,

$\alpha$ -zein reveals two components with apparent molecular weight of 19 and 22 kD. The other groups,  $\beta$ ,  $\gamma$  and  $\delta$ -zein, are less studied due to less commercial significance. The  $\beta$ -zein, a fraction that is soluble in 60% ethanol but insoluble in 95% ethanol, contains methionine-rich polypeptides, which was suggested to be oligomers of  $\alpha$ -zein linked by disulfide bonds (Shukla and Cheryan, 2001). Besides, a band at about 14 kD was often seen on the reducing SDS-PAGE gel of  $\beta$ -zein. In  $\gamma$ -zein, three components at 16, 27 and 50 kD was found. Another minor polypeptide with apparent molecular weight of about 10 kD, which was originally classified to the group of  $\alpha$ -zein, is now commonly defined as the  $\delta$ -zein (Woo et al., 2001). This research will focus on the  $\alpha$ -zein which is the group with the most scientific and industrial significance.

Stimulated by the scientific interests in the synthesis and structure of storage protein in cereal seeds, the cDNA and the amino acid sequences of zein have been determined. With nearly identical molecular weight, the  $\alpha$ -zein proteins are encoded by large multi-gene families into polypeptides with various amino acid contents. The non-polar amino acid residues in zein proteins are abundant which is responsible for the high hydrophobicity of zein. Meanwhile, zein is deficient in essential amino acids, such as lysine and tryptophan, which decreases the nutritional value. It is notable in Table 1 that the proportions of the chemically active amino acid residues in the  $\alpha$ -zein proteins are different, which possibly leads to different behaviors of  $\alpha$ -zein proteins in chemical reactions (Wu and others 2003).

### *Solvents*

Choosing suitable solvents for zein in research and industry is a scientific topic. Mixture of ethanol and water (aqueous ethanol) is conventionally used to prepare zein

solutions. However, zein could also be conveniently dissolved in many primary organic solvents. Evans and Manley have extensively studied the solubility behavior of zein in different solvents at different temperatures. In one of their publications, 69 likely primary solvents for zein are listed, such as acetic acid, glycerol and so on. Based on the nature of all primary solvents reported, they believed that due to the presence of hydroxyl, amide, amine and carboxyl groups, all single solvents of zein are amphoteric, meaning that they could form hydrogen bonds through donating or accepting electrons, although not all organic compounds containing these groups are zein solvents. Compared to in aqueous ethanol, zein in the primary solvents was found to show much better stability (Evans and Manley, 1941). Evans and Manley further made another comprehensive investigation on the binary solvents for zein, suggesting two types of solvents from mixture of water and a lower aliphatic alcohol, or of two anhydrous organic compounds (Manley and Evans, 1943). In the first type of solvents, example of alcohol includes acetone, methanol, ethanol, isopropanol and so on. The second type of binary solvents covers a much larger group of compounds than the aqueous alcohol solvents, and the resulting zein solutions have remarkably different properties regarding the stability and rheological characters (Manley and Evans, 1943). Some ternary solvent systems, such as acetone-water-formaldehyde mixture, tend to stabilize the zein solutions against gelation; however the ternary solvent systems are considerably too complicated for scientific and manufacturing purposes (Evans and Manley, 1944).

### *Structure*

The capability of zein to self-aggregate, to form fibers and films stimulates the scientific interest in the molecular conformation of zein. However, conclusion is still in

the air. Analysis to the amino acid sequence in zein reveals a series of tandem repeats. Most of researches on zein structure have been based on solution in alcoholic solutions. Empirical observations suggested that zein adopts a rod-like conformation. Based on a circular dichroism analysis of zein in 70% Methanol suggesting high content of helices in zein structure, and amino acid analysis, Argos and his colleagues proposed a model where 9 homologous adjacent, anti-parallel helices clustered into a capsule-like molecules which were arranged into zein planes by intra- and inter-molecular hydrogen bonds, as shown in (Figure 1A). The abundant glutamines in zein located at the turns and the two ends of the capsule stabilize the stacking of the zein planes (Argos and others 1982). Garratt et al. investigated the sequence homology between the repeat motifs in  $\alpha$ -prolamine proteins and suggested that majority of the helices show six faces composed of polar and non-polar amino acids alternatively (Figure 1B). The non-polar amino acids forming hydrophobic face on helices (shaded) induced the arrangement of the helices on a two-dimensional hexagonal net (Garratt and others 1993). This model easily accommodated the infinite oligomerization of  $\alpha$ -prolamine proteins with different numbers of tandem repeats, and allowed the efficient packing of storage proteins in protein body. With the advance of techniques, one SAXS analysis on zein done by Tatham et al. suggested that if the zein molecule in 70% aqueous methanol adopted a prolate ellipsoid conformation, then the two semi-axes were calculated to be 9.9 nm and 0.35 nm; or if in a rod conformation, the length and the diameter were calculated to be 15.3 nm and 0.69 nm (Tatham and others 1993). Matsushima and his colleagues obtained a similar result of zein in 70% aqueous ethanol by SAXS analysis. In addition, based on the assumption of Garratt model, they suggested a prism model with the three dimensions

of 13 nm (length of the prism with 9 or 10 helices), 3 nm (height of a helix with averaging 20 amino acid residues) and 1.2 nm (diameter of  $\alpha$ -helix) (Figure 1C) (Matsushima and others 1997). The concepts of prism model and previous mentioned hexagonal packing were employed by Wang et al. to explain the structural change of zein during processing (Figure 1D) (Wang and others 2005). Other 3D structure models, such as coiled-coil and hairpin, has been developed as well (Forato and others 2004; Momany and others 2006). However, none of these published models are perfectly consistent with the experimental data. Therefore, a clear and confirmative understanding about the molecular structure of zein is still in the air.

### **Applications of zein**

Application of zein has been explored in many aspects, including coatings, fibers, inks, adhesives, chewing gums, and delivery systems (Hurtado-Lopez and Murdan, 2005; Liu and others 2005; Gao and others 2007; Parris and Dickey, 2003; McGowan and others 2005). Coatings and forming fibers of zein seems to be the most promising applications.

#### *Films & Coatings*

One of the most important and traditional application of zein is films and coatings. Zein can steadily form transparent, glossy, tough and hydrophobic films simply by solvent evaporation. However, without modification and plasticization, the zein films are generally too brittle to withstand the industrial processing in most applications. In addition to the previously mentioned modifications on zein that can strengthen the mechanical properties, some low-weight molecules, such as some polyols and fatty acids, could improve the mechanical properties of zein films (Parris and Coffin, 1997;

Ghanbarzadeh and others 2006b; Ghanbarzadeh and others 2007a; Selling and others 2004; Tillekeratne and Easteal, 2000; Lawton, 2004; Lai and Padua, 1997; Lai and others 1997; Santosa and Padua, 1999). Besides standing alone forming films, zein was mixed into starch resulted in stiffer edible films with lowered water vapor permeability and higher water-resistance (Corradini and others 2006; Ryu and others 2002; Parris and others 1997). Zein also improves the biodegradable properties to plastics formed by synthetic polymers, such as ethylcellulose, polypropylene and polyethylene (Lee and others 2008; Romero-Bastida and others 2004; Herald and others 2002).

Zein-based coatings have a long history of industrial applications in food, cosmetic and paper products. The main utilization of zein currently is to coat paper cups, pharmaceutical tablets, nuts, candies to resist water uptake and/or oxidation (Council, 2000; Trezza and Vergano, 1994; Trezza and others 1998). With the unique hydrophobic nature of zein, studies reveal that zein-coated paper is suitable for the packaging of high-fat foods and sandwiches (Andersson, 2008). In addition, zein films are freeze/thaw stable, allowing its use in packaging to frozen foods (Padua and others 2000). Nevertheless, there are still limited studies on developing zein-based functional film. In the literature, only antimicrobial and antioxidant films have been prepared by incorporating lysozyme, albumin proteins, disodium EDTA and phenolic compounds in zein films (Gucbilmez and others 2007; Mecitoglu and others 2006; Arcan and Yemenicioglu).

## II. Biogenic ice nucleators

### Freezing and Ice Nucleation

Freezing is a process of solidification in which a uniform liquid turns to solid when the temperature decreases to a certain point. Most liquid, including water, freezing by forming crystals follows the first order thermodynamic phase transition, which predicts that at the presence of both liquid and solid, the temperature remains constant. For water this temperature is 273.16 Kelvin and well known to define the 0 °C. The whole process of water freezing consists of two related but distinct stages, nucleation and growth of ice crystals.

#### *Ice nucleation*

A homogeneous nucleation is the step that the water molecules spontaneously cluster to form an interface at the boundaries of the water and ice, where further water molecules can attach onto. This homogeneous ice nucleus is so small that the energy does not favor the proceeding of nucleation. The free energy barrier of homogenous ice nucleation leads to a significant supercooling status, during which the temperature a liquid is decreased to below its freezing point without it becoming a solid. Homogeneous ice nucleation happens in absolutely pure water in a container with perfectly smooth surface. With advanced techniques to obtain this ideal condition, the water can supercool to as cold as -70 °C (Jeffery and Austin, 1997). At this low temperature, stable ice lattices starts to form, and when the lattices reaches a critical size, it can serve as templates for the further attachment of water molecules (Zaritzky, 2006).

In most case, the presences of impurities or preferential sites provide the active surfaces for the formation of ice nuclei, and this process is called heterogeneous

nucleation. On the active surfaces, the free energy barrier is diminished, which means less supercooling accompanied by elevated nucleation temperature, as shown in the curves of typical time-temperature relationship (Figure 2).

#### *Growth of ice crystals*

Ice nucleus provides a template for further addition of water molecules in the growth of ice crystals (Figure 3). This process is an exothermic process and its macroscopic rate is controlled by the heat transfer, i.e. the rate of removal of the latent heat, as well as the mass transfer, i.e. the diffusion of water molecules and any solutes if present, since the water molecules need to diffuse towards the ice crystals while the solutes must be excluded from the ice (Zaritzky, 2006).

The rate of the whole freezing process, thus, is added up from the nucleation process and growth of ice crystals. It can be simply defined as the rate of temperature decrease. At high freezing rates, larger number of ice nuclei form into more and smaller ice crystals and vice versa, a few big ice crystals form at low freezing rates.

#### *Food freezing*

Water is the most fundamental component for live organisms, and this raises the importance of understanding the freezing process in food system. Compared to the aqueous system, the spatial heterogeneity inside live organisms and the existence of barriers to the water movement such as cell membranes, make the freezing process much more complicated in food system. In plant or animal tissue, it is generally believed that the freezing is initiated in the extracellular fluid whereas the intracellular fluid remains at a supercooled condition, because a lower nucleation temperature could be expected from the much higher concentration of various solutes inside the cell. As the extracellular fluid



freezes, the water activity outside cells continues decreasing to lower than the intracellular space. Consequently, the water diffuses from the cells to the extracellular space, resulting in the dehydration and shrinkage of the cells. In contrast, at lower temperature during a rapid freezing process, both intra- and extracellular fluids experience a quick freezing and minimum dislocation of water.

While freezing is an effective method for food preservation, it is also well known to damage the food quality through physical and chemical changes. As previously mentioned, a slow freezing process leads to dehydration and shrinkage of cells. However, if the cells are frozen too quickly, the retained water inside cells expands dramatically and causes mechanical damage to the fragile cellular structures. During frozen storage, certain part within the food system can still lose the moisture due to the moisture migration along the temperature gradients and sublimation on the surface which leads to the freezer burn. In addition, recrystallization of ice is another issue in long-term frozen storage because smaller ice crystals are less thermodynamically stable than large ones, and the net outcome is less amount of but larger ice crystals which cause mechanical damages.

Besides the physical damage, freezing also alters the chemical environment inside food system as a direct result of the increase in solute concentrations in the unfrozen phase since they are more likely to be excluded from the ice crystals. The consequent change of pH and ionic strength mostly affects the physiochemical status of the food macromolecules, such as protein denaturation, as well as the chemical reactions. Though, the temperature decrease slows down many enzymatic and non-enzymatic reactions, the increase of the reactant concentration in unfrozen phase plays the opposite role, which is

the reason that the reaction rate in some frozen systems reaches a maximum at a subfreezing temperature (Lee and Jiang, 2004). Freezing is less effective in food preservation than the thermal processing, partially because the activity of many enzymes is not deactivated in freezing. On the other hand, the mechanical damage to cellular structure can release enzymes and chemical substances from naturally enclosed environments, possibly facilitating the protein denaturation, lipid oxidation, degradation of pigments and nutrients, flavor deterioration, and enzymatic browning (Zaritzky, 2006).

Preserving food by freezing is energy demanding due to both thermodynamic and kinetic requirements. On thermodynamic aspect, it initially requires decreasing the temperature to the freezing point of food, followed by the removal of latent heat for ice nucleation and then further decrease to the temperature of ice before reaching the storage temperature, usually at -15 to -20 °C, which is required for quality retention and microbial safety of frozen foods. Because of the large values of specific heat and latent heat of water, the energy requirement solely for freezing process is large and increases as the water content of food increases. Kinetically, the supercooling of food set up a free energy barrier for ice nucleation to occur in addition to prolonging the time of freezing. More energy is required to decrease the temperature of water to nucleation temperature that could be far below the freezing point (Figure 2). To meet this requirement, most commonly used air-blast freezers are operated at -20 to -40 °C, while some tunnel and plate freezers are operated at conventional temperatures of -40 °C. In addition to freezing, the cold storage, transportation as well as pre-processing such as blanching and packaging are all significant contributors to high energy consumption (North and Lovatt, 2006).

Researches on food freezing techniques generally suggest four ways to reduce the energy consumption: 1) Identify new feasible technologies to reduce freezing time; 2) select optimal operation temperature of freezer and cold chamber for specific food; 3) good management practices in operating the cold chain in freezing, storage and transportation; 4) increase energy efficiency of freezer, cold chamber and other facilities; and 5) recycle the waste heat (Hall, 2011).

### **Characteristics of bacterial ice nucleators**

Many live organisms are able to tolerate prolonged periods at subzero temperatures. Most of them produce cryoprotectants such as anti-nucleating proteins (AFPs) and polyols. Whereas some other organisms produce specialized materials serving as heterogeneous ice nucleators. Ice nucleation materials have been reported in the leaves of plants such as winter rye and prunus, animals such as turtle, insects and lichens, and some microorganisms (Li and Lee, 1995; Duman, 2001; Brush and others 1994; Costanzo and others 2003; Duman and Horwath, 1983; Kieft and Ruscetti, 1990). Notably, many Gram-negative ice-nucleating (IN) active strains of bacteria have been isolated from the surface of plant leaves and confirmed to express strong protein-based heterogeneous nuclei which introduce the formation of ice, thus initiating frost injury to plants at temperatures above -5 °C. These IN active strains belong to the genera of *Pseudomonas*, *Erwinia* and *Xanthomonas* which are phytopathogen of scientific interest in preventing frost damage in agriculture. In addition to the natural IN active microorganism, the ice nucleation genes from *Pseudomonas* and *Erwinia* have been transformed into *Escherichia coli* and *Saccharomyces cerevisiae*, enabling these microorganisms to express the ice nucleation activities (Hwang and others 2001; Ohgama

and others 1992). An interesting feature among these bacterial ice nucleators is the quantitative and qualitative variability. Different species and strains of ice nucleation active bacteria differ greatly in their ice nucleation activity (INA). Even a population of cells in a strain contains ice nuclei that are active from -2 °C to -12 °C, which is called the threshold temperature. This phenomenon strongly suggests variability of the ice nucleation sites among bacteria.

### *Protein sequence and Structure*

A particular protein encoded by a single *ina* gene in each strain of the bacteria is essential for the ice nucleation activity and referred as the ice nucleation protein (INP). At least five homologous *ina* genes have been discovered and sequenced, including *inaZ* from *Pseudomonas syringae*, *inaW* from *Pseudomonas fluorescens*, *inaA* from *Erwinia ananas*, *iceE* from *Erwinia herbicola* and *inaX* from *Xanthomonas campestris* pv. *Translucens*. The encoded amino acid sequences of the ice nucleation proteins (INPs) are more strongly conserved than the DNA sequences, each with a molecular weight of around 120 kD. All of these INPs contain three domains: an N-terminal domain, a C-terminal domain, and a central domain composed of repeated units. The central repeating domain is rich in neutral, hydrogen-bonding residues, such as serine and threonine, and is moderately hydrophilic (Wolber and Warren, 1989). The repeating units consist of a large number of a consensus sequence (Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr). The N-terminal domain contains several moderately hydrophobic stretches and interacts with the phospholipids moiety of the outer membrane. The C-terminal domain is highly hydrophilic and exposed to the outermost membrane. The N- and C-terminal domains are essential to anchor the INPs on the surface of outer membrane (Wu and others 2006).

Until now, there are no experimental data on the tertiary structure of INPs. Three models that describe the repeating units forming into a repetitive template with lattice-matching surfaces seem to be most plausible (Figure 4). Two of models have been proposed by Warren and coworker, based on the prediction of mostly  $\beta$ -sheets with turns from the primary sequences and that many amino acids in the central domain naturally tends to form hydrogen bonds with water molecules (Warren and others 1986). However, the construction of these two models was unusual and did not compromise the principles of protein structures. Yet there is no counterpart to these models from existing protein structures. Mizuno suggested another 3-D structure model through energy calculation and based on the assumption that each octa-peptide repeat formed a helix (Mizuno, 1989). However, the Mizuno's models have a shortcoming of the premised assumption of helical conformation, which totally ignored predictions of correlative methods. Thus, the determination of a true ice nucleation protein structure will have to rely on the advanced analysis such as X-ray diffraction, nuclear magnetic resonance and so on.

#### *Formation of Ice Nucleation Sites*

Ice nucleation proteins do not equal to ice nucleation sites or ice nuclei. The protein is a necessary component but not sufficient. Ice nucleation proteins need post-translational modification to form functional ice nuclei. Three major related but chemically distinct types of ice nuclei were identified on the surface of various bacteria such as *P. syringae*, *E. herbicola*, and various strains of *ina+* recombinant *Escherichia coli*. The most effective group, called class A, is also the smallest population, which nucleates supercooled water at temperatures above  $-4.4\text{ }^{\circ}\text{C}$ . A second class, class B, is found on a larger portion of the cells and nucleates supercooled water between  $-4.8$  and -

5.7 °C. Most ice nuclei are effective at temperature at or lower than -7.6 °C, which is grouped into class C (Turner and others 1990). A similar classification is to define Type I, II, and III ice nuclei as those that are active at temperatures of above -5 °C, between -5 and -7 °C, and between -7 and -10 °C, respectively (Phelps and others 1986). Biochemical analysis revealed that the class A ice nuclei were composed of ice nucleation protein linked to phosphatidylinositol and mannose. The class B ice nuclei have the protein components presumably linked to the mannose and glucosamine moieties, but definitely lack the phosphatidylinositol. The class C ice nuclei appear to contain mainly the protein linked to a few mannose residues and to be partially imbedded in the outer cell membrane (Turner and others 1991).

$\gamma$ -radiation analysis indicated that the minimum mass of a functional ice nucleus, active only between -12 °C and -13 °C, was about 150 kD for all strains *P. syringae* and *E. herbicola* and *ina+* recombinant *E. coli*. Obviously, this ice nucleus belongs to the class C (Type III), with a mass approximately the same as a single ice nucleation protein. The studies also found that the threshold temperature of a nucleation site increased logarithmically with its size and the largest ice nucleation site measured active between -2 °C and -3 °C, possibly included an oligomer of 53 such subunits (Govindarajan and Lindow, 1988). The viewpoint that aggregation of ice nucleation proteins is essential for the substantial activity was also supported after obtaining the size of purified cell-free ice nuclei (Wolber and others 1986). Kozloff et al. summarized the whole presumable formation of the ice nucleation site as Figure 5.

### *Measurement of Ice Nucleation Activity*

Quantitative evaluation of the activity of ice nuclei might be a problem, due to the broad range of threshold temperatures of various types of ice nuclei, as well as that a massive supercooled liquid freezes too rapidly for sampling. Scientists realized that, when a sample was divided into large numbers of drops of equal volume and these droplets were simultaneously cooled at a fairly low rate of temperature change, two parameters could be observed to describe the activity, i.e. the threshold temperature,  $T$ , and the frequency of frozen droplets in total number of droplets at this temperature,  $f$ . Based on this theory, Vali generated the following equation to express the concentration of ice nuclei units at a given temperature:

$$\ln a(T) = -\frac{\ln(1-f)}{V_d} \cdot D \quad \text{Equation (1)}$$

Where besides  $T$  and  $f$ ,  $V_d$  is the volume of individual droplet, and  $D$  is the dilution factor. Plotting  $\ln a(T)$  against  $T$  in a semi-log graph gives the cumulative ice nucleation spectrum, which reveals the number of ice nuclei which are active at all temperature at and warmer than  $T$  in a unit volume of water. In some instance, the derivative of  $\ln a(T)$  is plotted in a semi-log graph, which is called the differential ice nucleation spectrum. The differential spectrum may be less commonly used, but it directly represents the variation of nucleus content with temperature (Vali, 1971).

Alternatively, ice nucleation activity may be expressed as the temperature at which 10%, 50% or 90% of droplets freeze or even simplified as the highest threshold temperature of the ice nuclei in the sample. Computer modeling has also been reported to analyze the ice nucleation activity.

### **Food application of bacterial ice nucleators**

Ina<sup>+</sup> bacteria initially demonstrate their important role in ecology and agriculture due to causing frost damage to forests and many economically important crops. The first commercial application of ina<sup>+</sup> bacteria was production of artificial snow. The *P. syringae*-based product is sold under the registered brand name of Snomax<sup>®</sup>.

Since freezing is the most commonly used method for food preservation, bacterial ice nuclei may play a functional role in the freezing control of foods. The food application of bacterial cell and cell-free ice nucleators was comprehensively reviewed by Li et al. (Li and Lee, 1995). In one set of experiment, bacterial cells of *P. syringae* and *E. herbicola* were incorporated into some model food systems, including sugar, protein solutions and oil/water suspensions. Addition of the ice nucleation active cells was proved to significantly raise the nucleation temperature and shorten the total freezing time (Li and others 1997; Zasytkin and Lee, 1999). Cell-free ice nuclei, extracellular ice nucleators (ECINs) protected fish actomyosin from denaturation during freeze/thaw cycling (Zhu and Lee, 2007). Ina<sup>+</sup> bacterial cells were also reported to improve the efficiency of freeze drying soy sauce, soybean paste (Watanabe and Arai, 1987). Ina<sup>+</sup> bacterial cells was studied as a tool for freeze texturing of raw egg white, bovine blood, soybean curd, milk curd, and so on, producing a flake-like texture (Arai and Watanabe, 1986).

However, a potential limit to the use of bacterial ice nuclei in foods comes from the safety concerns. All natural ina<sup>+</sup> microorganisms are Gram-negative bacteria. They may confound the assays for screening the bacterial contaminations in food. More importantly, these bacteria are classified as plant pathogens because they cause frost



damage to plants using their ice nucleation activity, although none of these bacteria has been found any mammalian toxicity (Li and Lee, 1995).

#### *Potential protection on frozen dough*

According to a market report by Packaged Facts released in earlier 2009, sales of frozen bread and frozen dough category in US sole share more than 10% of the frozen food market for the last five years (Porjes, 2009). Freezing technique offers many advantages, including extended shelf life and sales beyond a baker's immediate fresh market distribution area. Yet, it also leads to loss of the quality during storage, such as the increase in liquid loss and in proofing time, and the decrease in the retention capacity of CO<sub>2</sub> and in bread specific volume. The major issue affecting the properties of frozen dough is the yeast's stability during freezing, which has been extensively studied (Gelinas and others 1993; Gelinas and others 1994; Ribotta and others 2003; Rosell and Gomez, 2007). At freezing storage, yeast cell walls were damaged due to the reasons discussed in introduction section, and consequently release glutathione which weakens the dough by cleaving disulphide bonds in the gluten. Besides, it is also commonly believed that the longer proofing time and less bread volume was also due to the destruction of yeast during freezing which loss the enzymatic activity of gas production. Freeze/thaw cycles during the storage and transportation leads to the increase of ice crystal size and consequently aggravate the frozen damage to yeast. Traditionally, to prevent the effect of such reducing substances as glutathione, oxidants like ascorbic acid and potassium bromate are commonly added into yeast-leavened frozen dough. However, the use of potassium bromate has been banned in many other counties and also declined in U.S. in recent years due to the potential harm to consumers (Selomulyo and Zhou,

2007). Many researchers focused on developing other additives or dough compositions to compensate the deteriorate effect of freezing, such as emulsifiers, enzymes and hydrocolloids to stabilize the dough network, or optimized processing conditions. However, many of these approaches were treating the symptoms, but hardly got down to the root cause, the yeast.

Though some efforts have been spent on isolating freeze-tolerant strains of yeast (Teunissen and others 2002; Takagi and others 1997), it would not be easy to overcome the intrinsic vulnerability of yeast to freezing injuries. According to my best knowledge, there is no direct experimental data explaining why yeast and many other plant and animal cells are rather sensitive to freezing conditions compared to the bacterial cells. The scientists who study cryopreservation techniques commonly accept the two reasons that the response systems to stress in eukaryotic cells are less efficient and the complicacy in the intracellular structure, especially the intracellular membrane, decrease the adaptive capacity of eukaryotic cells to stress. Apparently, intracellular freezing is most lethal and direct damaging, whereas more often extracellular freezing leads to the hypertonic stress, under what condition membrane function becomes irreversibly lost. The dehydration in terms of either absolute water loss or active water loss is associated with the cell volume reduction, referred as the “Minimum Volume Theory”. Further, a mechanical stress builds up while the cellular skeleton including the intracellular and cytoplasmic membrane resist the volume reduction. When a critical point has been reached, this stress eventually causes progressive membrane damage, resulting in leakage and release of lysozyme, which was proposed to be the primary factor in freezing injury (Meryman, 1974). A recent published study on freeze-tolerant yeast implicitly supported

the above explanation. It found a strict relationship between the expressions of Aquaporin, a membrane channel protein involved in the transport of water, and the freeze-tolerance of yeast.

Given the above possible mechanism of freezing injury, the potential cryopreservation method on yeast should deal with the dramatic cell volume reduction by one means or another. This is actually how the so-called extracellular cryoprotectants function. The antifreeze agents, protein-based or polyol-based, were proposed to lower the extracellular freezable water content to diminish the build-up of osmotic gradient across the cell membrane (Zhang and others 2007; Panadero and others 2005). In several other researches, proline was shown to relieve the osmotic stress by stabilize the membrane structure (Takagi, 2008).

One interesting observation previously was that if seeding ice into a supercooling yeast suspension at subzero temperature, e.g.  $-7^{\circ}\text{C}$  or  $-2.5^{\circ}\text{C}$ , the yeast survival rate was increased by 3 folds or 6 folds respectively, compared to freezing initiated at about  $-15^{\circ}\text{C}$  (Nakamura and others 2009; Mazur, 1961). The advantages of initiating freezing at a higher subzero temperature are that the moderate fast ice formation allows the living cells to make metabolic adjustments resulting in smaller osmotic shock (Storey and Storey, 1996). Bacterial ice nucleators are adept in initiating ice nucleation at subzero temperatures. In fact, the cryoprotective function of bacterial ice nucleators was recently revealed on a fungal starter, *Geotrichum candidum* (Missous and others 2007). Given the widely utilization of yeast in food industry, if the bacterial ice nucleators could successfully protect the cells during freezing, the potential application will drive more

comprehensive and extensive investigation toward commercialization the bacterial ice nucleators.

Table 1 Comparison of amino acid compositions in mature zein proteins based on GenBank

Name	GenBank	Calculated mol wt	Number of amino acid in mature protein							
			Cys	Lys	His	Tyr	Asp	Glu	Gln	Non-polar
19kD $\alpha$ -zein B1	<a href="#">AAL16985.1</a>	25437.6	3	1	2	8	0	1	41	64.5 %
19kD $\alpha$ -zein B2	<a href="#">AAL16986.1</a>	29206.2	3	1	5	9	1	1	47	65.2 %
19kD $\alpha$ -zein B3	<a href="#">AAL16987.1</a>	26280.6	3	1	1	8	1	1	43	62.9 %
19kD $\alpha$ -zein D1	<a href="#">AAL16983.1</a>	26560.9	1	1	4	10	0	1	45	64.2 %
19kD $\alpha$ -zein D2	<a href="#">AAL16984.1</a>	26734.1	1	1	1	9	1	2	47	63.5 %
22kD $\alpha$ -zein 1	<a href="#">AAL16989.1</a>	28452.2	1	1	3	8	0	1	50	63.1 %
22kD $\alpha$ -zein 3	<a href="#">AAL16990.1</a>	28866.6	1	1	3	7	0	1	51	61.7 %
22kD $\alpha$ -zein 4	<a href="#">AAL16991.1</a>	29040.7	1	1	3	8	0	2	53	62.2 %
22kD $\alpha$ -zein 5	<a href="#">AAL16992.1</a>	28780.4	1	1	2	8	1	2	50	61.7 %

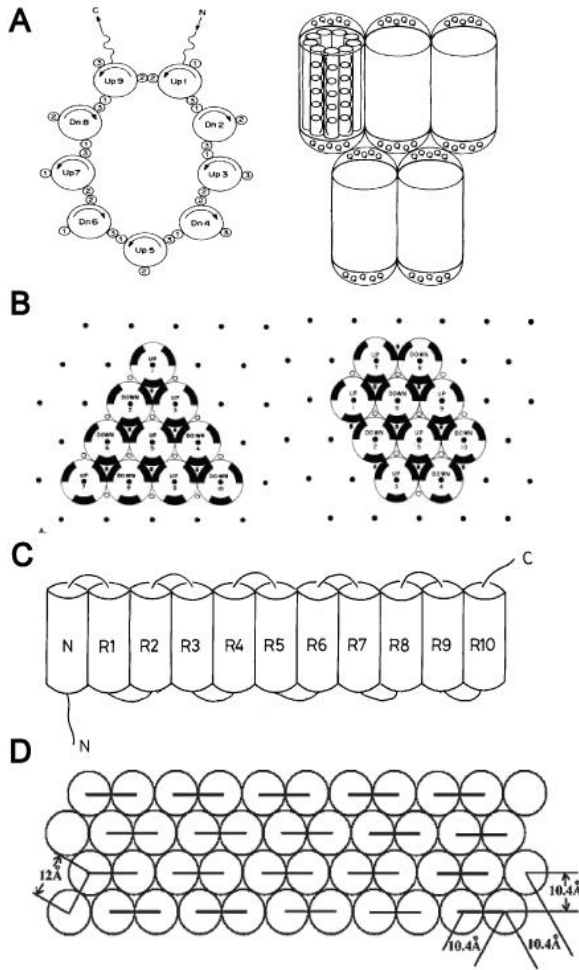


Figure 1 Conformation models of zein: A. nine-helical structure of zein molecule (left) and zein planes (right) (Argos et al., 1982); B. structural model for helix packing (Garratt et al., 1993); C. elongated prism model (Matsushima et al., 1997); D. hexagonal packing of zein prism (Wang et al., 2005).

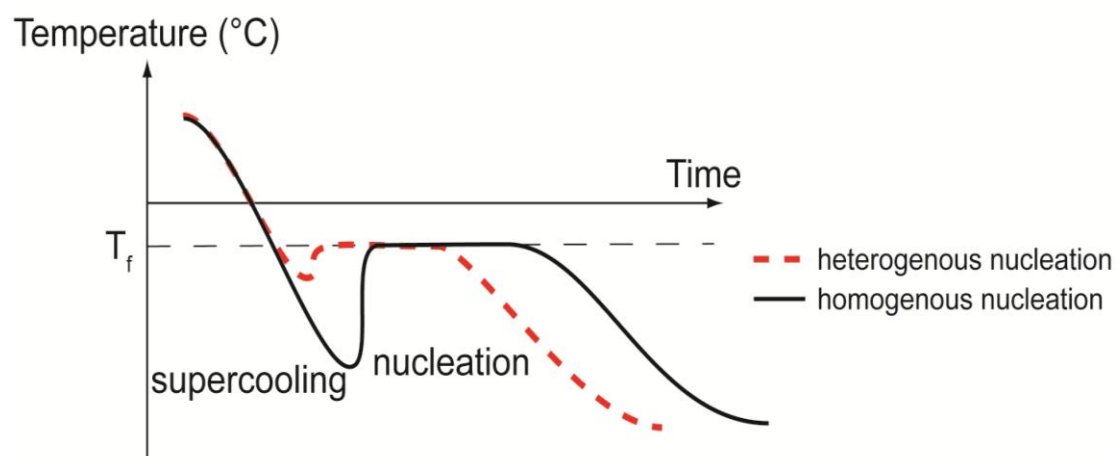


Figure 2 freezing curves of homogeneous and heterogeneous ice nucleation.

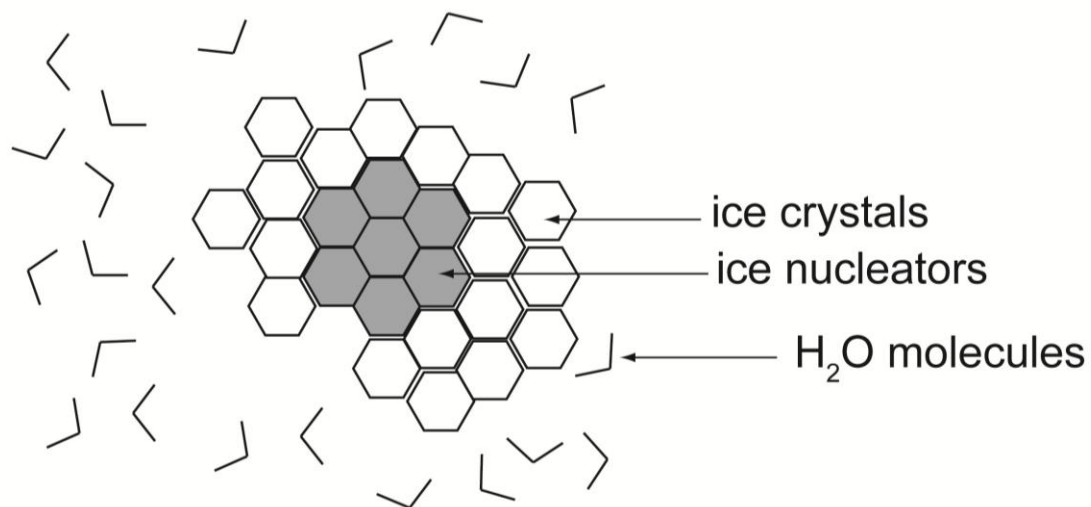


Figure 3 Ice nucleators and growth of ice crystals

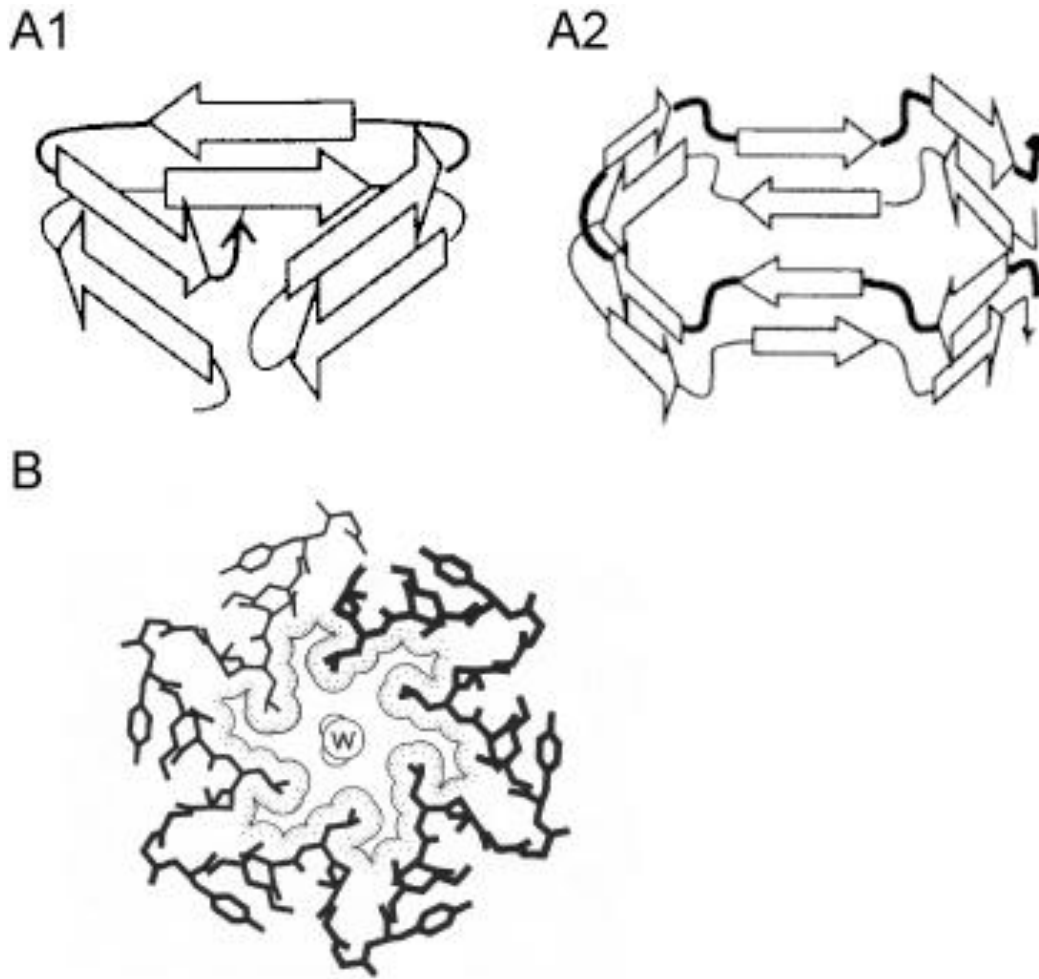


Figure 4 Structural models of bacterial ice nucleation proteins: A1. Triangular model of 48-mer forming three pairs of  $\beta$ -sheets; A2. Anti-parallel double-helix model of two 48-mers combination; proposed by Warren and Wolber (Hew and Yang, 1992); B. Skeletal axial view of helices proposed by Mizuno (Kajava, 1995).



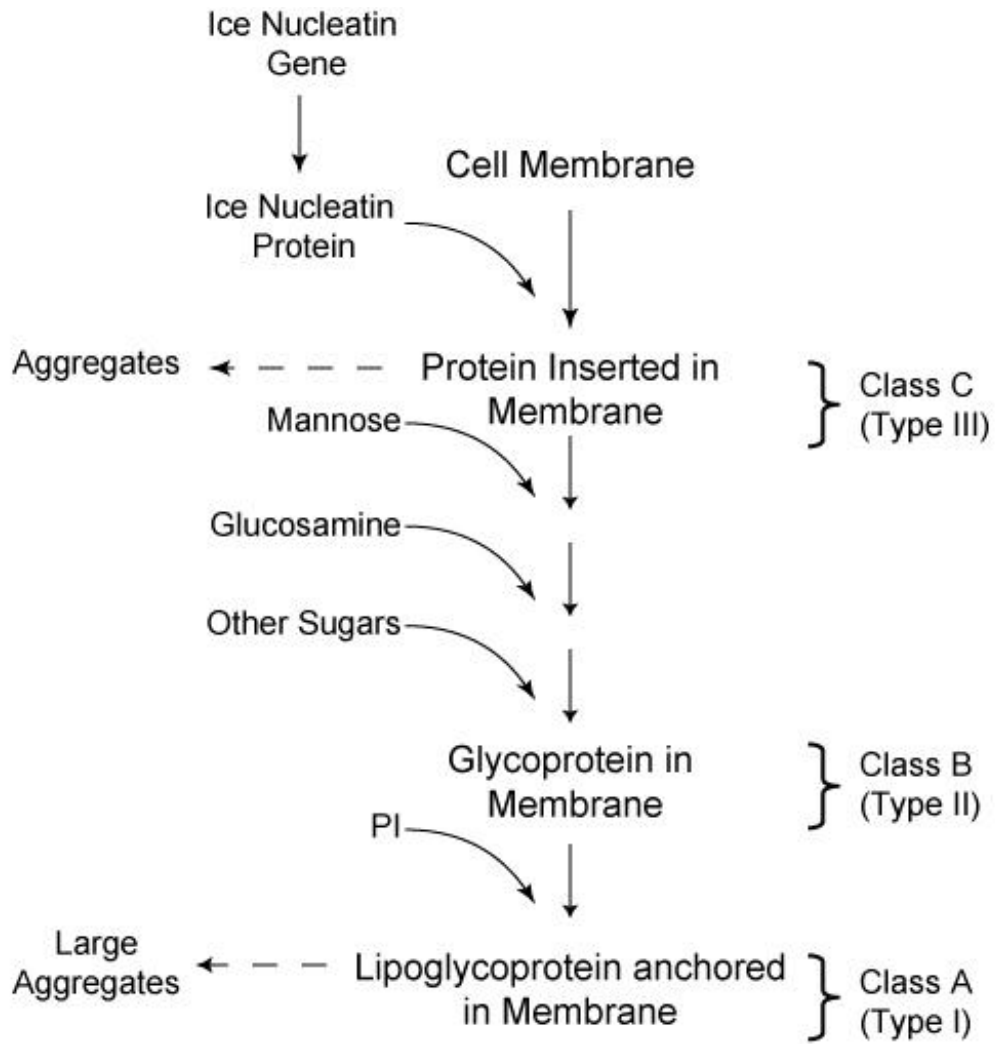


Figure 5 Suggested sequential formation of the ice nuclei (adopted from (Kozloff and others 1991))

## SCIENTIFIC RATIONALE

### Why use biogenic ice nucleators?

Water is the most fundamental component in food system and is involved in multiple types of reactions and processes. Freezing to food lowers the temperature as well as the water activities and therefore is among the most effective methods of food preservation. However, freezing, especially long-term frozen storage and failure of the cold chain, leads to deterioration in food qualities. Specifically in frozen bread dough, injured yeast cells lose their capability of gas production, resulting in longer proofing time, reduction of bread volume and crumb hardening. In addition, injured yeast cells release glutathione, a reducing agent which weakens the gluten network and consequently decreases the gas retention by gluten network.

Biogenic ice nucleators, such as extracellular ice nucleators (ECINs) from *Erwinia herbicola*, have been demonstrated to effectively reduce supercooling by initiating ice nucleation at subzero temperatures. Previous studies have revealed its promising prospects in food processing such as freeze drying, freeze concentration, freeze texturing and increasing efficiency of freezing (Li et al., 1997; Arai and Watanabe, 1986; Watanabe and Arai, 1987; Honma and others 1993). However, very few work studied the cryoprotective effects of biogenic ice nucleators on frozen foods. Notably, it was revealed that ECINs protected the fish actomyosin from denaturation in freeze/thaw cycles (Zhu and Lee, 2007).

In addition to food quality deterioration, another disadvantage of freezing technique is its high energy consumption during freezing, cold storage, and

transportation. Reducing time of freezing is considered a feasible effective method to reduce energy consumption. Time of freezing is simply defined as the time needed to decrease the temperature of food from a certain degree above freezing point to the steady temperature in a cold chamber. Previous work revealed that ECINs significantly decreased the time of freezing for model and real foods (Li et al., 1997; Li and Lee, 1998; Zasytkin and Lee, 1999). Thus, application of ECINs to frozen food may also save energy in freezing.

Application of biogenic ice nucleators in food freezing suffers from the safety concerns. All natural ice nucleating microorganisms are Gram-negative phytopathogenic bacteria. Although none of these bacteria has been found to cause any mammalian toxicity, they may confound the assays for screening the bacterial contaminations in food, and some bacterial components may be potential allergens. Therefore, mixing either ice nucleating bacteria or even purified cell-free ice nucleators is often unfeasible, inconvenient and unfriendly to formulation and labeling.

### **Why functional packaging instead of solely biogenic ice nucleators**

In recent years, improving functionality and using biopolymer are two emerging trends in food packaging, where nanotechnology may have its most promising prospects. Functional food packaging made by incorporation of active compounds into packaging matrix is a simple and flexible way to introduce new function to food system rather than mixing active compounds in. More importantly, the intensity of functional effects can be modulated and the duration of functional effects can be prolonged. Besides, the functional packaging may be reusable. In the case of ECINs, due to lack of food safety approval, mixing ECINs into food is currently not allowed. In addition, mixing ECINs

into solid food, such as meat, vegetable and fruits, is sometimes unfeasible. Yet mixing ECINs into processed food is inconvenient and unfriendly to formulation and labeling. Developing ice nucleation films provide a more versatile and convenient way to apply biogenic ice nucleators in frozen foods. In addition, the ice nucleation films allow immobilized ECINs to be reusable.

### **What's the advantage of zein?**

Using biopolymer in food packaging is the other trend because of their abundant resources and environmental-friendly nature. Zein, a prolamine protein enriched in the endosperm of maize, is a main co-product of the bio-ethanol industry. Zein film attracts a lot of scientific interests due to its unique thermoplasticity and hydrophobicity. In the area of food packaging, zein films and coatings are revealed to be suitable for packaging high-fat foods (Andersson, 2008). Zein can also tolerate low temperature and its film is freeze/thaw stable, allowing the film to be used for packaging frozen food (Padua et al., 2000). Furthermore, zein was made into functional food packaging bearing antimicrobial and antioxidant activities by incorporating lysozyme, albumin proteins, disodium EDTA and phenolic compounds into zein films (Gucbilmez et al., 2007; Mecitoglu et al., 2006; Arcan and Yemenicioglu).

### **What needs improvement to zein for ideal packaging of frozen foods?**

Appropriate packaging is important for frozen food, primarily serving the purpose on detaining dehydration, providing mechanical protection and preventing contamination. The most common problem for frozen food is the moisture loss through sublimation ("freezer burn"). Other special considerations for the frozen storage of food items include limited space for storage, low temperature effects on packaging materials and so on

(Krochta, 2006). Therefore, the properties of ideal packaging for frozen food should include:

- Made of food-grade materials, both the polymer and the plasticizers
- Water-resistant and moisture/vapor-proof: low water absorption and low water vapor permeability at high relative humidity (water activity)
- Does not become brittle and crack at low temperatures: freeze/thaw-stable of the matrix between polymer and plasticizers
- Durable: good mechanical properties on strength and flexibility
- Grease- and oil-proof
- Prefer low oxygen permeability and also prevent transmission to off-flavor and odor

As previously reviewed, zein has a GRAS status and can form grease-proof film that serve as a good oxygen and odor barrier and are freeze/thaw-stable. However, zein has intrinsic weakness in the mechanical properties and water sensitivity like most other biopolymer. When contacting food with high water activity, zein films could absorb the water from food and lose its integrity as a film. Also, solvent-cast zein films are too rigid and brittle for food packaging. Therefore, modification to zein films would be necessary to improve the mechanical properties and water resistance of zein films.

### **How to bring ice nucleation properties to zein film?**

Biologically active ingredients could be either blended into the film matrix or immobilized on the surface of films, depending on how the active ingredients play their functions. Biogenic ice nucleators facilitate the formation of ice by serving as heterogeneous ice nucleating seeds; therefore deposition onto the surface is desired. The

main challenge will be understanding properties of ECINs and the effects of physicochemical conditions on the association between the ice nucleators and surface of zein films.

Beside, the ice nucleation activity of developed film needs to be quantified. The Vali method is a relatively accurate method to quantify the activity of heterogeneous ice nuclei (Vali, 1971). However, the method is limited to test liquid which could be diluted. Because the ice nucleation films are unable to be diluted, a novel method needs to be develop for quantifying their activities

### **What food model to test the application of our functional packaging?**

According to a market report by Packaged Facts released in earlier 2009, sales of frozen bread and frozen dough category in US sole share more than 10% of the frozen food market for the last 5 years (Porjes, 2009). However, the shelf-life of frozen dough is limited and very vulnerable to any failure of the cold chain during storage and transportation. The primary factor is attributed to the cryosensitive nature of bakery yeasts, which are easily injured during prolonged cold storage and/or temperature fluctuations (Rosell and Gomez, 2007). Cold injury to yeast cells consequently leads to long proofing time, small bread volume and coarse crumb texture (Rosell and Gomez, 2007).

In my Ph.D. study, frozen bread dough serves as an example food system for the application of ice nucleation films. Frozen bread dough is chosen not only because improving its quality has realistic significance given bread is among the most important staple foods, but also because frozen dough is a solid system whose composition could be

simplified and well-defined. In addition, frozen dough is a comprehensively studied system whose mechanisms of freezing damage are well examined and understood.

## OBJECTIVE

The objective of this study is to develop nanotechnology-enabled zein-based ice nucleation films for the applications of frozen foods for the purposes of energy saving and quality improvement on frozen foods, i.e. frozen bread dough.

The specific aims of this study are:

- Development of methods for modulating the surface hydrophilicity of zein films.
- Improvement of the mechanical properties and water resistance of zein film by plasticization, so as to make them applicable on frozen dough.
- Development of zein films with immobilized extracellular ice nucleators from *Erwinia herbicola* on the surface, with their ice nucleation activity quantified.
- Examination of the cryoprotective effects of extracellular ice nucleators from *Erwinia herbicola* on the quality of frozen bread dough and of the corresponding mechanisms.
- Analysis of the efficacy of zein-based ice nucleation films on the quality of frozen bread dough.



## OUTLINE

To fulfill the objective and meet the specific aims listed in previous section, this study is separated into two initially parallel researches on zein films and on extracellular ice nucleators (ECINs), which later merge at developing ice nucleation films and then examining the frozen bread dough quality when the films were applied.

In Chapter One, obtaining flawless surface of cast zein films by using acetic acid as solvent is described. In addition, effective approach to control surface hydrophilicity of zein films, i.e. UV/ozone treatment, is demonstrated. This part of work prepares the zein films with suitable surface properties for later work on immobilization of ECINs

In Chapter Two, the issues of cast zein films on poor mechanical properties and water sensitivity are addressed by incorporating tributyl citrate as plasticizers. This part of work allows zein film to show acceptable flexibility for wrapping frozen dough and to maintain the film integrity against water absorption during frozen storage in the later experiments.

In Chapter Three, the potential of ECINs on improving the baking quality of frozen bread dough is revealed and the mechanisms of cryopreservative functions of ECINs are explored. This part of work confirms the expected effects of ECINs on frozen bread dough and provides preliminary understanding on ECINs function as cryoprotectants.

In Chapter Four, the development of optimum conditions to immobilize ECINs on the surface of zein film is described. Meanwhile, a novel method to quantify the ice nucleation activity of films is explained and verified.

In Chapter Five, the application of zein-based ice nucleation films on frozen bread dough is examined. The positive effects of ice nucleation films on energy saving and improvement of frozen food quality are demonstrated.

The research works that related to the current study are included in appendix as a reference for future study. In Appendix A, a new type of zein-based biomaterial was synthesized by chemical modification to zein with lauryl chloride through an acylation reaction, in order to address the issue on poor mechanical properties of cast zein films. In Appendix B, a work on isolation and characterization to biogenic ice nucleators from *Xanthomonas translucens* is demonstrated. In Appendix C, purification to the ice nucleation proteins by ice affinity was demonstrate with the defects and potential improvement of the method discussed.

# **CHAPTER ONE. ENGINEERING ZEIN FILMS WITH CONTROLLED SURFACE MORPHOLOGY AND HYDROPHILICITY**

*The work in this chapter has been published in the title of “Engineering Zein Films with Controlled Surface Morphology and Hydrophilicity” in the Journal of Agricultural and Food Science (Volume 57, Issue 6, Pages from 2186 to 2192) on February 23, 2009.*

## **Abstract**

A new method to engineer zein films with controlled surface morphology and hydrophilicity has been developed. It was observed that zein films cast from acetic acid showed much smoother surfaces compared to those from aqueous ethanol solutions. Furthermore, zein films with controlled surface hydrophilicity have been engineered by using UV/ozone treatment, which efficiently decreased the water contact angles of zein films from 80 ° to less than 10 ° within 130 s. Analysis indicated that the change in surface hydrophilicity was due to the change in surface elemental composition, and UV/ozone treatment converted portion of the surface methyl groups to carbonyl groups. This part of study prepared zein film with an appropriate surface for the immobilization of ECINs.

## **Introduction**

Biodegradable polymers from renewable resources have attracted a lot of interest in recent years because they have positive impacts on the economy and environment. Among the biopolymers, zein, a major protein found in the endosperm of corn, as a main co-product of the bio-ethanol industry, has been of scientific interests for decades.

Zein forms unique film that is tough, glossy, hydrophobic, greaseproof, and resistant to microbial attack, simply by casting (Shukla and Cheryan, 2001). These physical properties grant zein film unique functional roles as coating and packaging materials in food and pharmaceutical industries (Shukla and Cheryan, 2001). However, the applications of zein films for functional coating and packaging are largely dependent upon their surface properties, including surface morphology and hydrophilicity. Previous research suggested that surface properties of zein-based microspheres significantly affect the encapsulation efficiency of active compounds (Wang et al., 2005; Muthuselvi and Dhathathreyan, 2006). Gao et al. revealed that the surface morphology of zein in-situ gels affected the release profile of pingyangmycin in that a smoother surface formed a better barrier on the surface of the gel to eliminate an initial burst of release (Gao et al., 2007). However, until now, little work has been done on direct modification of zein film surfaces to achieve controlled surface morphology and hydrophilicity.

Studies showed that certain solvents, such as acetic acid (AcOH), could alter the secondary and tertiary structures of zein in solutions (Selling and others 2007). It has also been reported that acidification to zein solution using AcOH produced fibers with an appearance distinct from those without AcOH (Torres-Giner and others 2008). However, a clear understanding of the effects of solvent types (i.e., AcOH versus EtOH) on the surface properties as well as the underlying mechanism, which is essential in designing effective methods to control the physical properties of zein films, is still lacking.

On the other hand, combination of ultraviolet light ( $\lambda = 184\text{--}257\text{ nm}$ ) and ozone gas (UVO) treatment has been recognized as an effective method for the chemical modification of synthetic polymer (Macmanus and others 1999; Phely-Bobin and others

2000), which leads to extensive and rapid oxidation of the very top surface of polymer, but is not likely to alter the bulk properties, such as thermal and mechanical properties as well as moisture permeability of the polymer. This convenient and promising surface modification technique is worthy trying on biopolymers like zein.

The objective of this part of study was to develop effective methods for modulating the surface hydrophilicity of zein films. For this purpose, the surface morphology and surface hydrophilicity of zein films prepared from using either EtOH/water mixtures or AcOH as solvents have been studied by tapping mode atomic force microscopy and static water contact angle analyses, respectively. UVO treatment has been applied to modify zein film surfaces with controlled surface hydrophilicity. The possible mechanism of UVO surface modification has been investigated by a combination of contact angle measurements and X-ray photoelectron spectroscopy analysis.

## **Materials and Methods**

### *Materials*

Zein (lot #F40006031C1, regular grade) was obtained from Freeman Industries, LLC (Tuckahoe, NY). Glacial acetic acid (AcOH) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). EtOH (95%) was purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Deionized water was used throughout the experiment.

### *Zein films preparation*

Zein was dissolved using 60–95% (v/v) EtOH or 60–100% (v/v) AcOH as solvents to the concentration of 0.5 (w/v) or 4% (w/v). All of the zein solutions were

shaken vigorously overnight, filtered through 0.45  $\mu\text{m}$  filters (Whatman Inc., Florham Park, NJ) to remove insoluble impurities, and then spin coated onto silicon (Si) wafers, which were pre-cleaned with piranha solutions, using a Laurell model WS-400A-6NPP/LITE spin coater (Laurell Technologies Corp., North Wales, PA). Zein in EtOH/water solutions was spun at 4000 rpm for 80 s, while zein in AcOH/water solutions was spun at 800 rpm for 20 s, followed by 3000 rpm for 20 s. Clear and homogeneous zein films immediately formed and were then dried in a 50  $^{\circ}\text{C}$  vacuum oven overnight to remove the residual solvent. Subsequently, these zein films were stored in desiccators at room temperature before further analysis. The zein films were denoted according to sample preparation methods. For example, the first letter A or E denotes that either AcOH or EtOH was used as the solvent, while the number that follows corresponds to the concentration of zein solution for film preparation.

#### *UVO treatment*

Si wafers bearing zein films were prepared by spin coating using 4% (w/v) zein in glacial AcOH solution. These zein films were exposed to ultraviolet emission from a low-pressure quartz-mercury vapor lamp at the distance of 1 in. in the cabinet of a UV/Ozone Cleaning System (model 42, Jelight Co. Inc., Irvine, CA) with an exposure time ranging from 0 to 180 s.

#### *Contact angle measurement*

The static water contact angle measurements were carried out using a VCA Optima XE Dynamic Contact Angle Analyzer (AST Products Inc., Billerica, MA) at ambient condition. The image was recorded by a CCD camera immediately after the

water drop was deposited onto the zein film surface. The contact angle was measured on the images.

#### *Tapping mode atomic force microscopy (TP-AFM)*

TP-AFM images were collected by NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA) with a Si-etched RTESP7 cantilever (Veeco Nanoprobe, Camarillo, CA) under ambient conditions. Images were analyzed using the software NanoScope DI v. 5.30 (Veeco Instruments Inc., Santa Barbara, CA).

#### *X-ray photoelectron spectroscopy (XPS)*

Elemental compositions of different zein films were characterized using a PHI5400 spectrometer (Physical Electronics, Inc., Chanhassen, MN) with a dual anode X-ray source (Mg and Al) for excitation. The electron takeoff angle was 45 °, and the analyzer was operated in the constant energy mode for all measurements. XPS survey spectra over a binding energy range of 0–1000 eV were acquired using analyzer pass energy of 160 eV and 40 eV for high-resolution elemental scans. The vacuum pressure was around  $7.5 \times 10^{-8}$  mbar during spectral acquisition.

#### *Statistical analysis*

All data were expressed as means  $\pm$  standard deviations (SDs) with  $n$  equal to 6. A single-factor analysis of variance (ANOVA) was performed to assess the statistical significance of results between groups using SigmaStat 3.5. The statistical analyses were performed at a confidence level of 95%.

## Results and Discussion

### Effects of solvent on the surface properties of zein films

Osborne reported that zein could be readily dispersed in 50–95% EtOH/water mixtures as well as in glacial AcOH and AcOH/water mixtures, but not in either pure alcohol or water (Osborne, 1897). In our study, the zein sample used contains mainly  $\alpha$ -zein and was readily dissolved in 60–100% AcOH and 60–95% EtOH at room temperature. To study the effect of solvent on the surface properties of zein films, 0.5% w/v zein solutions were used to prepare zein films. Even though all solutions were transparent, zein in AcOH solutions exhibited yellowish green color compared to the yellow-orange color of zein in EtOH solutions. Also, zein in AcOH solutions was less viscous than zein in EtOH at the same concentration. The reason for the lower viscosity of zein in AcOH solutions has been reported as the plasticization effects of the carboxylic acid (Selling and Sessa, 2007). Considering the viscosity of the zein solutions and the different evaporation rates of AcOH and EtOH, a slightly lower spin speed was adopted for film preparation from AcOH-based solutions. The thicknesses of the obtained zein films were  $> 1 \mu\text{m}$ , as estimated by ellipsometry. Within this thickness range, the surface confinement effects from the substrate (Si) on the surface properties of zein films were negligible because surface confinement effects usually occur with film less than 50 nm thick.

Two representative TP-AFM images of zein films cast from glacial AcOH and 95% EtOH solutions are shown in Figure 6. The surface of zein film prepared from AcOH solution appeared to be smooth and featureless, while the surface prepared from 95% EtOH solution was rough and composed of granules with diameters ranging from 30



to 80 nm. Root mean square (RMS) roughness calculated by the software indicated that zein films prepared from EtOH solutions were 20~32 times rougher than those from AcOH solutions (Figure 7). In addition, the increasing EtOH concentration in zein solution made rougher surface of zein films. In contrast, the concentration of AcOH did not affect the RMS roughness of films.

The surface morphology of zein films depends upon how zein molecules aggregate on the Si wafer surface. However, as previously discussed, there is no clear or confirmative understanding about the molecular conformation of zein molecules. Nevertheless, the zein conformation in acetic acid solution was investigated recently through rheological analysis and SAXS analysis. The result suggested that in dilute solution (<43 mg/mL), zein stay in its original folding and conformation; whereas in concentrated solution, the zein molecules overlap and/or penetrate each other and form intermolecular associations. This is very different behavior from ethanol solution, where the conformation of zein molecules does not change as concentration increases (Li and others 2011). Besides, at a same concentration, the apparent viscosity of zein in acetic acid solutions was at least 160 times lower than the zein in ethanol solutions (Fu and Weller, 1999; Li et al., 2011), suggesting that the zein oligomers are much larger in ethanol solutions than they are in acetic acid solutions. Based on the rheological analysis to zein in the two different solvent environments, a reasonable hypothesis is that during film formation from acetic acid solution, zein protein chains stretched out and overlap each other which forms a continuous network resulting in smoother surface; whereas from ethanol solution, zein molecules keep large oligometric aggregate resulting in the observed patterned surface of film.

To better understand the solvent effect, the surface hydrophilicity of the spin-coated zein films was analyzed in contact angle measurements. Figure 8 shows the effects of solvent concentration on the static contact angles of zein films prepared from AcOH and EtOH solutions. The water contact angles of zein films from the EtOH solutions were consistently lower than those from AcOH solutions, suggesting that the zein films cast from AcOH solutions are more hydrophobic than those from the EtOH solutions.

Very often, the surface hydrophilicity of a film is affected by its functional group or elemental composition on the surface. Therefore, XPS was used to investigate the surface elemental compositions of different zein films (Table 2), where the ratio of elemental oxygen to elemental carbon of film prepared from 95% EtOH solution (E95) was slightly higher than from glacial AcOH solution (A100). However, this ratio was significantly higher for film prepared from 60% EtOH solution (E60) than from 60% AcOH solution (A60). Such a difference in surface elemental composition correlated very well with the water contact angle results.

### **Modification to the surface hydrophilicity of zein film by UV/ozone treatment**

UVO treatment can cause oxidation to the surface groups via a sequence of reactions. Molecular oxygen excited by UV light dissociates to form atomic oxygen,  $O(^1D)$ , and ozone,  $O_3$ , which are very reactive and can further generate hydroxyl radicals,  $\bullet OH$ . UV light also excites the film and generates free radicals. All of these reactive radical species subsequently react with the film surface and eventually oxidize the surface. Through the reactions, different kinds of polar functional groups could be generated, including hydroxyl, peroxy, carboxyl, and ester groups, depending on the

extension of oxidation, which eventually alters the surface hydrophilicity (Macmanus et al., 1999).

In the present study, zein films prepared from 4% (w/v) zein in glacial AcOH solutions were exposed to UVO treatment as they were intrinsically most hydrophobic as previously shown. Surface water contact angles were measured to monitor the modification process (Figure 9). The result indicated that UVO treatment was able to quickly convert the zein film surfaces from highly hydrophobic to highly hydrophilic over a very short period of exposure (2 min), evidenced by the decrease of the water contact angle from about 80 ° to less than 10 °. There were three stages during the UVO treatment: (1) from 0 to 60 sec, there was a slight increase in the water contact angle of the zein film, which may be due to the change of surface morphology resulting from the cross-linking reactions in protein molecules (25); (2) from 60 to 120 sec, a dramatically decrease in water contact angle from 78 to 10 ° was detected; and (3) after 120 sec, further UVO exposure had negligible impact on the surface contact angle. The surface elementary compositions of zein films at the three stages were also studied by XPS (Table 3). It was noted that during UVO treatment, the carbon content decreased, while the oxygen content increased with prolonged UVO exposure time. To further clarify the elemental changes on surface, XPS high-resolution scans on carbon *1s* were performed on zein films. By decomposing the high-resolution carbon *1s* spectra, the chemical structure of the oxidized functional groups as well as the oxidation mechanism on the zein film surface were elucidated (Figure 10). There were three components in all of the carbon *1s* high-resolution spectra: peak 1, which was located at around 284.1 eV, with binding energy characteristic to aliphatic carbons (C–H and/or C–C) showed a slight

shift toward the higher energy level after UVO treatment; peak 2, which was located at around 285.6 eV, originated from either C–O–C or C–OH; and peak 3, which was from 287.1 to 287.4 eV, corresponded to –COOH. The area for each peak, which corresponded to the composition of relevant functional groups, was calculated by fitting to a Gaussian function and was summarized in Table 4. The results indicated that, during the UVO treatment, the composition of carboxyl groups on film surface increases from 19.5% to 30.0%, whereas the composition of aliphatic carbons decreased from 68.1% to 56.9%, which correlated with the decrease in surface hydrophilicity of films. It should be emphasized that the UVO-induced oxidation occurred only at the very top surface of the zein protein film, and –OH or –COOH groups formed have no obvious detrimental side effects. Therefore, this surface modification method shows tremendous promise for food applications as compared with many other chemical modification methods.

In summary on this part of study, effective new approaches to control the surface morphology and surface hydrophilicity of zein films were demonstrated. Alternation of the solvents from EtOH to AcOH for film preparation led to distinct surface morphology and hydrophilicity. Zein film cast from AcOH revealed a much smoother and flawless surface resulted from molecular arrangement of zein protein in a more organized manner. On the other hand, zein films prepared from EtOH solutions were more hydrophilic due to their higher percentage of polar functional groups on the surface. As discussed, the smooth surface of zein films like prepared from AcOH is preferable for packaging applications. On the other hand, this work also demonstrated that the surface of zein films

could be effectively and efficiently modified by UVO treatment, which converted some of the  $-C-H$  groups into  $-COO-$  groups. With controllable surface properties, the potential to immobilizing biogenic ice nucleators on zein films could be readily exploited.

Table 2 Surface elemental compositions of zein films prepared from 0.5% (w/v) zein solutions using different solvents \*

Solvents for zein films preparation	Surface elemental compositions				RMS roughness (nm)	Water contact angle (degree)
	C (%)	N (%)	O (%)	O/C		
60% AcOH	56.8	13.7	29.5	0.519	$0.95 \pm 0.07$	$73.7 \pm 3.0$
100% AcOH	63.1	13.4	23.5	0.372	$1.51 \pm 0.20$	$79.4 \pm 2.7$
60% EtOH	27.2	5.94	66.9	2.455	$19.2 \pm 0.4$	$40.9 \pm 2.0$
95% EtOH	57.4	14.4	28.2	0.491	$32.1 \pm 0.3$	$73.2 \pm 0.7$

\* For comparison, the surface roughness and surface water contact angle of each zein films were also included.

Table 3 Surface elemental compositions of zein films prepared from 4% (w/v) zein in AcOH solution after UVO exposure for various time\*

UVO exposure time (s)	Surface elemental compositions				Water contact angle (degree)
	C (%)	N (%)	O (%)	O/C	
0 s	71.4	12.0	16.6	0.23	82.4
90 s	65.1	12.7	22.2	0.34	41.9
180 s	62.2	14.2	23.6	0.38	11.1

\* For comparison, the surface water contact angles of zein films after each exposure were also included.

Table 4 Compositions of carbon *1s* of zein films after UVO exposure for various time by deconvolution

UVO exposure time (s)	Composition and binding energy of C <i>1s</i> signals		
	C-C, C-H	-C-OH	-COOH
0 s	68.1% (284.0 eV)	12.4% (285.6 eV)	19.5% (287.1 eV)
90 s	61.0% (284.1 eV)	12.3% (285.6 eV)	26.7% (287.1 eV)
180 s	56.9% (284.3 eV)	13.1% (285.7 eV)	30.0% (287.4 eV)



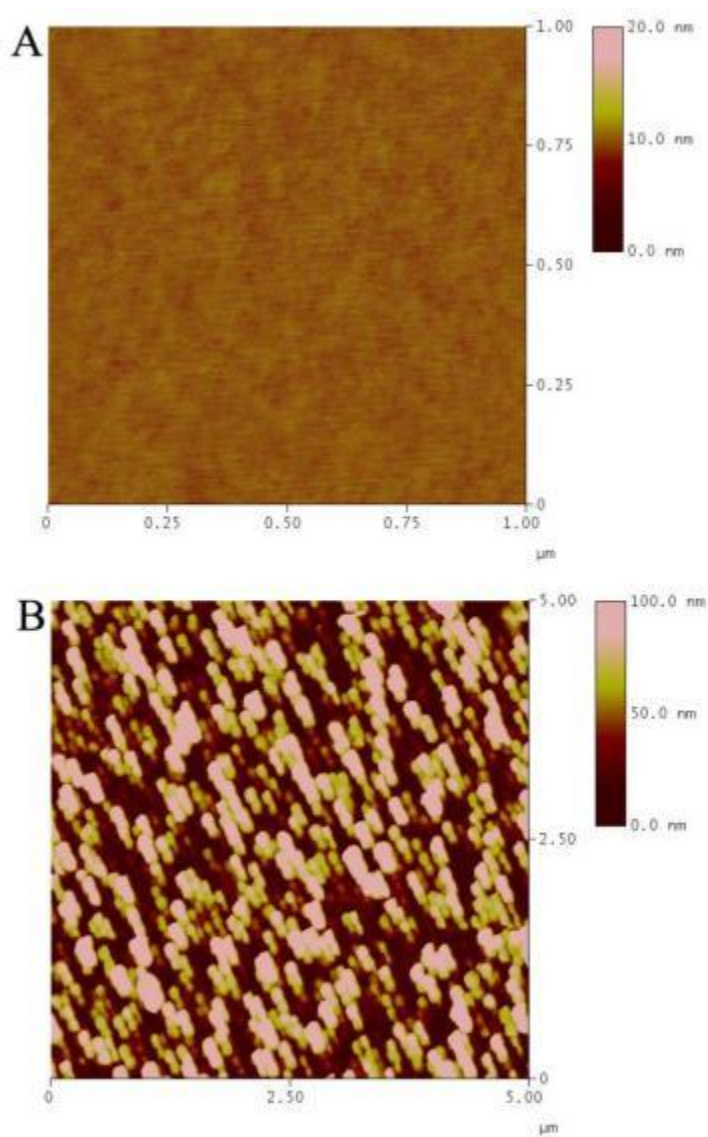


Figure 6 TP-AFM images of zein films prepared from 0.5% (w/v) zein in (A) glacial AcOH and (B) 95% EtOH solutions\*.

\* The scale size is 1  $\mu\text{m} \times 1 \mu\text{m}$ .

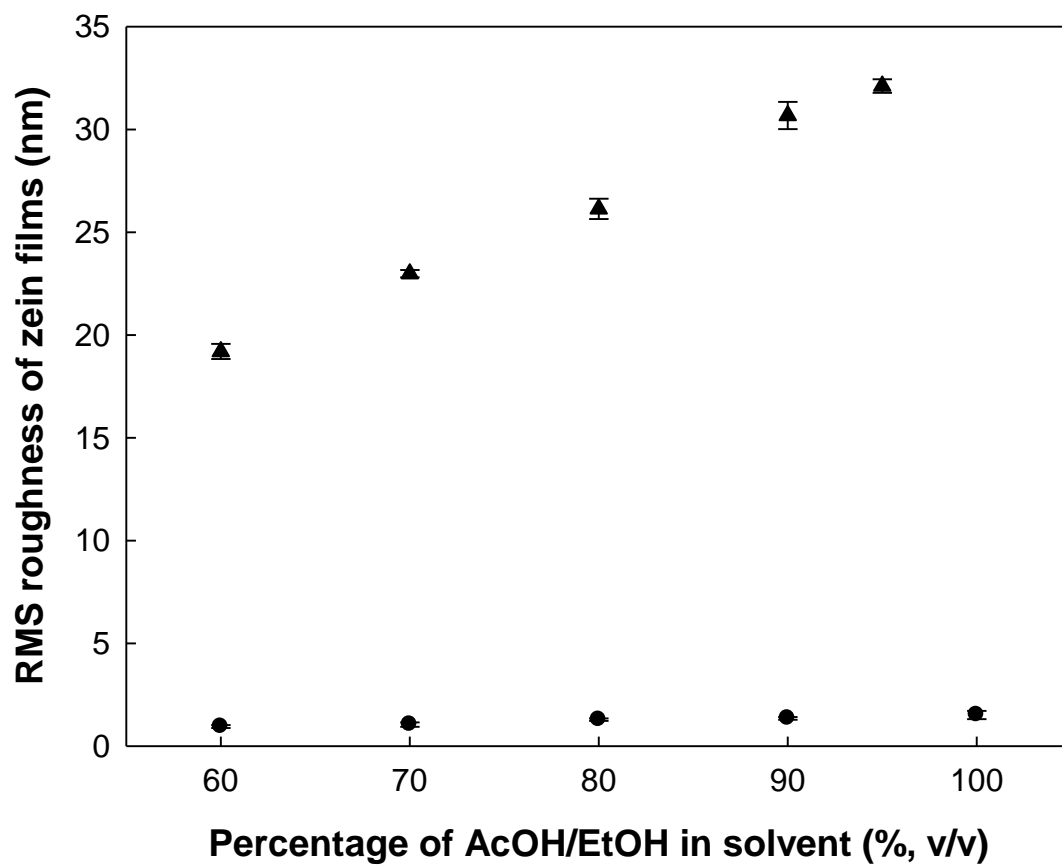


Figure 7 Effects of solvent on the surface roughness of zein films prepared from 0.5% (w/v) zein in AcOH (●) or EtOH (▲) solutions.

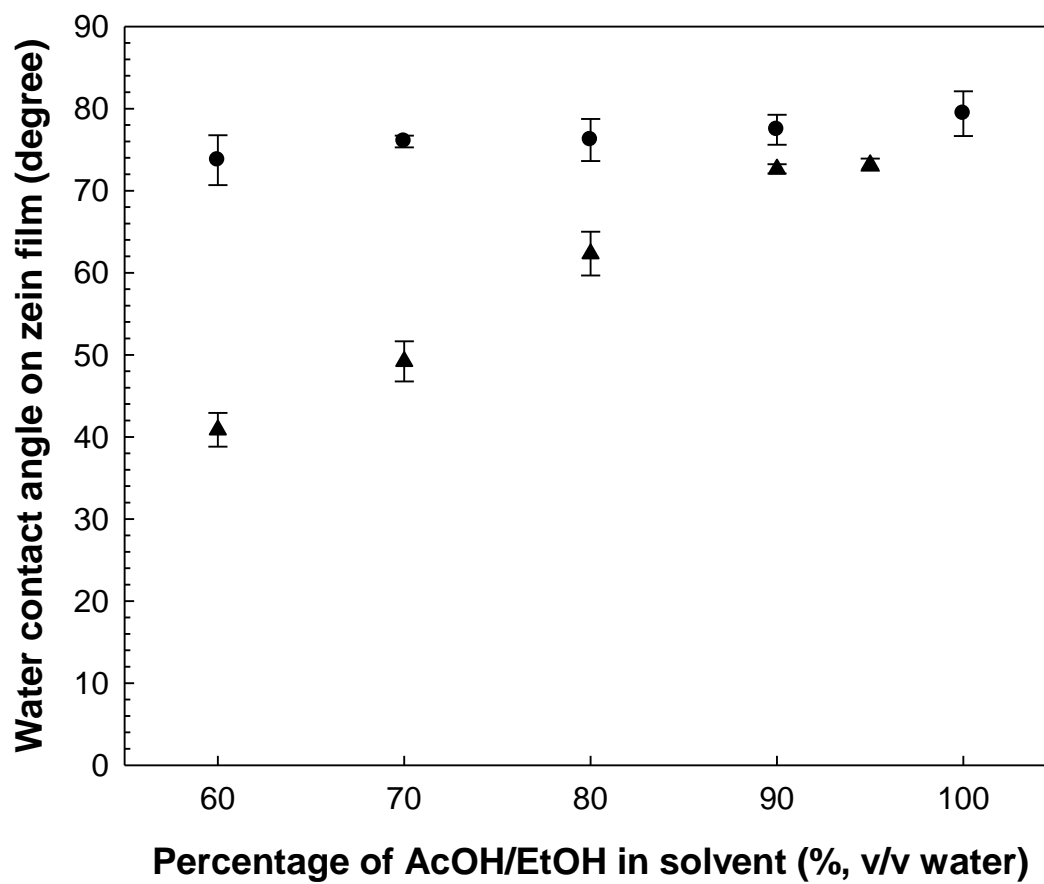


Figure 8 Effects of solvents on the water contact angles of zein films prepared from 0.5% (w/v) zein in AcOH (●) and EtOH (▲) solutions.

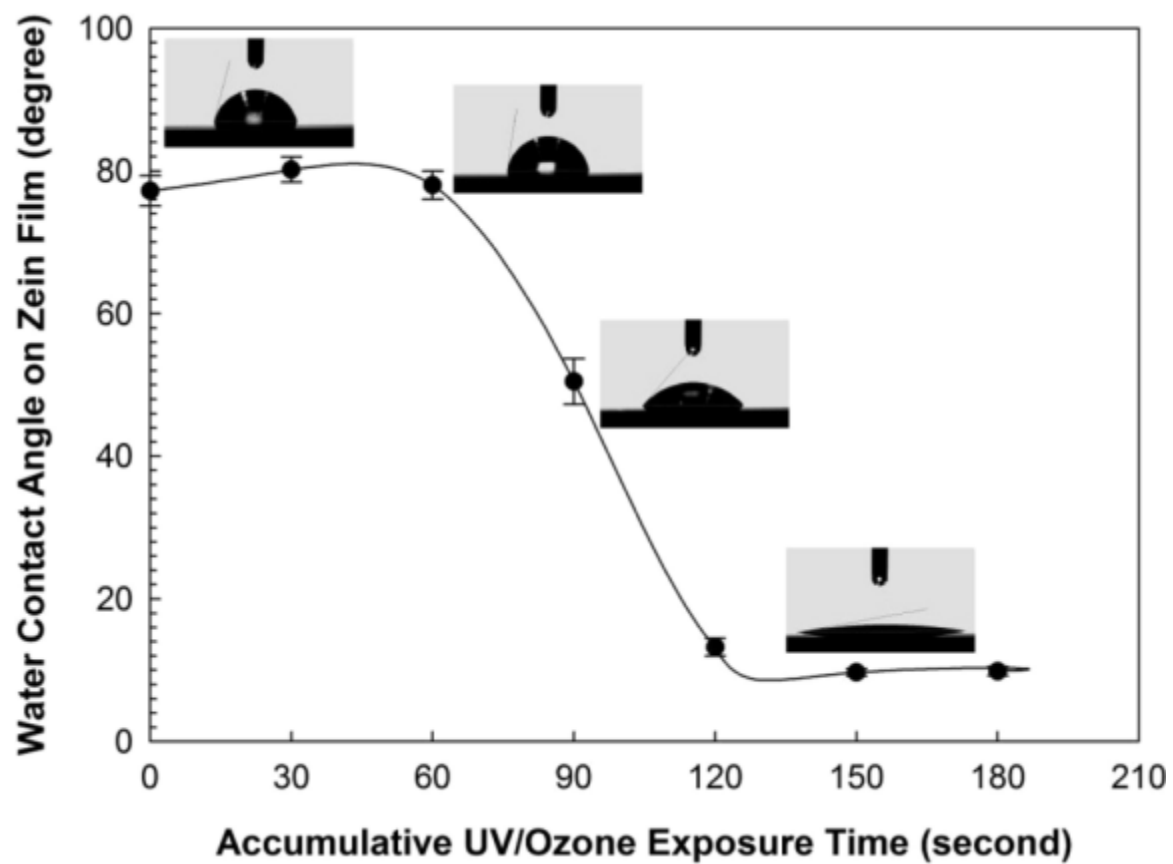


Figure 9 Effect of UVO exposure time on the water contact angles of zein film prepared from 4% (w/v) zein in AcOH solution.

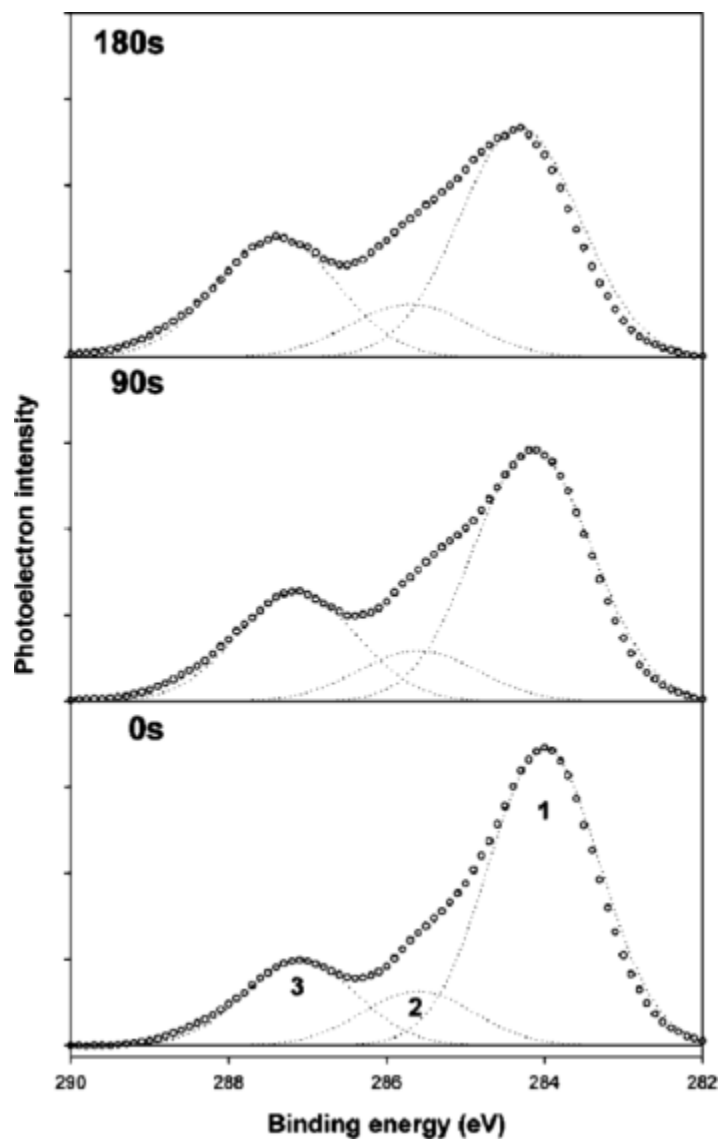


Figure 10 High-resolution XPS carbon  $1s$  spectra of zein films treated by UVO for 0, 90, and 180 sec\*.

\* The circles represent original XPS data, and dots represent deconvolution peaks fitted from three Gaussian functions.

## **CHAPTER TWO. IMPROVED MECHANICAL PROPERTY AND WATER RESISTANCE OF ZEIN FILMS BY PLASTICIZATION WITH TRIBUTYL CITRATE**

*The work in this chapter has been published in the title of “Improved Mechanical Property and Water Resistance of Zein Films by Plasticization with Tributyl Citrate” in the Journal of Agricultural and Food Science (Volume 60, Issue 23, Pages from 5988 to 5993) on May 8, 2012.*

### **Abstract**

This part of work aimed at addressing the issues of zein film on poor mechanical properties and water sensitivity. These two weaknesses of pure zein film make it inapplicable for wrapping frozen dough and limit any other applications as food packaging. For these purposes, tributyl citrate (TBC) was added into zein film to the mass ratios from 10% to 50%. It was observed a significant decrease in Young's modulus from 409.86 MPa in pure zein films to 136.29 MPa in zein films with 50% TBC. Among all films, those containing 10% TBC is most flexible and toughest. Both DSC and microscopy methods suggested that the TBC may be loaded up to 20% to avoid micro-sized phase separation. Through modeling with experimental data, incorporating 50% TBC reduced the water absorption capacity to 12.94% compared to 31.78% by pure zein film. More importantly, the integrity of zein/TBC film was maintained at high relative humidity and even after immersed in water. However, more than 20% TBC in zein films led to micro-sized phase separation which was harmful to mechanical properties. This

part of study prepared zein films with acceptable mechanical properties and water resistance to be applied on frozen food items in the following work.

## **Introduction**

Zein, a prolamine protein enriched in the endosperm of maize, is a main co-product of the bio-ethanol industry. Zein film is unique in its thermoplasticity and hydrophobicity. However, solvent-cast zein films are rigid and brittle, and thus, plasticizers are needed to improve their flexibility (Shukla and Cheryan, 2001).

Some polyol compounds, such as glycerol, sorbitol, polypropylene glycol, polyethylene glycol and monosaccharides have been blended into zein as plasticizers. Addition of these hydrophilic plasticizers generally decreased the strength and stiffness while increased the flexibility of zein films. However their effects on water vapor barrier properties are controversial and are dependent on the methods of film preparation. Among the hydrophilic plasticizers, addition of polyols increased the water vapor permeability, as a result of their hydrophilic nature and phase separation due to incompatibility between hydrophilic plasticizers and hydrophobic zein matrix (Parris and Coffin, 1997; Ghanbarzadeh et al., 2006b; Ghanbarzadeh et al., 2007a; Ghanbarzadeh and others 2006a; Ghanbarzadeh and others 2007b). Fatty acids, such as palmitic acid, oleic acid and others have been investigated on mixing into and/or coating the films prepared from zein resins. They surpassed those hydrophilic plasticizers in maintaining the film strength while significantly improving film flexibility as well as water resistance (Wang and Padua, 2005; Lai et al., 1997; Santosa and Padua, 1999). Citrate esters, derived from citric acid, are a large group of hydrophobic plasticizers commercially used

in food packaging and medical products, because they are non-toxic and biodegradable, which allows the packaging material contact food items and will not harm the biodegradability of the base material (Andreuccetti and others 2009). Tributyl citrate and acetyl tributyl citrate were used to plasticize poly (lactic acid) films, resulting in significantly increased elongation at break and lowered water vapor permeability (Labrecque and others 1997; Wang and others 2008).

The objective of this part of study was to improve the mechanical properties and water resistance of zein film by plasticization with tributyl citrate. For this purpose, the effects of tributyl citrate as a hydrophobic plasticizer on mechanical properties and water resistance of solvent cast zein films was investigated, with their tensile properties, glass transition temperature, water absorption and water vapor permeability evaluated.

## **Materials and Methods**

### *Materials*

The  $\alpha$ -zein of biochemical-grade purity was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Tributyl citrate (TBC,  $\geq 97\%$ ), potassium sulfate ( $>99\%$ ) were obtained from Aldrich Chemical Company. Glacial acetic acid was obtained from Fisher Scientific, Inc. (Pittsburgh, PA). Calcium chloride (Anhydrous, 8 meshes, Spectrum Chemical Corp., New Brunswick, NJ) was obtained from VWR Corp. (Radnor, PA).

### *Zein film preparation*

Zein and TBC solutions were prepared by dissolving corresponding amount of TBC and zein in 70 °C glacial acetic acid to the concentration of 2 g total substances per



10 mL Acetic acid. In the total substances, the mass ratio of TBC was 10%, 20%, 30%, 40% and 50%. To make zein films, the solutions were magnetically stirred at 70 °C for 30 min, and then 20 mL solution was cast on dia. 60 mm Teflon Petri dishes and dried in oven set at 25 inchHg and 40 °C for 48 hours. Transparent zein films were obtained for each solution. Films were stored at ambient condition at  $23 \pm 2$  °C and relative humidity 40% - 50%.

#### *Differential scanning calorimetry (DSC)*

Differential scanning calorimetry analysis was performed on a DSC 823E Thermal Analyzer (Mettler-Toledo Inc., Columbus, OH) supplied with liquid nitrogen and compressed nitrogen gas. Approximately 5 mg of film sample, packed into aluminum crucibles with lid, were heated from -120 °C to 210 °C at a rate of 10 °C/min. Glass transition temperature ( $T_g$ , the midpoint of the glass transition) and the melting temperature ( $T_m$ , the lowest point of melting peak) were automatically recognized by the STARe software (Mettler-Toledo Inc., Columbus, OH) from at least two thermal curves.

#### *Mechanical properties measurement*

Zein film strips ( $40 \times 10 \times 0.47 \pm 0.12$  mm) were prepared as specified in ASTM D882-10 and stored in 50% relative humidity desiccators under  $23 \pm 2$  °C for one month to reach equilibrium before tensile test. The moisture content of zein films was measured using Denver Moisture Analyzer IR-200 (Denver Instrument, Bohemia, NY). Mechanical properties were measured on a TA.XT2 Texture Analyzer (Texture technologies, New York, US) at a test speed of 6 mm/min and initial distance between grips of 18 mm. At least three replications were used to compare the ultimate tensile strength (UTS, the maximum loading stress), elongation to break (ETB, the strain at the moment the sample

breaks), Young's modulus (E, the slope of extended initial stress-strain curve) and the toughness (T, the area under the stress-strain curve before breaking point).

#### *Water absorption*

Water absorption was determined by a modification of the ASTM D870 (ASTM, 2010a) and similar as previously described (Lai et al., 1997). Films were preconditioned at 25 °C and 50% relative humidity were cut into three 30 × 30 mm specimens, which were then conditioned at 25 °C and 50% relative humidity for two days to reach equilibrium. Beakers were pre-dried at 60 °C for 24 hours and weighted. The specimens were submerged in distilled water in beaker at 25 °C for up to two hours, during which the specimens were removed at 15-min intervals and weighted after the surface water was wiped off. Water Absorption was calculated as a percentage ratio of water absorbed to initial dry weight. Loss of soluble matter from the sheets after two hours of immersion was monitored by evaporating the soaking water in the beaker at 60 °C for 24 hours and then weighing the change of the beaker.

#### *Water vapor permeability (WVP)*

Water Vapor Permeability was determined at 25±2 °C according to the ASTM E96/E96M-10 method (ASTM, 2010b). The inside of aluminum cells were filled with anhydrous calcium chloride. The cells were sealed with the zein films, and placed in desiccators containing a saturated potassium sulfate solution (97% relative humidity at 25 °C). At least three specimens for each sample were tested by weighing, twice everyday for a period of 10 days. Controls were set up similarly except that the cells contained no desiccant. Data were recorded in a weight gain vs. time graph. The slope of the straight

line during steady state of permeation, obtained by linear regression, was determined and the WVP was calculated by Equation (2).

$$WVP = \frac{G/t}{A \cdot P_s (RH_1 - RH_2)} \times thickness \quad \text{Equation (2)}$$

where  $G/t$  - the slope of the linear regression

*thickness* - average thickness of zein films

$A$  - permeation area

$P_s$  - the saturated water vapor pressure at 25 °C

$RH_1 - RH_2$  - difference of relative humidity between the two sides of zein films.

#### *Atomic force microscopy (AFM)*

The images of zein films surface were collected by tapping mode atomic force microscopy (TP-AFM) on a NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA) under ambient conditions. The Root-Mean-Square roughness (RMS) was calculated by the NanoScope software (Veeco Instruments Inc., Santa Barbara, CA) from three  $10 \times 10 \mu\text{m}$  images.

#### *Optical microscopy*

Microscopic images of films were obtained using a Nikon TE-2000 inverted microscope (Nikon Instruments Inc., Melville, NY).

#### *Statistical analysis*

One-way ANOVA analysis and modeling were performed using the SigmaPlot 12 (Systat Software GmbH, Erkrath, Germany) to determine any significant difference between values at confidence level of 95% ( $P < 0.05$ ).

## Results and Discussion

Transparent light-yellowish films were obtained from pure zein and zein with addition of 10% to 40% TBC, whereas zein films containing 50% TBC was slightly opaque. The surface of zein films containing 30% or more TBC was slightly greasy.

### Thermal properties

Thermograms of zein films measured by differential scanning calorimetry suggested the compatibility between zein and the plasticizer and effectiveness of plasticization. As shown in Figure 11, the thermogram of unplasticized cast zein film revealed two second order transitions at temperature around 110.4 °C and 180.5 °C, interpreted as glass transition temperature, which were slightly higher than unprocessed zein protein with two glass transitions at 94.4 °C and 173.6 °C (Shi et al., 2011). The changes suggested that during formation of the zein film, the protein chains established inter- and/or intra-molecular interactions that restricted the chain mobility. A first order transition peak was found at -20 °C in the heating scan of pure tributyl citrate which was attributed to its melting process. In the cast plasticized zein films, endothermic peaks related to the melting of tributyl citrate were only observed in plasticization level from 30% to 50%, but not in 10% or 20%. This indicated that the <20% TBC and zein remained a homogenous mixture after casting. However, it was still possible that the peaks were too small to be detected with the current DSC method, and further analysis such as Dynamic Mechanical Analysis could provide more information on this. If the polymers and plasticizers are immiscible, the mixture would reveal several distinct glass transition temperatures corresponding to individual glass transition of each component. The glass transition temperature for pure tributyl citrate was detected at -90.2 °C, which

was consistent to previous study (Ljungberg and Wesslen, 2005). For plasticized zein films, several glass transitions at the range between 70 °C and 180 °C were observed, taken as evidence of plasticization. In the case of film containing 10% tributyl citrate, the lowest glass transition was observed at 72.1 °C. According to the Gordon and Taylor equation (Gordon and Taylor, 1952):

$$T_g = \frac{x_1 T_{g1} + k_{zein} x_2 T_{g2}}{x_1 + k_{zein} x_2} \quad \text{Equation (3)}$$

where,  $x$  is the mass percentage; subscript 1 and 2 refer to zein and tributyl citrate respectively. If  $T_{g1}$  uses the value of 180.5 °C as measured,  $T_{g2}$  uses the value of -90.2 °C,  $k_{zein}$  uses the value of 6.24 (Madeka and Kokini, 1996), the calculation for films containing 10% tributyl citrate suggests a glass transition temperature of 69.7 °C, which is close to observed result, indicating a good miscibility at this plasticization level. However, the equation did not work for the films containing higher level of plasticizer: the glass transition temperatures did not decrease to a point lower than 72 °C. The calculation implies that using casting method, the tributyl citrate can be mixed homogenously into zein to a possibly maximum level of about 10% and further addition of tributyl citrate would not contribute to the plasticization effect.

### **Tensile properties**

The mechanical properties of zein films are influenced by the plasticizers and the preparation methods. A plasticizer is a substance incorporated into a polymer to increase its flexibility, workability or distensibility. A plasticizer works by spacing the chains of polymer, allowing the chains to move more flexibly and thus the plastics being softer.

A good method to evaluate mechanical properties of films is to study the stress-strain curves from tensile test. Typical tensile curves of pure and plasticized zein films were included in Figure 12. Without addition of TBC, the zein film (black solid curve) was brittle, suggested by a linear relationship between the loading stress and the strain curves during a small elastic deformation. With addition of TBC, the stress-strain curves revealed a curving toward plastic behavior at the end of the proportional region. The films with 10% TBC experienced a larger elastic extension. With films containing 30% to 50% TBC, yielding points could be observed.

A useful parameter to characterize a polymeric material is the young's modulus, which measures the normalized force necessary to make an initial elastic deformation to polymers. Compared to the pure zein film, the young's modulus of plasticized zein films was first increased and reached the highest value when 10% TBC was incorporated. It was then dramatically decreased as the mass ratio of TBC increased from 25% to 50% (Table 5), suggesting that addition of TBC at low level (10%) made the film even more rigid, whereas at higher level ( $\geq 20\%$ ) plasticizer increased the motility of zein molecules. Similar trends have been observed with other hydrophobic plasticizers such as palmitic acid and stearic acid (Lai et al., 1997). The observation was possibly a result from the anti-plasticization effect, which is usually observed at low plasticizer concentrations. Plasticizers at low level reinforce the intermolecular interactions in polymer matrix which become more rigid (Zhong and Jin, 2009). On the other hand, Lai et al. suggested the reason as the higher water contents in the pure zein films than plasticized films due to incorporation of hydrophobic plasticizers (Lai et al., 1997). Here, it was detected that pure zein films contains 6.52% water, which was higher than 10%-

TBC-plasticized films (3.79% water content). It was previously known that water was a good plasticizer for zein films (Gillgren and others 2009). It is possible that the diminished water content in zein films led to the increases in the rigidity of the films. TBC at percentage of 20% to 50% seemed to be as effective on reducing the rigidity of zein films as other hydrophobic plasticizers, such as oleic acid (Santosa and Padua, 1999).

The other two parameters that can be extracted from the tensile curves are the ultimate tensile strength (UTS) and the elongation to break (ETB). Incorporation of TBC at least retained the UTS while slightly increased the ETB. Among various level of plasticization, 10% was found to be optimal in that it increased the UTS to 17.8 MPa, which was about 3 folds compared to pure zein films. The extensibility (ETB) of zein films with 10% TBC was 4.53% which doubled the extensibility of pure zein films. In fact, tensile strength is influenced by intermolecular association in polymer matrix, which is often weakened by incorporation of plasticizer. However, some hydrophobic plasticizers, such as palmitic acid and stearic acid, showed exceptional beneficial effects on the strength of zein films (Lai et al., 1997), which was explained by strong interactions between the polymer and certain plasticizers (Santosa and Padua, 1999). With enhancement on both UTS and ETB, the zein films with 10% TBC was six times tougher than pure zein films, suggested by the toughness calculated as the area under the curve. In the films containing 30% or higher TBC, micro phase separation may exist as the DSC result suggested. This disturbed the molecular structure and led to loss of toughness compared to films containing 10% TBC.

### Water absorption

To investigate the effects of TBC on the water absorption of zein films, samples were immersed in deionized water for two hours and the weight gains were recorded at interval of 15 minutes. After two-hour soaking, pure zein films and zein films with 10% TBC turned to slightly opaque because of excessive absorption of water. The films became much softer and stickier on the surface; whereas, the films containing 20% TBC or more generally maintained their yellowish color and integrity.

The water absorption was calculated as the percentage ratio of weight gain to original weight of films and plotted again the duration of immersion in Figure 13. No significant loss of soluble composition into water (averagely 0.6%) was observed. When the mass ratio of TBC increased from 10% to 50%, the amount of absorbed water decreased. A previous study adopted the concepts from drying operations and developed the below equation to describe the kinetics of water absorption by zein films (Lai et al., 1997):

$$y(t) = M_s(1 - e^{-t/T}) \quad \text{Equation (4)}$$

where,  $y(t)$  – percentage of weight gain by water absorption (a percentage ratio of water absorbed to initial dry weight)

$t$  – time

This equation was applied to fit the data points in Figure 13, shown as the solid lines. The values of parameter and the correlation coefficient,  $R$ , for all zein films were included in Table 6, where the  $M_s$  reflects the water absorption capacity, and the  $M_s/T$



reflects the initial rate of water absorption. The  $R$  values for all groups are higher than 0.989, indicating this equation can provide a valid fitting for the current experimental data. The  $M_s$  value suggested that an initial addition of TBC up to the level of 10% did not decrease the capacity of zein films on water absorption, because statistical analysis indicated no significant difference between  $M_s$  values of pure zein films and that of zein films with 10% TBC. However, 10% TBC slowed down the initial water absorption rate, suggested by a smaller  $M_s/T$  values. As further increase of TBC content, both the water absorption capacity and the initial absorption rate of zein films were reduced. Statistical analysis suggested that TBC has dramatic effects on limiting the water absorption by zein films, which may be due to the hydrophobic nature of TBC. Also as described previously, those films containing 30% or more TBC bear a slightly greasy surface, which prevented the film from absorbing water (Lai et al., 1997).

Hydrophilic plasticizers are shown to be especially effective in improving the extensibility and reducing the rigidity of zein films at ambient conditions because those hydrophilic plasticizers absorb water which adds to the plasticization. However, in a environment where excessive free water is available, such as in food where the water activity is normally high, these zein films plasticized with hydrophilic plasticizers absorbed too much water and became weak (Parris and Coffin, 1997; Lawton, 2004). In contrast, hydrophobic plasticizers better maintained the film strength even at high relative humidity (Lawton, 2004). TBC is a molecule containing hydrophobic butyl chains. As expected, TBC could maintain the film integrity even after immersed in water. This is especially important for biopolymer-based films to be applied in food packaging.

### **Water vapor barrier properties**

The barrier properties of zein films were measured as the water vapor permeability with the results included in Table 7. The water vapor permeability of polymer is usually affected by the hydrophilic/hydrophobic nature of the polymer and the homogeneity of the polymer matrix, especially the surface hydrophobicity and homogeneity. Statistical analysis by One-way ANOVA suggested that addition of TBC lowered the water vapor permeability of zein films at a high relative humidity of 97% ( $P=0.020$ ). However the difference on the WVP between pure zein films and films with 20% was insignificant ( $P=0.053$ ). Usually, moisture absorption at high relative humidity made polymer films soft and swollen. The absorbed water serves as a plasticizer for the polymer films and increased the flowability of polymer chains (Lawton, 2004). This less dense structure as well as more flexible polymer chains in the matrix allows the water molecules to diffuse quickly, resulting in high water vapor permeability (Lai and Padua, 1998). It was expected that incorporation of TBC could reduce the WVP of zein films due to its hydrophobic nature limited the water absorption. However, with the present result, it is hard to determine the effects of TBC on WVP, and further research is needed to clarify this.

### **Surface microstructure**

The surface of pure zein films bears many cracks, voids and wrinkles shown in the optical microscopic images and AFM images (Figure 14). It probably resulted from the high surface tension during the solvent casting of pure zein films. There could also be cracks and voids inside the films which caused the pure zein films to be brittle and less tough. When the TBC was added to the mass ratio of 20%, the surface tension may be

released and the film surface was much smoother under the examination of optical microscopy. Root-Mean-Square (RMS) roughness calculated from AFM images indicated a decrease of roughness on zein films from 66.3 nm on pure zein films to 24.1 nm on films containing 20% TBC (Table 8). However, the roughness was again increased to 45.5 nm at the level of 50% incorporated TBC. From the optical images, a micro phase separation was obvious in films containing 50% TBC but not in films with 20% TBC. AFM images also revealed small soft spherical structure which could be separate domain formed by TBC. As mass ratio of TBC increased from 0 to 20%, film surface appeared to be much smoother. The cracks and voids on the surface of pure zein films could be the site for water binding during moisture absorption, which explained the slightly higher water vapor permeability. However, when the loading of TBC reached the limitation, possibly about 20%, TBC started to form phase separation from zein matrix. Therefore the heterogeneous films with 50% TBC were again less effective as a moisture barrier.

In summary, the effects of TBC on the mechanical properties, water resistance, water vapor barrier properties and surface microstructure were investigated. Films incorporated with 10% TBC showed optimally improved toughness and flexibility. The plasticized films were also more resistant to water shown as lowered water absorption capacity and/or rate. In our following study entitled “Developing zein films with ice nucleation function and its application in frozen dough”, zein film plasticized with TBC was adopted for the application on frozen food. Incorporating TBC allowed the cast zein films to be flexible enough for wrapping, and water-resistant enough to retain integrity through freezing storage.

Table 5 Effect of tributyl citrate (TBC) on the tensile properties of zein films\*

	<b>Modulus (MPa)</b>	<b>UTS (MPa)</b>	<b>ETB (%)</b>	<b>Toughness (MPa)</b>
<b>Pure zein</b>	409.86 ± 7.62 <sup>b</sup>	6.70 ± 0.37 <sup>b</sup>	1.96 ± 0.18 <sup>c</sup>	0.071 ± 0.007 <sup>b</sup>
<b>+ 10% TBC</b>	556.29 ± 29.42 <sup>a</sup>	17.80 ± 4.26 <sup>a</sup>	4.53 ± 0.54 <sup>a</sup>	0.414 ± 0.079 <sup>a</sup>
<b>+ 20% TBC</b>	255.28 ± 27.36 <sup>c</sup>	5.47 ± 0.64 <sup>b</sup>	3.03 ± 0.20 <sup>b</sup>	0.092 ± 0.004 <sup>b</sup>
<b>+ 30% TBC</b>	256.64 ± 33.57 <sup>c</sup>	5.36 ± 1.33 <sup>b</sup>	3.26 ± 1.01 <sup>b</sup>	0.112 ± 0.072 <sup>b</sup>
<b>+ 40% TBC</b>	268.03 ± 48.20 <sup>c</sup>	4.34 ± 0.19 <sup>b</sup>	2.44 ± 0.12 <sup>bc</sup>	0.061 ± 0.005 <sup>b</sup>
<b>+ 50% TBC</b>	136.29 ± 32.05 <sup>d</sup>	3.25 ± 0.94 <sup>b</sup>	3.53 ± 0.17 <sup>b</sup>	0.066 ± 0.017 <sup>b</sup>

\* Values with no letters in common are significantly different  $p < 0.05$  using the Holm-Sidak multiple-comparison method.

Table 6 Kinetic parameters of water absorption TBC-plasticized zein films

	$M_s$ (g)	$M_s / T$ (g/min)	$R$
<b>Pure zein</b>	31.7771 $\pm$ 0.2393 <sup>a</sup>	1.6461	0.9992
<b>+ 10% TBC</b>	32.2883 $\pm$ 0.9538 <sup>a</sup>	0.7233	0.9973
<b>+ 20% TBC</b>	25.8862 $\pm$ 1.4324 <sup>b</sup>	0.5643	0.9908
<b>+ 30% TBC</b>	20.0006 $\pm$ 1.1732 <sup>c</sup>	0.4420	0.9893
<b>+ 40% TBC</b>	17.2580 $\pm$ 0.6539 <sup>d</sup>	0.4176	0.9945
<b>+ 50% TBC</b>	12.9417 $\pm$ 0.1972 <sup>e</sup>	0.3378	0.9990

\* Values with no letters in common are significantly different  $p < 0.05$  using the Holm-Sidak multiple-comparison method.

Table 7 Effect of TBC on the water barrier property of zein films

	WVP (g.mm/(h.m <sup>2</sup> .mmHg))
Pure zein	0.0225 ± 0.0099 <sup>a</sup>
+ 10% TBC	0.0220 ± 0.0049 <sup>a</sup>
+ 20% TBC	0.0086 ± 0.0021 <sup>a</sup>
+ 30% TBC	0.0109 ± 0.0042 <sup>a</sup>
+ 40% TBC	0.0133 ± 0.0031 <sup>a</sup>
+ 50% TBC	0.0199 ± 0.0020 <sup>a</sup>

\* Values with no letters in common are significantly different  $p < 0.05$  using the Holm-Sidak multiple-comparison method.

Table 8 Effect of TBC on the surface roughness of zein films analyzed by AFM

	<b>RMS (nm)</b>
<b>Pure zein</b>	66.3 $\pm$ 2.2
<b>+ 20% TBC</b>	24.1 $\pm$ 1.2
<b>+ 50% TBC</b>	45.5 $\pm$ 3.3

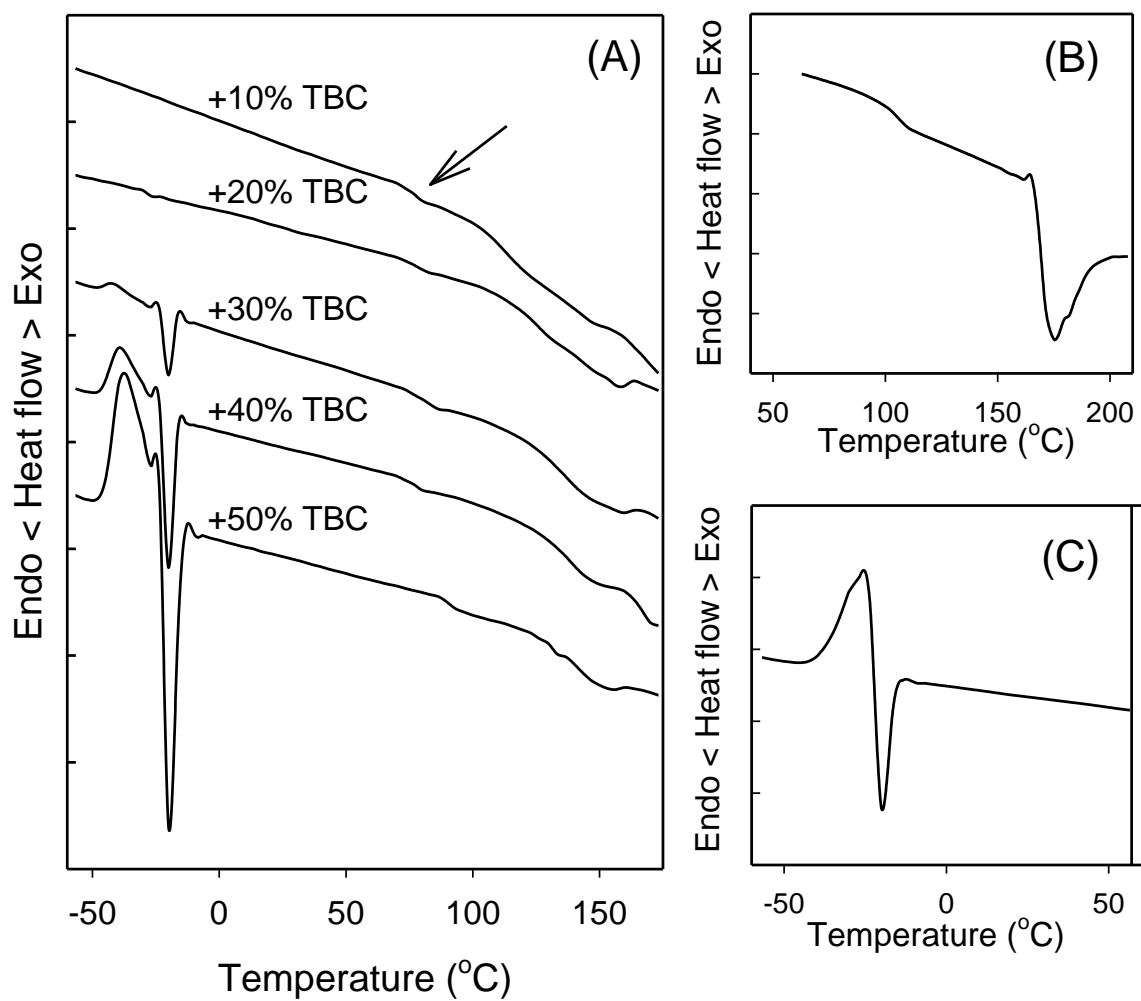


Figure 11 thermograms for (A) zein films plasticized with 10% - 50% TBC, (B) pure zein films and (C) TBC. The heat flow between each tick in all three graphs is 0.1 W/g



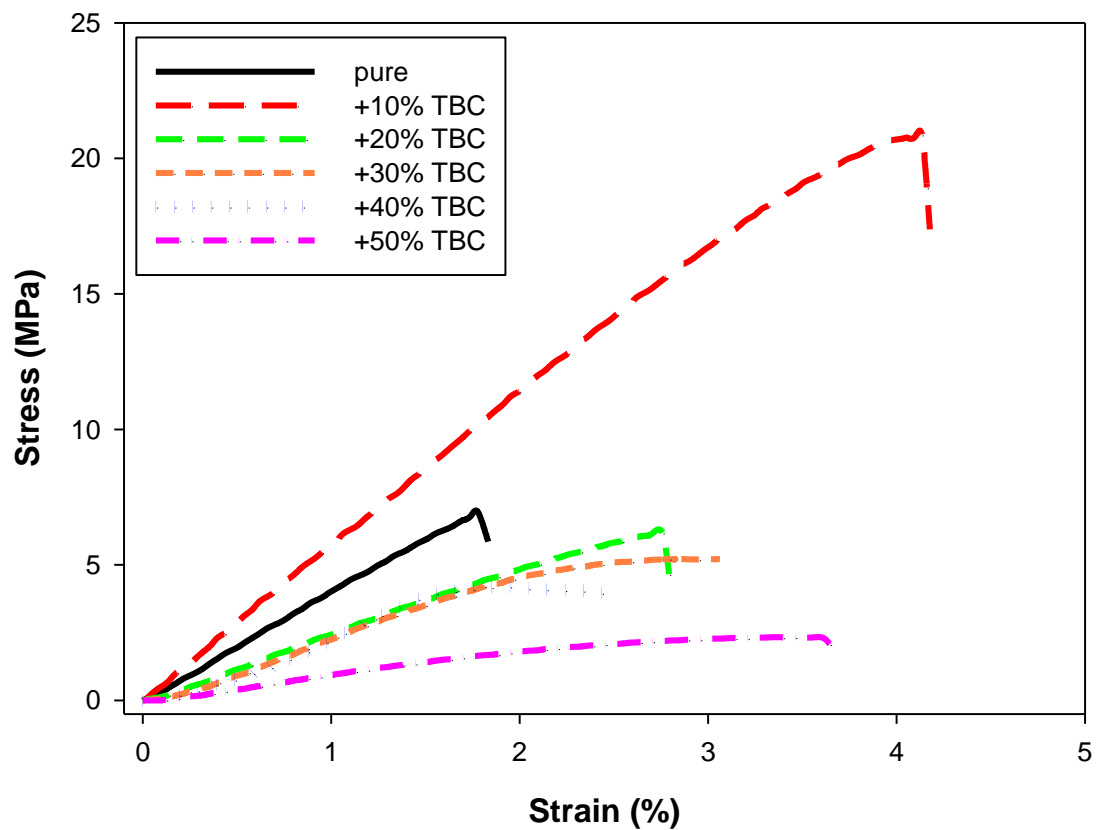


Figure 12 Representative stress-strain curves for pure zein sheets and zein sheets plasticized with TBC

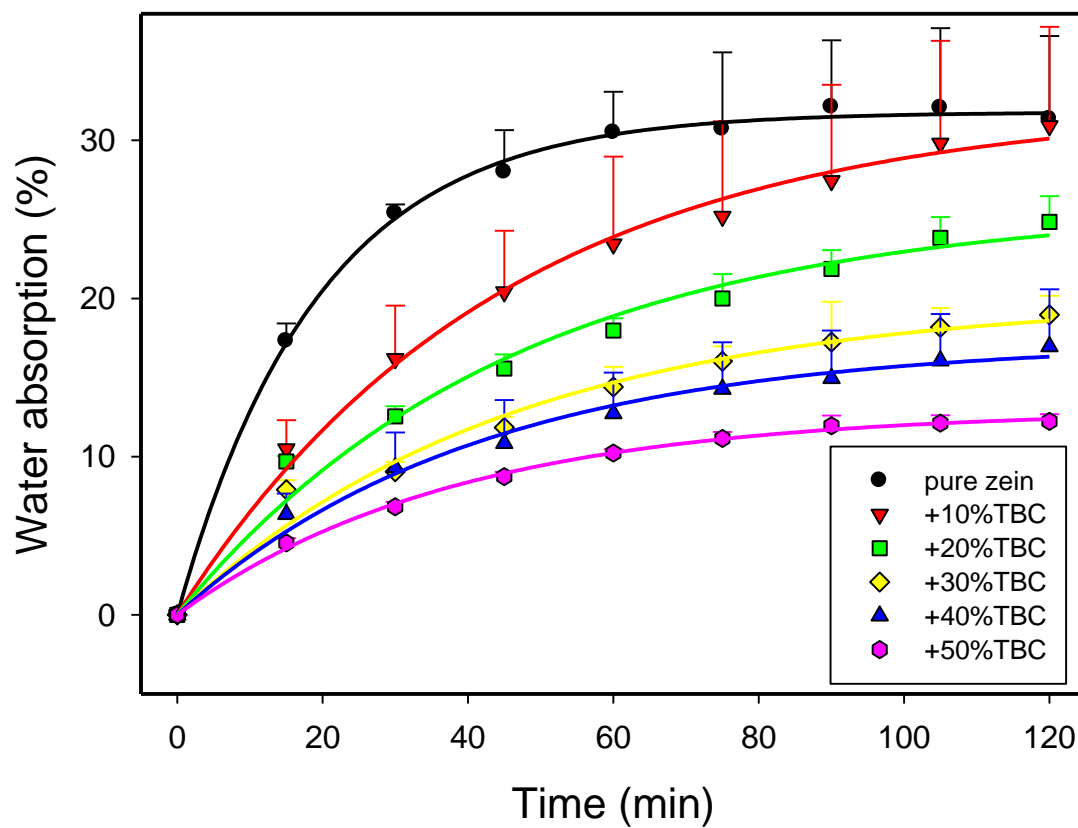


Figure 13 Effect of incorporated TBC on the water absorption of zein sheets

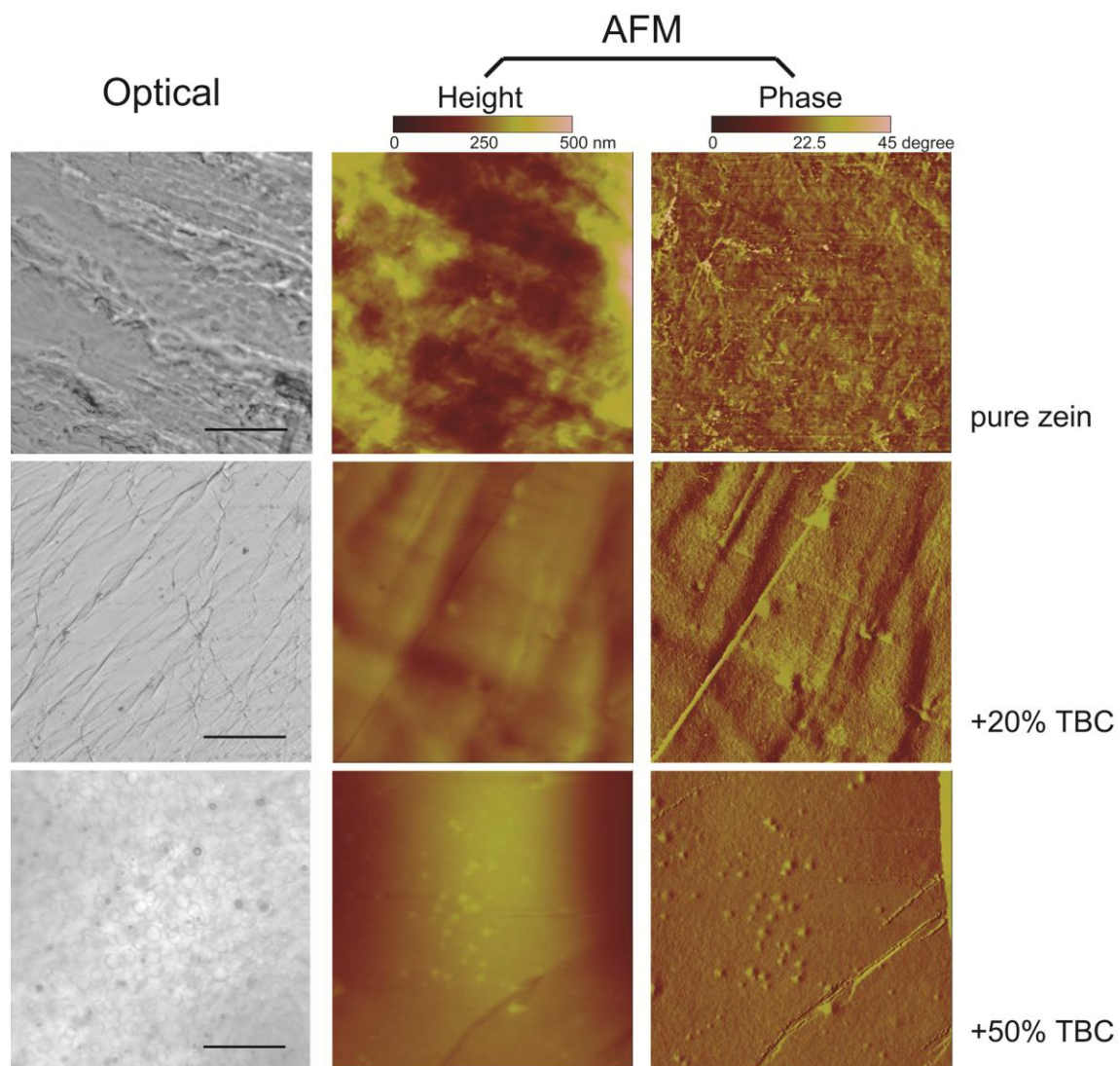


Figure 14 Optical microscopy and AFM to pure zein films and TBC-plasticized zein films\*.

\* Bar in optical microscopy = 50 µm; AFM image size: 10×10 µm.

# **CHAPTER THREE. A NOVEL APPROACH FOR IMPROVING YEAST VIABILITY AND BAKING QUALITY OF FROZEN DOUGH BY ADDING BIOGENIC ICE NUCLEATORS FROM *ERWINIA HERBICOLA***

*As of submission of this dissertation, the work in this chapter has been submitted in the title of “A Novel Approach for Improving Yeast Viability and Baking Quality of Frozen Dough by Adding Biogenic Ice Nucleators from Erwinia Herbicola” to the Journal of Cereal Science for consideration of publication.*

## **Abstract**

Freezing deteriorates the baking quality of frozen bread dough by causing lethal injury to yeast cells and gluten network (depolymerization, reduction and denaturation). To investigate the potentials of biogenic ice nucleators in frozen food applications, the effect of extracellular ice nucleators (ECINs) from *Erwinia herbicola* on the baking quality of frozen dough upon three freeze/thaw cycles were investigated. With addition of ECINs to the activity of  $2.4 \times 10^6$  units per gram of dough, hardening of bread crumb caused by freeze/thaw cycles was alleviated by about 50% compared to the control. Additionally, the crumb of ECINs-incorporated frozen dough showed 50% larger specific volume compared to the control. The effect of ECINs was explained by their protection to yeast cells. ECINs were able to improve the viability of log-phase and stationary-phase yeast cells in suspensions by about 100 and 10 fold, respectively, and viability of yeast in frozen dough by 17%. Yet, ECINs did not reveal protective effects on the gluten

composition or the rheological property of frozen dough. Addition of ECINs reduced the time of dough freezing by 20%, which potentially saved 1250 kJ energy in the current case. This work revealed the functions of ECINs on protecting baking quality of frozen bread dough and viability of yeast cells, as well as on energy saving.

## Introduction

Ice nucleation materials function as heterogeneous ice nucleators to minimize the supercooling of water. Ice nucleation proteins (INP) have been found in the leaves of plants such as winter rye and prunus, animals such as turtles, and lichens, and some microorganisms such as strains in the genera of *Pseudomonas*, *Erwinia* and *Xanthomonas* (Costanzo et al., 2003; Duman, 2001; Brush et al., 1994; Duman and Horwath, 1983; Kieft and Ruscetti, 1990).

The food application of INP-expressing (*Ina*+) bacterial cells and cell-free ice nucleators was comprehensively summarized (Li and Lee, 1995). Notably, addition of the *Ina*+ cells was shown to significantly raise the ice nucleation temperature and shorten the total freezing time (Li et al., 1997; Hwang et al., 2001; Zasytkin and Lee, 1999). Extracellular ice nucleators (ECINs) from *Erwinia herbicola* revealed a protective effect on gel-forming capacity of fish and textural stability of fish actomyosin during freeze/thaw cycles (Zhu and Lee, 2007). *Ina*+ bacterial cells were also reported to improve the efficiency of freeze drying of soy sauce and soybean paste (Watanabe and Arai, 1987) and to modify the texture of raw egg white, bovine blood, soybean curd and milk curd after freezing (Arai and Watanabe, 1986).

Introduction of freezing technique to bakery industry was revolutionary. For manufacturer, freezing storage largely extends the shelf life of dough, which allows the industry to expand their sales from local to nationwide. For consumer, frozen dough offers convenience in addition to warm bakery goods that have comparable qualities to freshly prepared ones. However, long term freezing storage is harmful to the qualities of frozen dough (Ribotta and others 2001b). The freezing damage to yeast was referred as the primary factor contributing to the quality loss (Rosell and Gomez, 2007). During freezing storage, yeast cells are injured, and consequently release glutathione which weakens the dough by cleaving disulphide bonds in the gluten. Additionally, the longer proofing time and smaller bread volume were also due to that the freezing-injured yeast cells lose the capability of gas production. Severe temperature fluctuation during the storage and transportation also leads to increase of ice crystal size and consequently aggravate the frozen damage to yeast (Rosell and Gomez, 2007). Traditionally, to counteract the released reducing substances such as glutathione, oxidants like potassium bromate are added into yeast-leavened frozen dough. However, the use of potassium bromate has been banned in many countries and also declined in U.S. in recent years due to the potential harm to consumers (Selomulyo and Zhou, 2007). Besides, selection and isolation of freeze-tolerant yeast strains attract much scientific effort (Shima and others 2010). Also, in order to compensate the deteriorating effect of freezing, many researchers focused on developing other improvers, such as emulsifiers, enzymes, hydrocolloids and ice-structuring proteins to stabilize the dough network (Al-Dmoor and others 2009).

Previous study suggested that after three successive freeze/thaw cycles, frozen dough revealed significant quality deterioration (Rosell and Gomez, 2007). In the present

study, the protective effect of ECINs on yeast-leavened frozen dough was investigated. Firstly, ECINs were blended into frozen bread dough which was baked after repeated freeze/thaw cycles. The qualities of these breads were characterized and compared to the bread from frozen dough without ECINs and fresh dough. Furthermore, to investigate the mechanism of ECINs' protective effects, the viability of yeast and structure of gluten network after freeze/thaw cycles were also examined. This study revealed the cryopreservative application of ECINs in frozen bread dough.

## **Materials and Methods**

### *Materials*

Malt extract broth and malt extract agar (BD, Franklin Lakes, NJ), sucrose (Fisher), Sodium Chloride (Fisher), L-serine (Aldrich), L-alanine (Aldrich), were used as received. Deionized water was used throughout the experiment. All purpose flour (10% protein, 73% carbohydrate, 12% moisture, General Mills Inc., Minneapolis, MN), Fleischmann's<sup>®</sup> ActiveDry<sup>™</sup> bakery yeast (ACH Food Companies, Inc., Cordova, TN) and shortening (92% fat, GreatValue<sup>®</sup>, Wal-Mart Stores, Inc., Bentonville, AR) for making dough were purchased from supermarket.

### *Preparation of Extracellular Ice Nucleators*

The *Erwinia herbicola* subsp. *ananas* was obtained from the American Type Culture Collection (ATCC Cat. No. 11530) and routinely cultured in yeast extract (YE) medium consisting of 2.0% yeast extract, 1.0% sucrose, 0.2% L-serine, 0.2% L-alanine, 0.086% K<sub>2</sub>SO<sub>4</sub>, and 0.14% MgSO<sub>4</sub> under 18 °C at shaking speed of 200 rpm.

Extracellular ice nucleators (ECINs) were isolated from *E. herbicola* Cells which were harvested by centrifugation at 10,000  $\times g$  for 20 min and then re-suspended in 20 mM Tris/HCl pH 8.0. Sonication of cells was done under output of 50 watts on ice, 10 seconds with 10 seconds interval for 3 times. Cells and debris were removed by centrifugation at 10,000  $\times g$  for 20 min and supernatant was subjected to filtration through 0.45  $\mu m$  filters. The filtrate was put on ultracentrifugation at 160,000  $\times g$  for 2 hours and pellet was re-suspended in 20 mM Tris/HCl with 20 mM  $MgCl_2$ , pH 8.0. Lyophilized ECINs in the form of powder were stored at -20 °C.

#### *Dough preparation and baking procedure*

A basic dough recipe was adopted from AACC method 10-10B (AACC, 2000). Two doughs were prepared for each condition. For each dough piece, 450 g flour, 252 g water, 7 g yeast, 27 g sucrose, 6.75 g NaCl, 27 g shortening was mixed and kneaded in KitchenAid® PRO 500 mixer under 4 °C to minimize the activity of yeast. Each dough was about 720 g. Optionally, ECINs (ice nucleation activity:  $6.9 \times 10^6$  units/mg) was dispersed in water and then mixed into dough to 0.35mg per gram of dough. After mixing, dough was sheeted and rolled into 20 cm long cylinder, immediately followed by freezing at -20 °C for 48 h and then thawed in a 4 °C refrigerator for 24 h as one freeze/thaw cycle. After three freeze/thaw cycles, dough pieces were fermented at  $30 \pm 2$  °C under  $80 \pm 5\%$  relative humidity for 60 min (fresh dough) or 2½ h (frozen dough). The dough was baked in 21.6  $\times$  11.2  $\times$  6.9 cm aluminum pan at 205 °C for 30 min. After baking, bread was cooled to room temperature and packed into plastic bags. Bread was left under ambient temperature and analyzed within 24 h.



### *Measurement of bread qualities*

Method for measuring bread properties was adopted from AACC method 74-9 (AACC, 2000). Bread loafs was weighed and the loaf volume was measured by rapeseed displacement. Each bread loaf was cut into 25 mm slices. At least four slices were characterized for the texture profile analysis carried out with a TA.XT2 texture analyzer (Stable Micro Systems, Ltd., London, UK), using a 40 mm cylindrical acrylic probe. The bread slides were compressed at speed of 1.7 mm/sec to total distance of 10 mm (40% strain) and withdraw at the same speed. The following textural parameters were recorded from the force-distance curves: firmness (g, force at 25% strain), fracturability (g, the force when detecting the first significant peak during compression), and resilience (% , area during the withdrawal of the penetration divided by the area of the first penetration). pH of bread was recorded in suspension of 1g bread homogenized in 10 ml dH<sub>2</sub>O. Water content was measured using Denver Moisture Analyzer IR-200 (Denver Instrument, Bohemia, NY). Photographs of sliced breads were analyzed using ImageJ software with respect to the number of gas cells, cell sizes, cell perimeter and ratio of gas cell area to total analyzed area. Crumb color was measured using Minolta CR-200 Colorimeters (Konica-Minolta) for values of *L* (lightness, white-black), *a* (color-opponent dimension, red-green) and *b* (color-opponent dimension, yellow-blue. The color difference, *dE*, was calculated as:

$$dE = \sqrt{(dL)^2 + (da)^2 + (db)^2} \quad \text{Equation (5)}$$

where *dL*, *da*, and *db* were the differences for *L*, *a*, and *b* values between the sample and the reference (a white ceramic plate having *L* value of 93.4, *a* value of -1.8, and *b* value

of 4.4). Pigment in crumb was extracted according to the AACC method 14-50 (AACC, 2000), by mixing 2 g crumb sample in 10 mL water-saturated *n*-butanol for 16 h. The mixture was then centrifuged and the absorbance at 436 nm ( $A_{436\text{nm}}$ ) was measured in a Cary-50 spectrophotometer (Varian Instruments, Walnut Creek, CA).

#### *Yeast survival counting in saline suspension*

Yeast cells were grown in malt extract (ME) broth at 30 °C under shaking. When  $OD_{600}$  of the broth reached 1.0, yeast cells at early log phase were harvested. When  $OD_{600}$  reached 3.5, yeast cells at stationary phase were harvested. Cells were then washed with saline solution (0.9% NaCl) and diluted with saline precisely to  $OD_{600}$  0.10. Different concentrations (0.001, 0.01, 0.1, and 1 mg/ml) of ECINs were added to aliquots of cell suspension. Subsequently, 1 ml aliquots of samples and control (yeast suspension without ECINs) in microcentrifuge tubes underwent five freeze/thaw cycles (-20 °C for 20 min and 25 °C for 10 min). Before and after 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> cycle, enumeration of viable yeast cells were performed on malt extract agar (MEA) plates.

#### *Yeast survival counting in dough*

In the fresh dough and frozen dough after three freeze/thaw cycles, 3 g specimens collected from the geometric center and surface of dough were shaken vigorously in 27 mL 0.9% saline for 3 h to extract the yeast cells. Enumeration was then performed as above. Three repeats were performed for each sample.

#### *Freezing curves*

Temperature changes in dough or yeast suspension during the freeze/thaw cycles were monitored using Omega Thermometer OM-CP-IF200 with a TTSS-HH

thermocouple (Omega engineering, Inc., Stamford, CT). The data were recorded by the software OmegaSoft® v.2.00.74 (Omega engineering, Inc., Stamford, CT).

#### *Analysis of gluten in dough by SDS-PAGE*

After thawing, gluten was extracted from 1.6 g (containing ~1g solid) dough from each sample using 20 ml of 2% sodium dodecyl sulfate (SDS) solution (Ribotta et al., 2003). After centrifugation at  $92,000 \times g$  for 3 min, supernatant was analyzed on discontinuous SDS-PAGE with 5% stacking gel and 8% separating gel in Tris-Glycine running buffer at 100 Volt. The gel was stained with Coomassie blue and the imaged.

#### *Rheological analysis to dough*

Frozen dough was thawed at 4 °C for 24 hrs prior to test. Small deformation oscillatory measurement was done using ARES rheometer (TA Instruments Ltd., Leatherhead, UK) under controlled stress mode. Serrated parallel plate with diameter of 25 mm and gap of 2 mm was employed for all tests. Excess dough was trimmed off with shape blade and the edge was covered by paraffin oil to prevent sample drying during tests. Dough was allowed to rest between plates for exactly 15 min before each measurement for relaxation and temperature stabilization. Preliminary dynamic strain sweep test from 0.01% to 10% at frequency of 1 rad/s was performed to determine the linear viscoelastic region. A strain of 0.1% within this region was used for oscillatory tests. Dynamic frequency sweep were conducted from 0.1 to 100 rad/s to evaluated the storage modulus ( $G'$ ) and loss modulus ( $G''$ ). Measurements were conducted in triplicate and average curves were plotted.

### *Reducing sugar content*

The content of reducing sugar in bread was measured according to AACC method 74-9 (AACC, 2000). The result was recorded as mg maltose per gram of bread.

### *Statistical analysis*

One-way ANOVA or two-way ANOVA was performed using Sigma-Plot v.12.0 with integrated SigmaStat module. Different letters indicate significant difference ( $P < 0.05$ ).

## **Results and Discussion**

### **Effect of ECINs on the bread quality of frozen dough**

The properties of breads baked from fresh dough and frozen dough with and without ECINs are summarized in Table 9 and the cross-sections of bread were taken for analysis on gas cell structure (Figure 15). Compared to the fresh dough, breads made from the frozen dough (without ECINs) showed lower specific volume. Addition of ECINs lessened this deteriorating change. Specific volume is defined as the volume per unit of weight. In this study, the difference on weight among samples was negligible, and the length and the width of the bread were restricted by the bakery pan. Therefore, the difference on specific volume among samples mainly came from the height that the dough rose to during fermentation and baking. Additionally, pH of the breads from frozen dough was lower than that from fresh dough, which may be due to the injury to yeast cells and release of acids from the cells (Ribotta et al., 2003). Addition of ECINs reduced the acidity of the bread, because of their protection on yeast cells as discussed below.

Bread texture profiles were also compared among the three types of breads. Freeze/thaw treatment to dough without ECINs made the bread crumb 5-fold firmer and less elastic (decreased resilience). Addition of ECINs reduced the crumb hardening by 50%. The crumb of bread with ECINs was also springier (increased resilience) than the ones without ECINs. Usually, water content and the crumb gas cell structure are the two most important factors affecting the crumb firmness. In this study, no statistical difference in either water content or water activity was detected among the three groups, whereas the difference in the crumb gas cell structure was more pronounced ( $P < 0.05$ ) (Table 9). There were fewer and larger gas cells in frozen-dough bread, indicated by lower cell density but larger mean cell area and mean perimeter. And this is commonly observed in frozen dough with a weak gluten network due to freezing damage. The total volume of gas cells also decreased in the frozen-dough bread suggested by smaller cells-to-total area ratio. In contrast, addition of ECINs allowed frozen-dough bread to have an improved gas cell structure that revealed no statistical difference in total cell area and cell size compared to fresh-dough bread (Table 9).

In terms of crumb color, freeze/thaw cycles made the bread crumb appear darker than those from fresh dough (larger value of  $dE$ ), and the crumb color in the ECINs-blended frozen-dough bread was slightly brighter than the frozen-dough bread without ECINs (smaller value of  $dE$ , Table 9). The apparent color change was not due to the pigment change in bread crumb, as homogenized breads showed no difference in color and the crumb extracts had similar absorbance at 436 nm (Table 9). Rather it reflected the change of the gas cells. An appealing bread crumb should show a white color, which is due to evenly distributed fine gas cells with thinner cell wall. This kind of gas cell

structure is more likely to reflect light rather than absorbing light (Pomeranz, 1960). After freeze/thaw treatment to the dough, the gas cells in bread crumb were enlarged with reduced number of cells and thicker cell wall, leading to more brownish crumb suggested by larger values of  $dE$  (off-white), more positive values of  $a$  (redness) and  $b$  (yellowness) (Table 9).

The mechanism of the compromised bread quality from frozen dough possibly includes damage to yeast cells and gluten network (Rosell and Gomez, 2007). Damage on yeast cells directly affects the number and size of gas cells in bread crumb and specific volume. The injured yeast cells also release reducing compounds, such as glutathione, which harms the gluten network. The network built-up by the inter- and intra-molecular association of gluten and starch is the most important parameter affecting the gas and water retention, as well as the crumb texture, whereas low temperature diminishes the hydrophobic force that holds these macromolecules in the matrix (Rosell and Gomez, 2007). All these mechanisms could explain the deteriorated properties in frozen dough observed in the present study.

### **Effect of ECINs on freeze/thaw survival of yeast in suspension**

Given that the damage to yeast cells and gluten network are two major possible reasons causing the deteriorated quality of frozen dough, in order to investigate the mechanisms for ECINs' protection on bakery qualities of frozen dough, the effects of ECINs on the viability of yeast and the integrity of gluten network upon freeze/thaw cycles were examined in the following sections.

Firstly, yeast at log phase and stationary phase, representing different metabolic stage, was subjected to five successive freeze/thaw cycles. The survival of yeast cells was compared to the initial values (Figure 16). The viability of log-phase yeast cells experienced a nearly three-magnitude loss after five freeze/thaw cycles, compared to less than one magnitude loss in the stationary-phase cells, suggesting that the cells at stationary phase were less vulnerable to freeze/thaw damage than the cells at log phase, whose metabolism is more active. Previous work also suggested that yeast cells at latent state (lag phase) were more resistant to freezing damage compared to metabolically active state (log phase) and this could be explained by the physiological changes including thickening of cell walls, varied membrane compositions as well as accumulation of trehalose and glycogen in lag phase (Park and others 1997).

The cryoprotective effect of ECINs on yeast cells was remarkable, especially on log-phase yeast cells. As shown in Figure 16A, even with 0.001 mg/ml ECINs, the viability was significantly increased ( $P < 0.05$ ) compared to the control. When the ECINs reached 1 mg/ml, the loss of cell viability was largely reduced to less than one magnitude. Moreover, 1 mg/ml ECINs were also effective on protecting stationary-phase yeast cells, which were more resistant to freeze/thaw stress (Figure 16B). In this case, the loss of viability after addition of 1 mg/ml ECINs was minimal, from 80% after one cycle to 58% after five cycles. Interestingly, when the concentration of ECINs was lowered to 0.01 and 0.001 mg/ml, addition of ECINs became harmful to the viability of stationary-phase yeast.

The record of temperature change during a freezing process (freezing curve) revealed detailed profiles of the freezing process. As shown in Figure 17, without

addition of ECINs, supercooling was observed in the freezing of the yeast suspensions in both log and stationary phase. However, the supercooling for stationary-phase cells were about 50 seconds longer than that for log-phase cells. And the nucleation temperature, defined as the lowest temperature that a supercooled liquid reaches, was  $-11.26^{\circ}\text{C}$  in log-phase cell suspension, in contrast to  $-12.33^{\circ}\text{C}$  in stationary-phase cell suspension. Following the supercooling, the log-phase cells showed an immediate temperature decrease, suggesting that phase transition of the suspension was super-fast after ice nucleation. In contrast, the temperature in stationary-phase cell suspensions remained at freezing point for about 40 seconds before further decrease, which corresponded to the period that ice and water were co-present. The longer supercooling, lower nucleation temperature and slower freezing in stationary-phase yeast may be due to the anti-freezing effects of trehalose produced by those cells (Park et al., 1997). Addition of ECINs at all test concentrations resulted in the minimized of supercooling in both log-phase and stationary-phase yeast cell suspension.

The time of phase transition, which is duration for a system to stay at freezing point, is essential for freezing damage and cryopreservation, as two different mechanisms of freezing damages have been proposed for super fast and slow freezing conditions (Karlsson and Toner, 1996; Mazur and others 1972). In a super-fast phase transition, formation of intracellular ice crystals is lethal and causes direct damage to cells. In contrast, slow freezing encourages the formation of large external ice, causing an osmotic stress between the two sides of cellular membrane and an efflux of water from cells to the environment. Consequently, dehydration and volume reduction of cells lead to



progressive membrane damage, resulting in leakage and release of lysozyme and eventually cell injury and death (Meryman, 1974).

In the present study, it was likely that ECINs function as a cryopreservative agent by controlling the rate of phase transition. Specifically, in the log-phase yeast suspension, addition of ECINs at all test concentrations reduced the supercooling (Figure 17A) and thus avoided the super fast freezing damage and preserved yeast cells (Figure 16A). In the literature, supercooling and nucleation at low temperature was demonstrated to be detrimental to yeast cells. Seeding ice into a supercooling yeast suspension at subzero temperature, e.g.  $-7^{\circ}\text{C}$  and  $-2.5^{\circ}\text{C}$ , the yeast survival rate was increased by 3-fold and 6-fold respectively, compared to freezing initiated at about  $-15^{\circ}\text{C}$  (Nakamura et al., 2009; Mazur, 1961). On the other hand, in stationary-phase yeast suspension, ECINs at concentration of 0.1 and 1 mg/mL accelerated the freezing rate which possibly avoided slow-freezing damage, thus preserved the yeast cells. But ECINs at 0.001 and 0.01 mg/mL prolonged phase transition and subsequently failed to preserve the yeast viability (Figure 16B and Figure 17B).

### **Effect of ECINs on yeast survival in frozen dough**

It was previously observed that the yeast cells become even more vulnerable to repeatedly freezing when blended into dough (Rosell and Gomez, 2007). In addition to revealing the cryoprotective effect of ECINs on yeast in suspension, further examinations on yeast in frozen dough were performed. The amount of viable yeast cells at the surface and center of fresh and frozen dough was evaluated with results plotted in Figure 18. The yeast concentrations at the surface and the center of each type of dough had no significant difference ( $P>0.05$ ). However, during three freeze/thaw cycles, yeast suffered dramatic

loss of viability, remaining one half of the original concentration before freezing. Addition of ECINs rescued the viability of yeast ( $P < 0.05$ ), resulting about 17% more viable yeast compared to that without ECINs. The increase in yeast viability in frozen dough with addition of ECINs was consistent with test on yeast in suspensions, and explained the higher specific volume of bread and the better gas cell structure in crumb shown in Table 9.

In the experiment on freezing yeast in suspension, it was observed that the higher yeast viability in suspension corresponded to a moderate rate of phase transition by addition of ECINs. However, the freezing process in dough was much more complicated and the freezing curve could only record the overall temperature change (Figure 19). Although supercooling was not detected, the freezing at  $-5^{\circ}\text{C}$  in dough without ECINs lasted for about 1.5 hours before temperature further decreased, in contrast to a faster freezing for about 20 minutes by adding ECINs. The exact mechanism of cryoprotective effect of ECINs on yeast in dough remains unclear.

Reducing sugars provide fermentative substances for yeast. In this study, their contents were measured to evaluate the extent of fermentation by yeast. As shown in Table 10, the difference in the content of reducing sugar in bread baked from fresh dough and frozen dough were statistically insignificant ( $P > 0.05$ ).

The cryoprotective function of bacterial ice nucleator could be extended to other microorganisms. It was revealed that addition of commercial ice nucleation protein (SNOMAX<sup>®</sup>) decreased the lethality of fungal starter *Geotrichum candidum* during freeze/thaw cycles (Missous et al., 2007). Ice nucleation gene has been cloned into yeast

cells which consequently possessed ice nucleation activity (INA) (Hwang et al., 2001). The INA+ yeast cells could very possibly be more tolerant to freezing damage, similar as those genetically modified yeast cells expressing anti-freeze protein (Yeh and others 2009), which have a prospective application in frozen dough and other frozen food industries.

### **Effect of freeze/thaw on gluten and dough rheology**

In addition to the damage to yeast cells, it was suggested that freezing also affects the gluten network which eventually harms the bread texture (Rosell and Gomez, 2007). Accordingly, the protein contents from fresh dough and frozen dough, with and without addition of ECINs were extracted and analyzed by SDS-PAGE and Coomassie blue staining (Figure 20).

Among the proteins extracted from fresh and frozen dough, the bands with molecular weight (Mw) lower than 30 kD belonged to the species other than gluten (Ribotta and others 2001a). The gluten fraction consisted of high-molecular-weight (HMW) subunits and low-molecular-weight (LMW) subunits with boundary at 60 kD. In this gel, clear bands of HMW subunits were not observed and it was very likely that many protein species with large molecular weight did not enter the separating gel. In the region of LMW subunits, one difference between the fresh and frozen dough was observed at about 50 kD (labeled as R1) and 47 kD (labeled as R2), where some protein shifted to lower molecular-weight region on the gel shown as broader bands. Besides, another protein band with Mw of about 32 kD, labeled as R3, appeared after freeze/thaw treatments. Bands at this region of molecular weight could belong to the single-chain

gliadin (Bietz and Wall, 1986). There was no evident difference between the frozen dough without and with addition of ECINs.

Gluten composition, especially the content of HMW subunits, largely affects the rheological properties of gluten, where lower strength and elasticity were expected for frozen dough. However, rheological analysis to fresh and frozen dough did not well support such expectation. Figure 21 shows the mechanical spectra of fresh and frozen dough. All samples exhibit dominant solid-like elastic properties as the storage modulus ( $G'$ ) values remained higher than the loss modulus ( $G''$ ). Since the values of moduli as well as the frequency dependence of moduli were statistically the same among the three samples, no clear difference has been detected regarding the rheological properties of fresh and frozen dough.

Several mechanisms have been proposed to cause the depolymerization of gluten and loss in rheological quality of dough during freezing: (1) Reducing agents, i.e. glutathione, released from the damaged yeast break the disulfide bonds in glutenin; (2) ice recrystallization ruptures the gluten structure; and (3) the hydrophobic force for stabilizing intermolecular association in gluten network was weakened by prolonged storage at low temperature (Rosell and Gomez, 2007; Kontogiorgos and others 2007). However, the rheological analysis in this study did not detect significant difference in gluten network between fresh dough and dough after three freeze/thaw cycles. The reason is unclear. But it's likely that the method is not sensitive enough to reveal changes in gluten network happened within three freeze/thaw cycles and further freeze/thaw treatments will cause a more detectable deterioration of gluten network. Thus, the effects of ECINs on gluten network could not be neglected even without support from

rheological analysis. SDS-PAGE suggested different protein composition among samples. In addition, it was observed that in bread baked from dough with ECINs, the crumb had more homogeneously sized and distributed gas cells than that in bread from control frozen dough, which implicated an improved gluten network by addition of ECINs.

### **Overview of the mechanism for ECINs' protection on frozen dough**

Based on the experimental results and analysis in the above sections, the possible mechanism for ECINs' protective effect on the quality of frozen dough is shown in Figure 22. ECINs, as biogenic heterogeneous ice nucleators, diminish or minimize the supercooling step and accelerate the freezing rate (Figure 17 and Figure 19), which ensures a moderately fast freezing rate. Furthermore, because of the moderately fast freezing rate without supercooling step, the viability of yeast cells is improved (Figure 16 and Figure 18). Consequently, the desirable qualities in pH, specific volume, size and number of gas cells, color, and texture profiles were partially retained (Table 9).

### **Potentials of ECINs in cryopreservation and energy savings**

Previous discussion demonstrated the potential in ECINs as a cryoprotectant, which is defined as those substances that, when added into the medium, protect biological materials from freezing damage (Hubalek, 2003). Common cryoprotectants, such as glycerol and DMSO, function by reducing the freezable water (Hubalek, 2003). Some other cryoprotectants function by replacing the water and forming hydrogen bonds with biological materials so as to stabilize the conformation and activity of biological material. However, this work suggested a novel mechanism of ECINs as a cryoprotectant which manipulates the freezing rate to reach a moderately fast freezing of yeast cells in both

suspension and frozen dough. With future studies on safety evaluation, ECINs isolated from *E. herbicola* would exhibit even more applications on preserving biological materials and foods. On the other hand, the ice nucleation gene from *E. herbicola* has been successfully cloned into *Saccharomyces cerevisiae* (yeast) which subsequently revealed ice nucleation activity (Hwang et al., 2001). There *ina+* yeast could probably show higher freeze/thaw stability that would have tremendous potential application in frozen dough industry.

As seen in Figure 19, addition of ECINs did not affect the initial cooling rate of the dough at about 16 °C/hr. However, with the presence of ECINs, the time of phase transition was shortened by 1.2 hours compared to the control without ECINs, if the time of phase transition was defined as the duration of temperature changes between  $-4.9 \pm 0.5$  °C, where -4.9 °C was the observed freezing point of dough and 0.5 °C was the sensitivity of the instrument. As result of the accelerated phase transition, the total time to reach complete freezing state (duration of temperature changes from 10 °C to -15.9 °C) for frozen dough blended with ECINs was shorter by 1.4 hours, which is a 20% reduction. With the freezer working at averagely 248 Watts, the energy potentially saved during freezing of dough reached about 1250 kJ. This indicates that ECINs, capable to significantly raise the ice nucleation temperature and shorten the total freezing time in model and real food system (Li et al., 1997; Hwang et al., 2001; Zasytkin and Lee, 1999), can potential reduce energy consumption for food freezing.

In a summary on this part of study, the protective effect of incorporation of ECINs on yeast viability and the bakery quality of frozen dough upon freeze/thaw cycles

was demonstrated. Without ECINs, baked bread from frozen dough revealed deteriorated qualities, including lower specific volume and harder texture. Addition of ECINs to frozen dough reduced the deteriorations on all of these qualities. The mechanism of the protective effect of ECINs is that ECINs improved the viability of yeast both in suspensions and in frozen dough. Additionally, ECINs reduced the time of freezing which result in decreased energy consumption. Based on the results in this investigation, ECINs are promising in frozen food application on both improvement of frozen food quality and energy saving.

Table 9 Characterization and texture profile analysis of bread from fresh dough and dough after freeze/thaw cycles with and without addition of ECINs

Parameter	Bread Quality		
	Fresh dough	Dough without ECINs, after three freeze/thaw cycles	Dough with ECINs, after three freeze/thaw cycles
pH	5.47 $\pm$ 0.15 <sup>a</sup>	4.90 $\pm$ 0.09 <sup>b</sup>	5.25 $\pm$ 0.11 <sup>a</sup>
Water content (%)	37.86 $\pm$ 0.02 <sup>a</sup>	37.31 $\pm$ 0.05 <sup>a</sup>	37.83 $\pm$ 0.64 <sup>a</sup>
Water activity	0.91 $\pm$ 0.01 <sup>a</sup>	0.90 $\pm$ 0.01 <sup>a</sup>	0.88 $\pm$ 0.01 <sup>a</sup>
Specific volume (cm <sup>3</sup> /g)	4.12 $\pm$ 0.03 <sup>a</sup>	2.07 $\pm$ 0.02 <sup>c</sup>	3.04 $\pm$ 0.02 <sup>b</sup>
TPA			
Firmness (g)	1061 $\pm$ 64 <sup>a</sup>	5634 $\pm$ 690 <sup>c</sup>	3446 $\pm$ 273 <sup>b</sup>
Fracturability (g)	1410 $\pm$ 83 <sup>a</sup>	6193 $\pm$ 746 <sup>c</sup>	4333 $\pm$ 328 <sup>b</sup>
Resilience	0.23 $\pm$ 0.01 <sup>a</sup>	0.18 $\pm$ 0.01 <sup>b</sup>	0.22 $\pm$ 0.01 <sup>a</sup>
Image analysis			
Cell-Total area ratio (%)	50.4 $\pm$ 1.9 <sup>a</sup>	42.7 $\pm$ 4.8 <sup>a</sup>	56.2 $\pm$ 4.3 <sup>a</sup>
Cell density (cells/cm <sup>2</sup> )	91 $\pm$ 4 <sup>a</sup>	25 $\pm$ 1 <sup>c</sup>	68 $\pm$ 9 <sup>b</sup>
Mean cell area (mm <sup>2</sup> )	0.56 $\pm$ 0.04 <sup>a</sup>	1.74 $\pm$ 0.23 <sup>b</sup>	0.84 $\pm$ 0.18 <sup>a</sup>
Mean perimeter (cm)	0.22 $\pm$ 0.01 <sup>a</sup>	0.57 $\pm$ 0.03 <sup>b</sup>	0.24 $\pm$ 0.01 <sup>a</sup>
Color analysis			
L	76.88 $\pm$ 0.86 <sup>a</sup>	67.15 $\pm$ 3.15 <sup>b</sup>	70.64 $\pm$ 0.54 <sup>b</sup>
a	-1.54 $\pm$ 0.05 <sup>a</sup>	-1.17 $\pm$ 0.18 <sup>b</sup>	-0.94 $\pm$ 0.19 <sup>b</sup>
b	13.91 $\pm$ 0.45 <sup>a</sup>	13.99 $\pm$ 0.49 <sup>a</sup>	14.43 $\pm$ 0.43 <sup>a</sup>
dE	19.07 $\pm$ 0.75 <sup>a</sup>	27.96 $\pm$ 3.09 <sup>b</sup>	24.89 $\pm$ 0.58 <sup>ab</sup>
Pigment (A <sub>436nm</sub> )	0.0358 $\pm$ 0.0046 <sup>a</sup>	0.0377 $\pm$ 0.0033 <sup>a</sup>	0.0372 $\pm$ 0.0022 <sup>a</sup>

\*The letters following data indicated the result of statistical analysis. Values with no common letter are significantly different (P<0.05).



Table 10 Reducing sugar content of bread baked from fresh and frozen dough

	Bread baked from		
	Fresh dough	Frozen dough w/o ECINs	Frozen dough w/ ECINs
Reducing sugar (mg per gram of bread)	11.7 $\pm$ 0.4 <sup>a</sup>	11.9 $\pm$ 0.2 <sup>a</sup>	10.7 $\pm$ 0.3 <sup>a</sup>

\*The letters following data indicated the result of statistical analysis. Values with no common letter are significantly different ( $P < 0.05$ ).

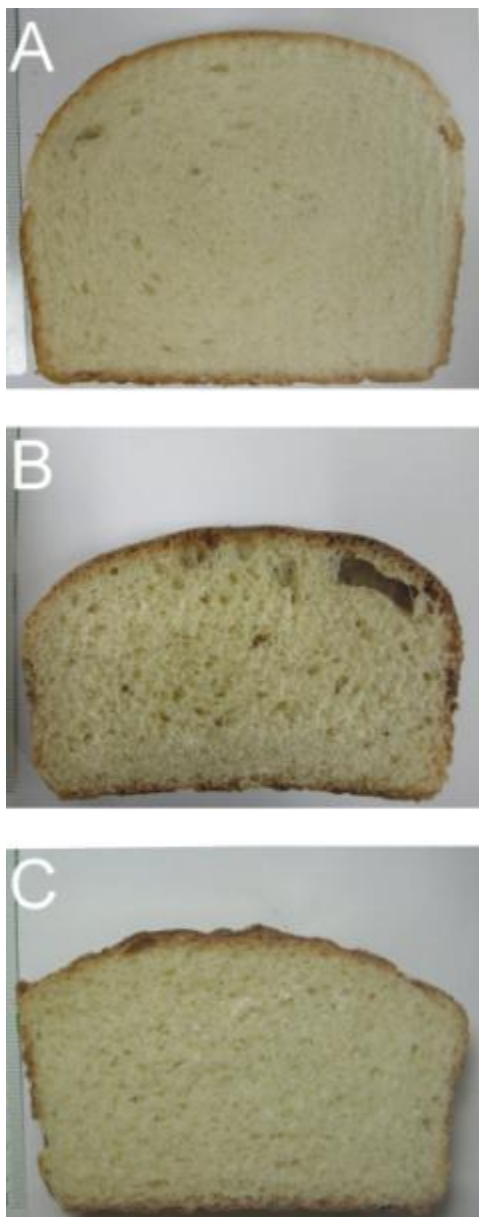


Figure 15 Cross section of bread baked from (A) fresh dough, (B) frozen dough, and (C) frozen dough blended with ECINs\*.

\* The width of each image was 14.9 cm.

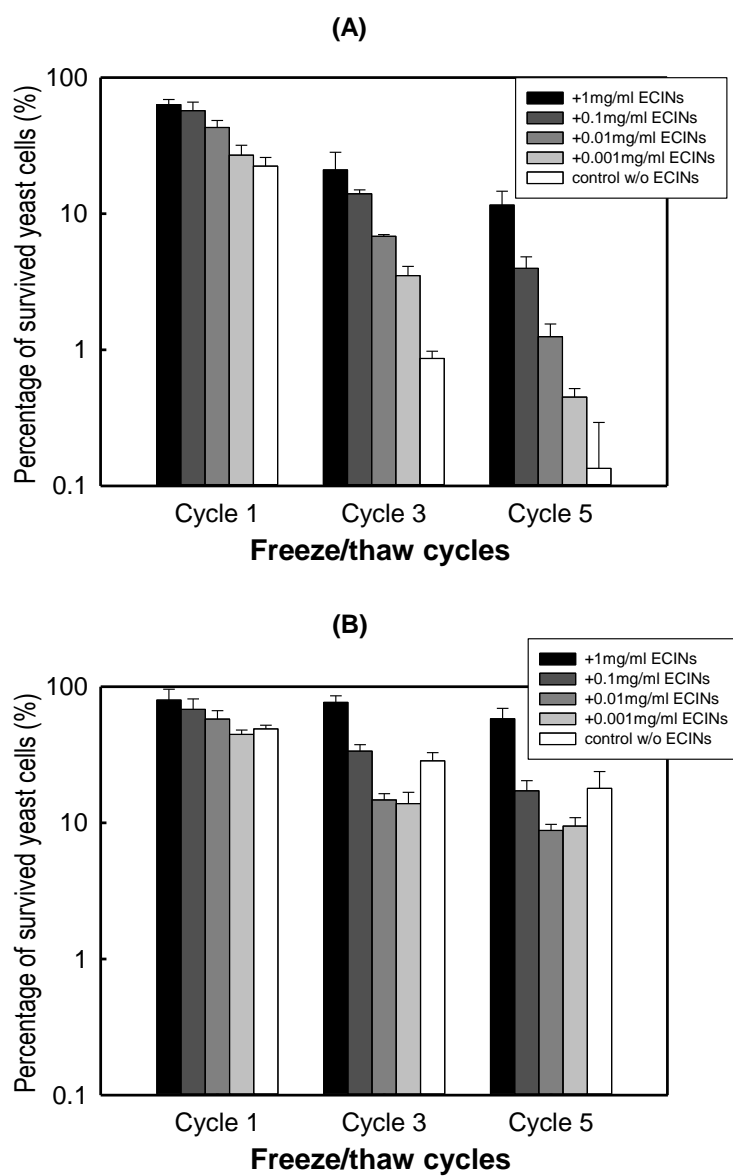
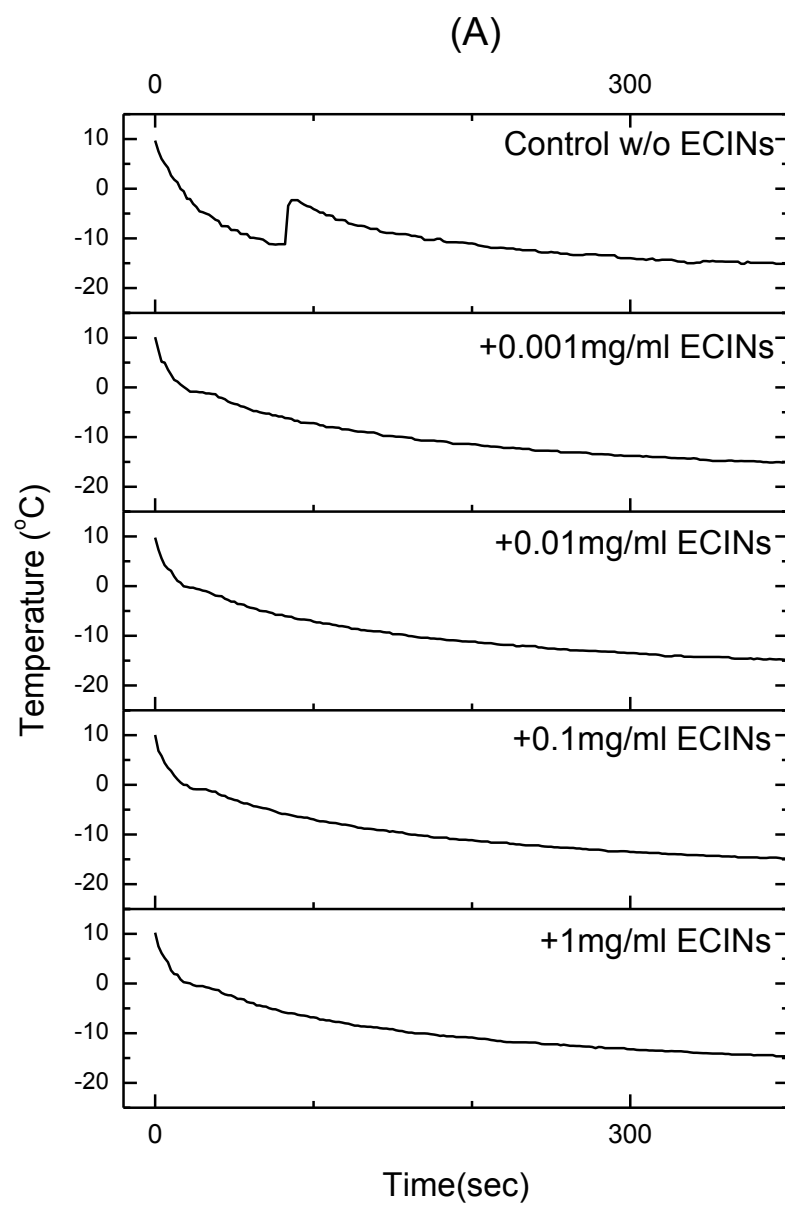


Figure 16 Survival ratio of (A) log-phase and (B) stationary-phase yeast cells in suspensions through freeze/thaw cycles without ECINs or with various concentrations of ECINs.



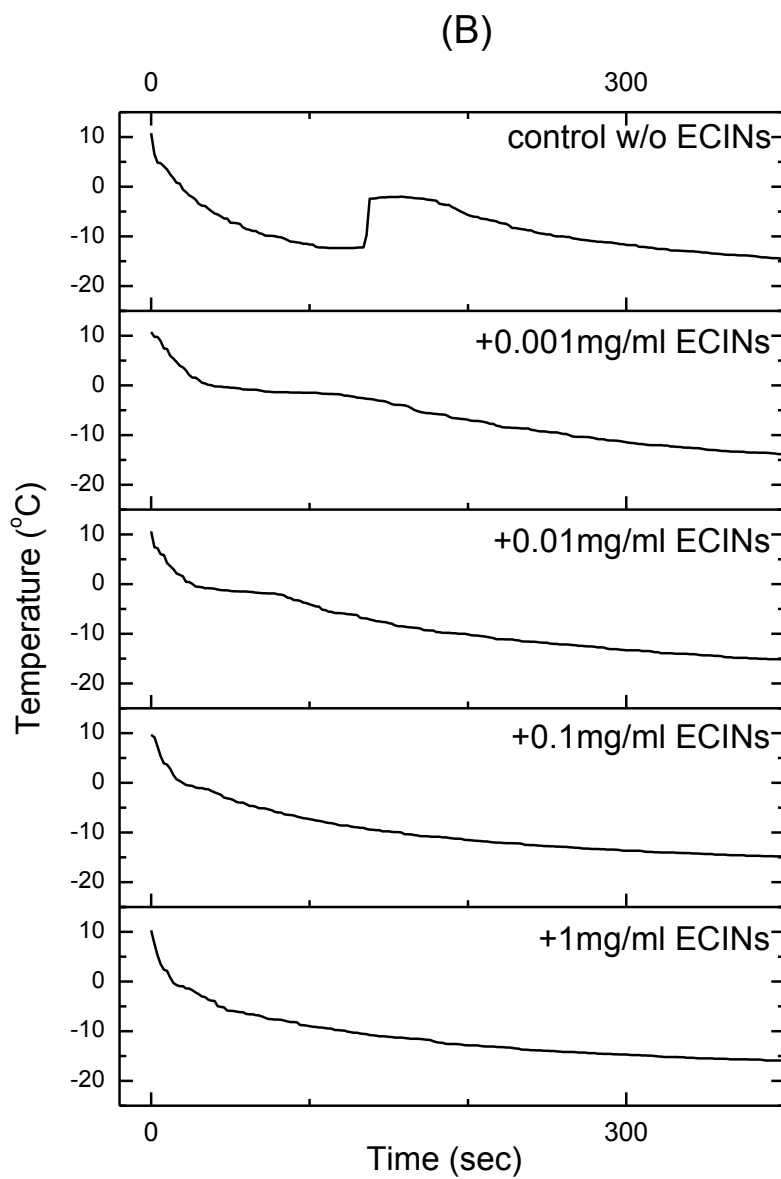


Figure 17 Effects of ECINs on the temperature changes of (A) log-phase and (B) stationary-phase yeast cells in suspensions during freezing.

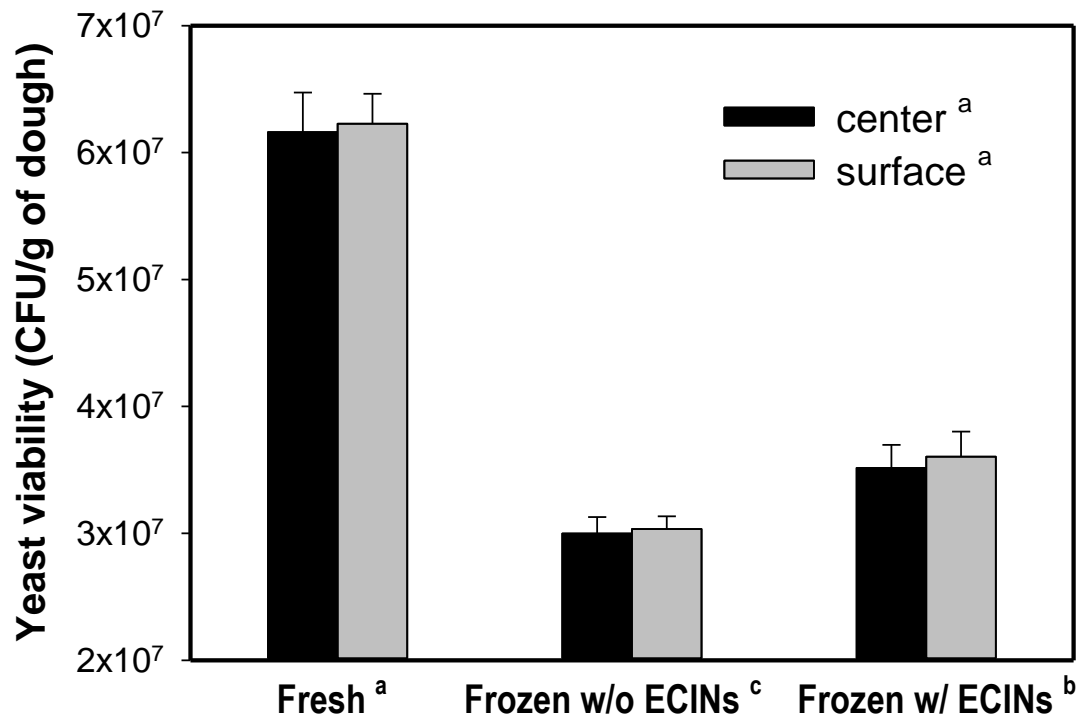


Figure 18 Concentration of viable yeast in fresh dough and frozen dough with and without blended ECINs\*.

\* The letters in superscript following the sample names and in the legend indicated the result of statistical analysis. Values with no common letter are significantly different ( $P < 0.05$ ).

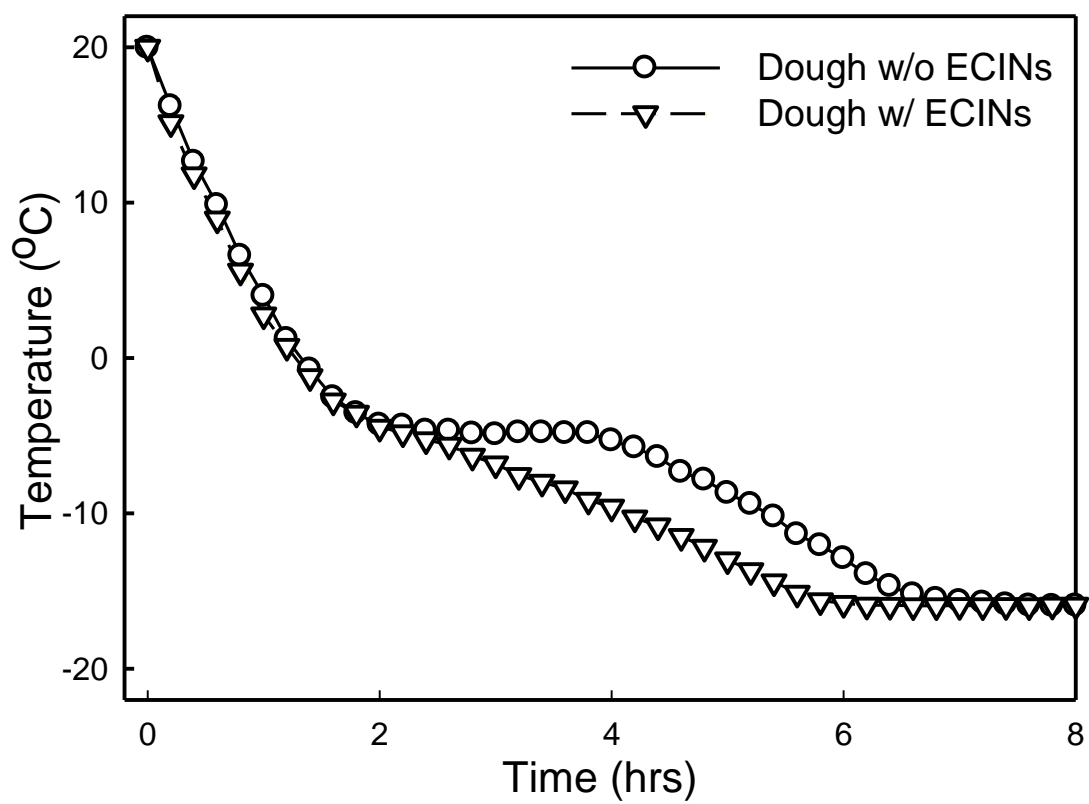


Figure 19 Effects of ECINs on the freezing process of dough.

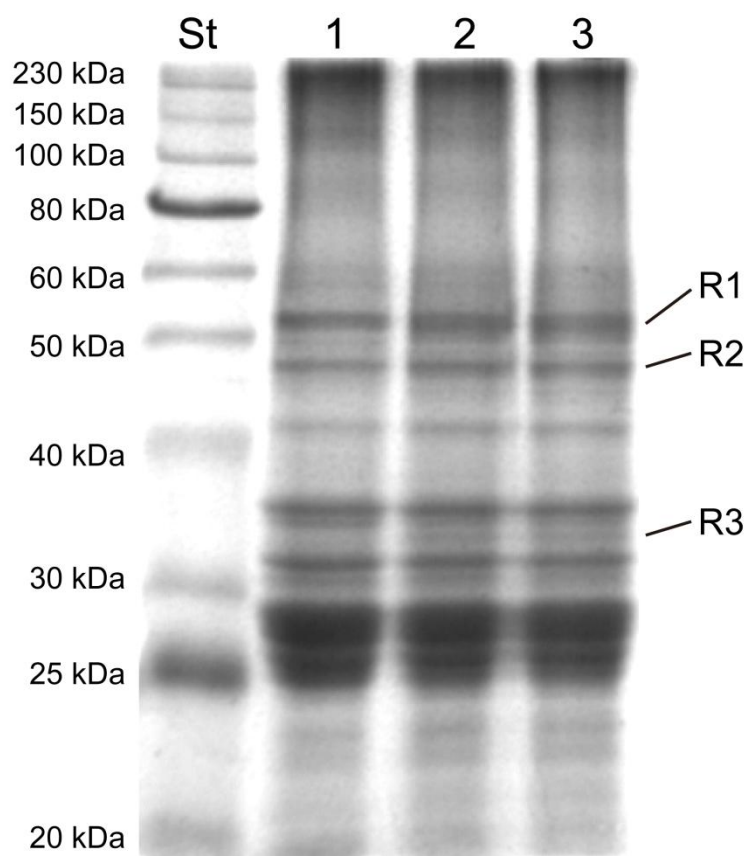


Figure 20 SDS-PAGE analysis of protein extracts from dough.

Lane St: protein standard; lane 1: fresh dough; lane 2: frozen dough without addition of ECINs; 3: frozen dough with ECINs.



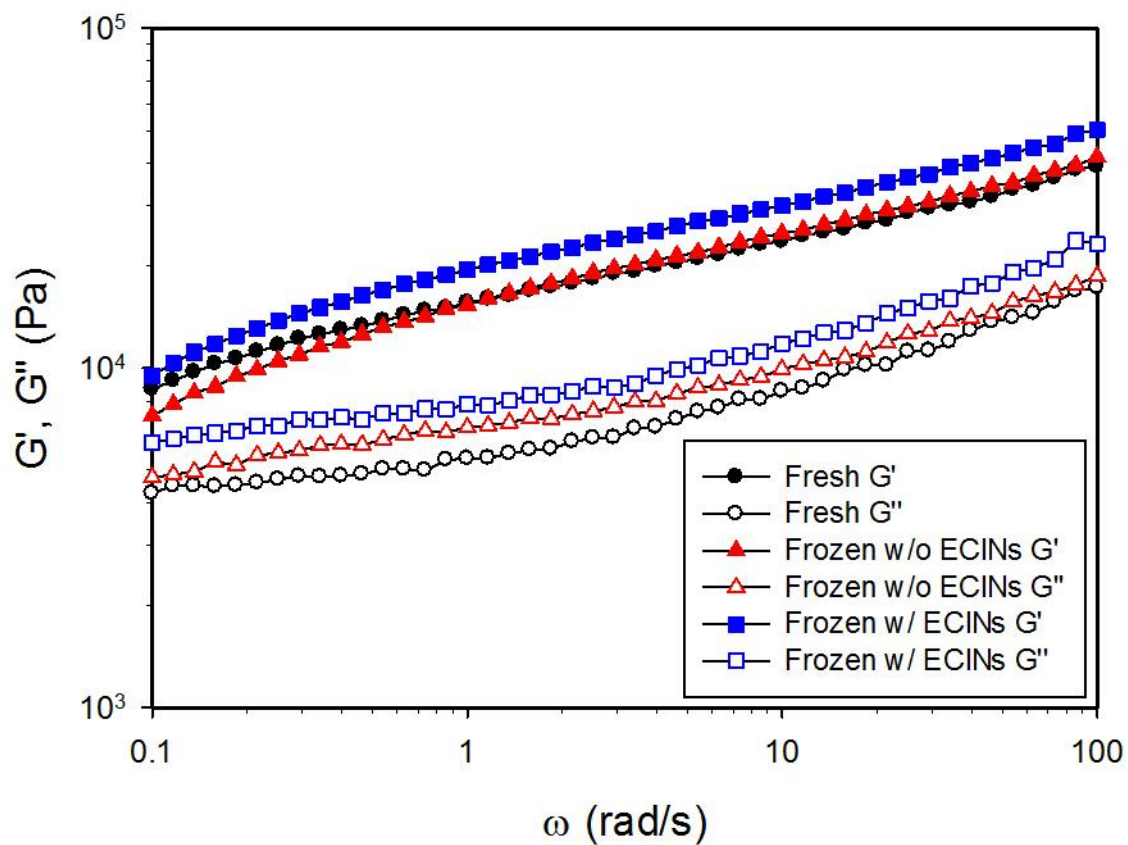


Figure 21 Frequency sweeps of  $G'$  and  $G''$  of fresh dough and thawed frozen dough that have been processed by three freeze/thaw cycles.

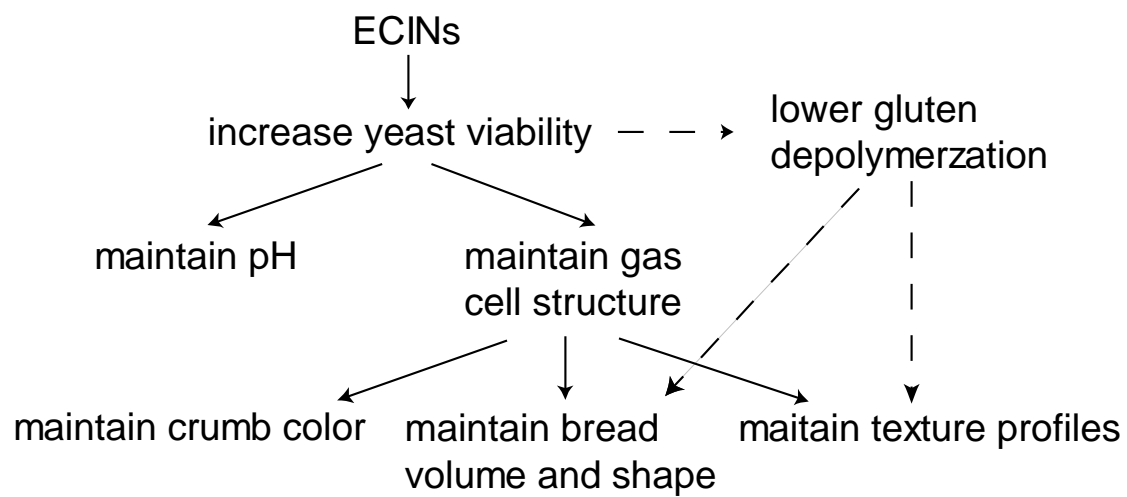


Figure 22 Summary on the mechanisms of protection by ECINs on frozen dough. Dash lines indicated unconfirmed mechanisms.

## **CHAPTER FOUR. PROMOTING ICE NUCLEATION ON ZEIN SURFACE THROUGH LAYER-BY-LAYER DEPOSITION OF EXTRACELLULAR ICE NUCLEATORS**

*As of submission of this dissertation, the work in this chapter has been submitted in the title of “Promoting Ice Nucleation on Zein Surface through Layer-By-Layer Deposition of Extracellular Ice Nucleators” to the Journal of Physical Chemistry for consideration of publication.*

### **Abstract**

This study aimed at developing a novel zein-based biopolymer film with ice nucleation activity through the adsorption of biogenic ice nucleators, i.e., extracellular ice nucleators (ECINs) isolated from *Erwinia herbicola*, onto zein film surface. The adsorption behaviors and mechanisms were investigated using quartz crystal microbalance with dissipation monitoring (QCM-D). On unmodified hydrophobic zein surface, the highest ECINs adsorption occurred at pH 5.0; for UV/ozone treated zein surface followed by deposition of poly(diallyldimethylammonium chloride) (PDADMAC) layer, the optimum condition for ECINs adsorption occurred at pH 7.0 and 0.05 M, where the amount of ECINs adsorbed was also higher than that on unmodified zein surface. QCM-D analyses further revealed a two-step adsorption process on unmodified zein surfaces, compared to a one-step adsorption process on PDADMAC-modified zein surface. In order to quantify the ice nucleation activity of ECINs-coated zein films, an empirical method was developed to correlate the number of ice nucleators

with the ice nucleation temperature measured by differential scanning calorimetry (DSC). Our results indicated that the highest ice nucleation activity (INA) of ECINs on ECINs-modified zein film was found to be 64.1 units/mm<sup>2</sup>, which was able to elevate the ice nucleation temperature of distilled water from -15.5 °C to -7.3 °C.

## **Introduction**

Biopolymers gain much attention due to their potentials as alternative biodegradable packaging materials to reduce the usage of petroleum-based synthetic polymers. The biopolymers such as polysaccharides, proteins and some natural gums show advantages over those petroleum-based synthetic polymers, such as polyethylene (PE) due to their abundant and renewable sources, as well as their environmentally-friendly and biodegradable nature. Using biopolymers in packaging materials is an emerging trends and will be a evolutionary progress in food packaging industry (Rhim and Ng, 2007).

Application of zein has been developed in many aspects, such as coatings, fibers, inks, tissue engineering and drug delivery (Wang and others 2007; Miyoshi and others 2005; Luo and others 2011; Luecha and others 2011). The most promising applications of zein are biodegradable films, coatings and plastics used for packaging. With the unique hydrophobic nature of zein, studies reveal that zein-coated paper is suitable for the packaging of high-fat foods and sandwiches (Andersson, 2008). In addition, zein films are freeze/thaw stable, allowing its use in packaging to frozen foods (Padua et al., 2000). Nevertheless, there are still limited studies on developing zein-based functional film. In the literature, only antimicrobial and antioxidant films have been prepared by

incorporating lysozyme, albumin proteins, disodium EDTA and phenolic compounds in zein films (Gucbilmez et al., 2007; Mecitoglu et al., 2006; Arcan and Yemenicioglu).

Biogenic ice nucleators, such as extracellular ice nucleators (ECINs) isolated from *Erwinia herbicola*, are demonstrated to minimize the supercooling while initiating ice nucleation at elevated subzero temperatures, measured by freezing curve and DSC (Zasytkin and Lee, 1999). The sources, characteristics and potential applications in foods of ECINs have been summarized by Li and Lee (Li and Lee, 1995). A protective effect from ECINs was demonstrated on fish fillet, which prevented the denaturation of fish actomyosin during freeze/thaw cycles (Zhu and Lee, 2007). A recent study in our lab also found that ECINs protected the bakery quality of frozen dough through freeze/thaw cycles.

This part of study aimed at developing a zein-based functional film with ice nucleation activity which can be used for frozen foods applications. This work was stimulated by previous research related to the immobilization of enzymes on polyelectrolyte film surfaces, which clearly indicated that polymer thin film could maintain the activity of the immobilized enzymes (Chaniotakis, 2004). In this paper, ECINs were immobilized on UV/ozone treated zein film surface through layer-by-layer deposition method. Quartz crystal microbalance with dissipation monitoring (QCM-D) technique was used to monitor the adsorption behavior of ECINs on zein films and to understand the deposition processes involved in zein films of different surface chemistry. Furthermore, a new empirical quantitative method was developed to assess the ice nucleation activity of zein-based films. The integration of extracellular ice nucleators (ECIN) into biopolymer thin films is expected to create novel edible films or novel

biodegradable packaging materials which can be very advantageous in biomedical (e.g. storage of cells in frozen environments) and frozen foods applications with regard to energy saving and quality improvement.

## Materials and Methods

### *Materials*

The  $\alpha$ -zein of biochemical-grade purity was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Poly (diallyldimethylammonium chloride) (PDADMAC, also abbreviated as PDDA) solution, average Mw 200,000-350,000 (medium molecular weight), 20 wt. % in H<sub>2</sub>O was purchased from Sigma Chemical Co. Milli-Q water was used throughout the experiment.

### *Preparation of extracellular ice nucleators*

The *Erwinia herbicola* subsp. *ananas* was obtained from the American Type Culture Collection (ATCC Cat. No. 11530) and routinely cultured in yeast extract (YE) medium consisting of 2.0% yeast extract, 1.0% sucrose, 0.2% L-serine, 0.2% L-alanine, 0.086% K<sub>2</sub>SO<sub>4</sub>, and 0.14% MgSO<sub>4</sub> under 18°C at shaking speed of 200 rpm.

Extracellular ice nucleators (ECINs) were isolated from *E. herbicola* Cells which were harvested by centrifugation at 10,000  $\times g$  for 20 min and then re-suspended in 20 mM Tris/HCl pH 8.0. Sonication of cells was done under output of 50 watts on ice, 10 seconds with 10 seconds interval for 3 times. Cells and debris were removed by centrifugation at 10,000  $\times g$  for 20 min and supernatant was subjected to filtration through 0.45  $\mu m$  filters. The filtrate was put on ultracentrifugation at 160,000  $\times g$  for 2 hour and

pellet was re-suspended in 20 mM Tris/HCl with 20 mM MgCl<sub>2</sub>, pH 8.0. Lyophilized ECINs in the form of powder were stored at -20 °C.

#### *Ice nucleation activity assay*

Ice nucleation activity (INA), defined as the activity units at -10 °C or higher was determined by the droplet-freezing assay (Vali, 1971) with some modifications. ECINs were dispersed into pH 7.0 Tris buffer to 10 mg/mL. Ten-fold dilutions were prepared with distilled water. Twenty droplets, 10 µL each, were transferred onto the surface of polished aluminum pan which was then placed in -10.00 °C bath. After 3 min, the number of frozen droplets was counted and the value between 5 and 18 was considered to be statistically valid and the corresponding dilution was used for calculation using Equation (1) as:

$$INA = -\frac{\ln(1-f)}{V_d \cdot D} \quad \text{Equation (1)}$$

$f$  - Fraction of frozen droplets, calculated as  $n/20$ , where  $n$  is the number of frozen droplets

$V_d$  - Volume of droplets (10 µL)

$D$  - Dilution which gives a frozen droplet number in the range of 5 to 18, out of the 20 droplets

To examine the environmental effect on ECINs' activity, solutions of different pH and ionic strength (IS) were prepared by using NaCl, HCl and/or NaOH.

### *Buffer preparation*

In the experiments that ECINs adsorbed on zein films, acetate buffers were prepared to pH 5.0 and pH 6.0. Tris buffers were prepared to pH 7.0 and 8.0. NaCl was used to adjust the IS in buffer to designated value.

### *Quartz crystal microbalance with dissipation monitoring (QCM-D)*

Gold sensor crystals (Q-Sense AB, Sweden) were pre-cleaned by UV/ozone treatment and wash liquid ( $\text{H}_2\text{O}:\text{H}_2\text{O}_2:\text{NH}_3$ , 5:1:1 v/v/v). Zein films spin-coated on gold sensors were prepared as described in previous work (Shi et al., 2009).

QCM-D measurements were performed with a Q-SENSE D300 system (Q-Sense AB, Sweden) at 25 °C. Samples and buffers were conducted into the temperature-controlled chamber as gravitational flow through a 5-mL polypropylene pipette tip. Buffers were first introduced into the chamber to reach steady state with the film. Ten minutes after the baseline was established, samples (ECINs or PDADMAC) were introduced into the chamber to adsorb onto the surface of film. Minimal 5-min steady state was held until proceeding to the next step. The threshold to recognize steady state was that change of frequency became slower than 1 Hz per 5 min. Each adsorption step was followed by a wash step using the corresponding buffer. During the steps, shifts in both frequency ( $\Delta F$ ) and energy dissipation ( $\Delta D$ ) were recorded simultaneously at the fundamental resonant frequency (5 MHz) along with the third (15 MHz), fifth (25 MHz), and seventh (35 MHz) overtones towards the end of each test.

Due to that biomolecules form viscoelastic layers which do not follow a rigid oscillation, the Voigt model was used to accurately estimate the mass of adsorption



(Voinova and others 1999). In this model, the  $\Delta F$  and  $\Delta D$  are expressed by below equations.

$$\Delta F \approx -\frac{1}{2\pi\rho_0 h_0} \left\{ \frac{\eta_3}{\delta_3} + h_1 \rho_1 \omega - 2h_1 \left( \frac{\eta_3}{\delta_3} \right)^2 \frac{\eta_1 \omega^2}{\mu_1^2 + \omega^2 \eta_1^2} \right\} \quad \text{Equation (6)}$$

$$\Delta D \approx \frac{1}{\pi f \rho_0 h_0} \left\{ \frac{\eta_3}{\delta_3} + 2h_1 \left( \frac{\eta_3}{\delta_3} \right)^2 \frac{\eta_1 \omega^2}{\mu_1^2 + \omega^2 \eta_1^2} \right\} \quad \text{Equation (7)}$$

where  $\rho_0$  and  $h_0$  are the density and thickness of crystal;  $\eta_3$  is the viscosity of the bulk fluid (buffer);  $\delta_3 [= (2\eta_3/\rho_3\omega)^{1/2}]$  is the viscous penetration depth of the shear wave in the bulk fluid;  $\rho_3$  is the density of the bulk fluid and  $\omega$  is the angular frequency of the oscillation. The four parameters for the adsorbed layer are the thickness ( $h_1$ ), the density ( $\rho_1$ ), the viscosity ( $\eta_1$ ) and the elastic shear modulus ( $\mu_1$ ). With  $\Delta F$  and  $\Delta D$  measured at the fundamental (1<sup>st</sup>) and the 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> resonant frequency simultaneously, eight sets of data in one test could be modeled using the Equation (6) and Equation (7).

Density and viscosity of water at 25 °C, 0.997 g/mL and 0.890 cP, were provided to the software as  $\rho_3$  and  $\eta_3$ . For ECINs, a common protein density of 1.22 g/cm<sup>3</sup> (Andersson and Hovmöller, 1998) was adopted as  $\rho_1$  for the software to calculate the thickness ( $h_1$ ) of ECINs layer. The adsorbed mass was calculated by multiplying  $h_1$  by  $\rho_1$ . The INA of ECINs used for adsorption on zein films is  $1.61 \times 10^6$  units/mg.

#### *Fourier transform infrared spectroscopy (FT-IR)*

The Fourier transform infrared spectra were collected under ambient conditions, using a Thermal Nicolet Nexus 670 FT-IR spectrometer (Thermo Fisher Scientific Inc.,

Waltham, MA) equipped with a Smart MIRacle™ horizontal attenuated total reflectance Ge crystal accessory. Each spectrum was averaged by 256 scans with 4 cm<sup>-1</sup> resolution.

#### *Differential scanning calorimetry (DSC)*

DSC analyses were performed on a DSC 823E thermal analyzer (Mettler-Toledo Inc., Columbus, OH) supplied with liquid nitrogen and compressed nitrogen gas. Three batches of ECINs were individual prepared as described previously. Their INA was determined as  $1.05 \times 10^6$ ,  $8.56 \times 10^5$  and  $3.57 \times 10^6$  units/mg, respectively. Each batch of ECINs was prepared into 1,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  mg/ml solutions. To measure the nucleation temperature of ECINs solutions, 10  $\mu$ L ECINs solution was carefully transferred into 40- $\mu$ L aluminum crucibles with lid. Temperature was decreased from 4 °C to -30 °C at a rate of -1 °C/min. Empty crucible was used as reference. To measure the nucleation temperature of water on films, the film was cut to exactly cover the bottom of crucible, and then 10  $\mu$ L dH<sub>2</sub>O was transferred carefully onto films and spread to cover the whole film surface. After lid was closed, the crucible with sample was subjected to the same freezing condition as above. Crucible with films but without water was used as reference.

#### *Statistical analysis*

One-way ANOVA, t-test and non-linear regression were performed using Sigma-Plot v.12.0 with integrated SigmaStat module.

## Results and Discussion

### Effects of pH and ionic strength on the INA of ECINs

ECINs were protein-based ice nucleators containing lipid and carbohydrate moieties (Li and Lee, 1995). To investigate the optimum adsorption conditions on zein films, the effect of environmental physicochemical factors (pH and ionic strength, IS) on the INA of ECINs was firstly examined. As shown in Figure 23A, ECINs retained the activity in a wide pH range from pH 4.0 to 9.0. In extreme pH beyond this range, the ECINs activity dramatically diminished ( $P < 0.05$ ).

In most cases, pH dependency of protein activity was determined by the pK values of certain amino acids involved directly in catalytic reactions or in maintaining the protein conformation (Nielsen and others 2001). As for ECINs, the INA depends on the overall structure of the lipoglycoprotein complexes (Hew and Yang, 1992), which may account for the relative insensitivity to pH variation.

In addition to pH, the effect of IS was also examined. The maximum INA was detected at low IS, from 0.01 M to 0.05 M (Figure 23B). Under higher IS, ECIN activity was inhibited ( $P < 0.05$ ). This finding was consistent with the previous report (Phelps et al., 1986).

Based on these findings, effects of pH from 5.0 to 8.0 and IS from 0.02 M to 0.10 M on the adsorption of ECINs on surface of zein films were investigated in the following sections.

### Adsorption of ECINs on hydrophobic surface of zein films

Original zein films have a hydrophobic surface. After UV-ozone treatment, the surface is hydrophilic, bearing negative charge (Shi et al., 2009). On the other hand, it was suggested that ECINs have both hydrophobic and hydrophilic patches on their surface (Mizuno, 1989; Hew and Yang, 1992). Therefore, it was hypothesized that ECINs could adsorb onto original zein surface via hydrophobic or onto UV/ozone-treated negatively-charged zein surface via electrostatic interactions. In order to find the optimum adsorption conditions to generate highest INA activity on zein films, the adsorption of ECINs on both original and UV/ozone-treated zein film surface were investigated.

Firstly, the adsorption of ECINs on original zein films was monitored in real time by QCM-D, which measured the frequency shift ( $\Delta F$ ) and dissipation shift ( $\Delta D$ ) while ECINs gradually adsorbed on the surface of zein films. Since the  $\Delta F$  and  $\Delta D$  at 1<sup>st</sup> resonant frequency were not used by the software for data fitting, the curves of  $\Delta F_3$ ,  $\Delta F_5$ ,  $\Delta F_7$ ,  $\Delta D_3$ ,  $\Delta D_5$  and  $\Delta D_7$ , obtained from a representative test were plotted in Figure 24. The curves revealed a real-time adsorption of ECINs: at  $t_1$ , ECINs solution flowed in and the adsorption caused a rapid decrease of frequency and increase of energy dissipation, which gradually slowed down until steady state. After 40 min, both  $\Delta F$  and  $\Delta D$  became stable, suggesting the adsorption and desorption reached equilibrium. Subsequently, the buffer flowed in at  $t_2$  for washing step, which mostly removed the reversibly adsorbed ECINs, shown as a slight increase of frequency. Meanwhile, the energy dissipation,  $\Delta D$ , further increased after the washing step, suggesting after washing, the ECINs layer became even more loosely bound.

The frequency shifts at the 3<sup>rd</sup> overtones for adsorption under various pH were plotted in Figure 25A. While increasing pH from 5.0 to 8.0, the frequency shifts decreased. Moreover, the pattern of adsorptions also changed. At pH 5.0, 6.0 and 7.0, there was only small frequency increase after washing step, indicating that most of the adsorption at these conditions was irreversible. In contrast, at pH 8.0, the adsorbed ECINs were rarely retained after washing step, indicating a mainly reversible adsorption at this pH. Voigt model was used to analyze the data to estimate the adsorbed mass (Figure 25B). The highest adsorption of ECINs occurred at pH 5.0 and increasing pH reduced the adsorption.

In order to investigate the adsorption behavior of ECINs on hydrophobic zein surface,  $\Delta D$  was plotted against  $\Delta F$  in Figure 25C. The plots for pH 6.0 to pH 8.0 shared similar slope values which are clearly larger than the plot for pH 5.0. Because a smaller slope of the plot corresponds to a more compact and rigid layer and vice versa (Rodahl and others 1997), this indicated that at pH 5.0, ECINs adsorbed onto zein films in a more compact molecular arrangement and formed a more rigid layer than the adsorption at higher pH. Also, plots for pH 6.0 to 8.0 showed a bending as the adsorption proceeded, suggesting a two-step adsorption at these conditions, where the first step revealed larger frequency change with a deeper slope compared to the second step. These multiple adsorption stages were commonly observed in adsorptions of biological materials, where the initial adsorption usually involved a quick addition of biomolecules which formed a loose layer, resulting in a quick change of  $F$  and larger slope of  $\Delta D$  to  $\Delta F$ ; in the second stage, the adsorption slowed down and the adsorbed biomolecules reorganized to form a more compact layer (Zhou and others 2004).

For untreated zein film, the surface hydrophobic. The pH-dependent adsorption of ECINs may be due to the change of net charge of ECINs under different pH. Using pH-dependent turbidimetric titration, we previously observed that the isoelectric point (pI) of ECINs was closed to 4.2. At pH 5.0, the net charge on the surface of ECINs proteins was close to zero, making the surface of ECINs more likely to build hydrophobic interactions with the non-polar groups of zein. This resulted in the formation of more compact and rigid ECINs layer on zein surface at pH 5.0 compared to higher pH under which the surface of ECINs was more negatively charged.

### **Adsorption of ECINs on UV/ozone-treated hydrophilic surface of zein films**

It was previously demonstrated that the surface of zein film could be modified by UV/ozone (UVO) treatment to form carboxyl groups (Shi et al., 2009). In this study, similar UV/ozone treatment was applied to zein film surface to make it negatively charged, and structural changes of zein film surface were monitored by ATR- FTIR as shown in Figure 26. UV/ozone treatments resulted in the formation of a broad shoulder peak in the wavenumber range between  $1710\text{ cm}^{-1}$  and  $1760\text{ cm}^{-1}$ , which corresponded to the formation of negatively charged carboxyl groups (Shi et al., 2009). When the UVO treatment reached 12 minutes, the integrity of zein film was damaged, as evidenced by the decrease of overall spectral intensity.

Considering that modified zein surface with carboxyl groups and ECINs both bear negative charges at pH 5 ~ 8, a positively charged layer was needed to establish electrostatic interactions. PDADMAC is a strongly dissociated quaternary ammonium polycation and low-molecular-weight polymer that was demonstrated to form compact, rigid and flat layer when adsorbed onto fiber web. It was previously demonstrated to

successfully mediate the adsorption of negatively-charged bacterial cells onto the negatively-charged surface of hemicelluloses and wood extractives (Leino and others 2011). In the present study, the adsorption condition of PDADMAC onto UVO-treated zein surface was firstly determined (Figure 27).

Subsequently, the PDADMAC-mediated adsorption of ECINs on UVO-treated zein film surface was monitored by QCM-D (Figure 28), which involved injection of PDADMAC at time  $t_1$  and a following washing step at  $t_2$ , in addition of injecting ECINs at time  $t_3$  and the second washing step at  $t_4$ .

The effect of pH on adsorption of ECINs was firstly examined (Figure 29). Among the tested pH conditions, the adsorption of ECINs at pH 5.0 revealed a distinct pattern, where the ECINs layers formed at pH 5.0 were more compact than those formed at higher pH, suggested by smaller slope of  $\Delta D$  to  $\Delta F$  in Figure 29C. However, in contrast to the maximum ECINs adsorption on original hydrophobic zein surface occurring at pH 5, in the case of hydrophilic surface, neutral pH was preferred under which the highest mass of adsorbed ECINs reached about  $4 \mu\text{g}/\text{cm}^2$  (Figure 29B). It was unclear why maximum adsorption occurred at neutral pH rather than higher pH under which ECINs were expected to bear more negative charge and formed stronger electrostatic forces with the PDADMAC polycation layer. Interactions other than simple electrostatic interaction might be involved in this process.

Changes of frequency and mass of ECINs adsorption on the surface of UVO-treated-zein/PDADMAC bilayer at various IS were plotted in Figure 30A and B. Lower IS is usually favored for electrostatic interaction due to lower salt shield effect. For

example, Lundin et al. revealed that when the IS reached 0.15 M,  $\beta$ -casein hardly remained adsorbed on hydrophilic silica and quartz surfaces after rinsing (Lundin and others ). In the current case where IS ranged from 0.02 to 0.10 M, adsorption of ECINs did not exactly follow the expected trend. Instead, a higher adsorption occurred at an intermediate level of IS at 0.05 M. On the other hand, decrease in the slope of  $\Delta D$  to  $\Delta F$  with increased IS suggested that higher IS drove the ECINs protein to form a more compact and rigid layer (Figure 30C), with the mechanism discussed in the following section.

Comparing the adsorption of ECINs onto both original hydrophobic and PDADMAC mediated UVO-treated hydrophilic zein surfaces, the maximum ECINs adsorption was achieved on the UV/ozone-treated hydrophilic zein surface at pH 7.0 and IS 0.05 M.

### **Summary on adsorption behaviors of ECINs on zein films**

In summary, the possible mechanisms involved in ECINs adsorption on the two types of zein films were illustrated in Figure 31. ECINs were suggested to contain large hydrophobic and hydrophilic patches on the surface for the water molecules to bind and to orient into conformations that facilitate the formation of hydrogen bonds in ice (Mizuno, 1989; Hew and Yang, 1992). Thus, ECINs have potential affinities with either hydrophobic surface or hydrophilic surface. Comparing the two situations, adsorption by hydrophobic force dominated in the case on hydrophobic surface, while mainly electrostatic forces were involved in the adsorption of ECINs on the UV/ozone-treated-zein/PDADMAC bilayer. Similar as observation with other amphiphilic biomolecules, when adsorbed on hydrophobic surface, monolayer coverage by ECINs was expected



with their hydrophobic patches facing toward the surface and the hydrophilic patches toward the buffer (Rodahl et al., 1997). When the surface was hydrophilic, the ECINs molecules turned around having the hydrophilic patches interact electrostatically with the surface. In this case more ECINs were needed to shield each other's hydrophobic patches from buffer (Rodahl et al., 1997). That's why relatively more ECINs adsorbed to hydrophilic surface than hydrophobic surface (Figure 25B and Figure 29B).

Regarding the pH effect, under pH 5 that was close to the pI of protein, the surface net charge of ECINs was minimized and therefore the adsorption on original zein surface driven by hydrophobic force was promoted (Figure 25B). At higher pH, under which the surface of ECINs became charged, the adsorption behaviors of ECINs also changed, as suggested by those distinctive curves of  $\Delta D$  to  $\Delta F$  at pH 6 to 8 in contrast to those at pH 5, on both hydrophobic and hydrophilic surfaces (Figure 25C and Figure 29C). In addition to pH effect, higher IS enhanced the hydrophobic force among ECINs molecules, thus drove the ECINs to form a close-packed layer as suggested by a smaller slope of  $\Delta D$  to  $\Delta F$  in Figure 30C (Rodahl et al., 1997).

### **Development of method for quantifying ice nucleation activity on film**

The Vali method is a relatively accurate method to quantify the activity of heterogeneous ice nuclei (Vali, 1971). Its procedure involves series dilution and intensive sampling as described in the method section, which is time-consuming and burdensome. Moreover, the method is limited to test liquid which could be diluted. Because the ice nucleators on films are unable to be diluted, a novel method is required to determine the activity of ice nucleators on a surface.

Relationship between nucleation temperature and number of ice nuclei in a defined volume has been observed in some previous studies (Vali, 1971; Bigg, 1953). Recently, Zhang and his colleagues described this relationship using below equation.

$$T_s = A + Bxe^{Kx} \quad \text{Equation (8)}$$

where  $T_s$  was the degree of supercooling (difference of nucleation temperatures with and without ice nucleators),  $x$  was the number of ice nucleators (Zhang and others 2009). However, when we plotted the nucleation temperature versus the number of ice nuclei, we found a modification of the Equation (8) to Equation (9) better fitted the data obtained from three independent batches of ECINs with varied ice nucleation activity (fitting correlation coefficient,  $R^2=0.98$ ).

$$T_s = A + B \log IN \cdot e^{K \log IN} \quad \text{Equation (9)}$$

where  $T_s$  is nucleation temperature measured by DSC;  $IN$  is the number of ice nucleators. By non-linear regression,  $A$ ,  $B$  and  $K$  were calculated as -9.69, 0.44 and 0.17, respectively. The difference between Equation (9) obtained in this study from Equation (8) is possibly due to two reasons. Firstly, this study examined a five-magnitude range in the number of ice nucleators from 1 to  $10^5$  units whereas Equation (8) was based on data from one-magnitude range from  $1 \times 10^4$  to  $17 \times 10^4$  units/mL. Secondly, this study used DSC to detect nucleation temperature of 10  $\mu$ L liquid, whereas the other study used freezing curves to determine the nucleation temperature in 6 mL liquid. Because heterogeneous ice nucleation is a stochastic process, the accurate measurement on nucleation temperature becomes more difficult in a larger volume (Vali, 1994; Vali,

1971; Wilson and others 2003). Due to the same reason, many studies on heterogeneous ice nucleators preferably tested several magnitudes of concentration of ice nucleators.

QCM-D method is well established to measure the mass of adsorption of substances on surfaces (Jonsson and others 2008). This method was used to verify our developed empirical equation and its applicability in quantifying INA on surface. Films were prepared using the optimum adsorption condition with ECINs solution at two concentrations, 1 and 0.1 mg/mL, and the activity of ECINs on the film was determined by our developed DSC method and by QCM-D method (Table 11). In one method, the INA of films was calculated from the nucleation temperature of water on films using equation (5). On the other hand, the mass of adsorption was measured by QCM-D and the activities of the films were then calculated by multiplying the mass of adsorption with the INA of ECINs ( $1.61 \times 10^6$  units/mg). With both loading concentrations of ECINs, the two methods gave consistent values for the INA on zein films, suggesting that the developed method based on nucleation temperature was applicable in quantifying the INA of films.

In summary on this part of work, to develop zein-based ice nucleation films, the effect of different conditions on ECINs adsorption was examined. Firstly, it was found that ECINs' activity was better retained at pH 4.0 to 9.0 and IS 0.01 to 0.05 M. Using QCM-D analysis, the adsorption behavior of ECINs were examined on both original hydrophobic and PDADMAC-mediated UVO-treated zein films. At pH 5.0, which was close to the pI of ECINs, the adsorption of ECINs on original zein films reached maximum compared to adsorption at higher pH, possibly because hydrophobic

interaction favored this pH. On the other hand, neutral pH and IS 0.05 M was preferred for immobilization of ECINs on the surface of UVO-treated-zein/PDADMAC bilayer. Comparing the adsorption of ECINs at all conditions, the maximum ECINs adsorption was achieved on the hydrophilic zein surface at pH 7.0 and IS 0.05 M.

In order to quantify the ice nucleation activity of films, a new empirical method was developed to quantitatively correlate the number of ice nucleators with the nucleation temperature measured by DSC. The ice nucleation activity (INA) of the ECINs-immobilized zein films was assessed using the method developed and verified by comparing to the values obtained from QCM-D method. The INA values obtained by our novel developed method were statistically same as the values from QCM-D method which has been well established in quantifying mass of adsorbed substances on surface.

Table 11 Comparison to the INA of zein films measured by DSC versus by QCM-D

ECINs Concentration in film preparation	DSC method			QCM-D method	
	Nucleation temperature (°C)	Number of ice nucleators * (units)	INA ** (units/mm <sup>2</sup> )	Mass of adsorption (×10 <sup>-7</sup> g/cm <sup>2</sup> )	INA*** (units/mm <sup>2</sup> )
1 mg/mL	-7.31 ±0.11	1508.2 ±337.9	64.1 ±14.4 <sup>a</sup>	41.3 ±1.0	66.5 ±1.6 <sup>a</sup>
0.1 mg/mL	-8.40 ±0.11	118.7 ±34.1	5.0 ±1.4 <sup>b</sup>	2.8 ±0.8	4.5 ±1.3 <sup>b</sup>

\* Calculated from Nucleation temperature using Equation (9)

\*\* Area of films for DSC test was 23.54 mm<sup>2</sup>

\*\*\* Calculated from mass of adsorption using INA of ECINs as 1.61 ×10<sup>6</sup> units/mg

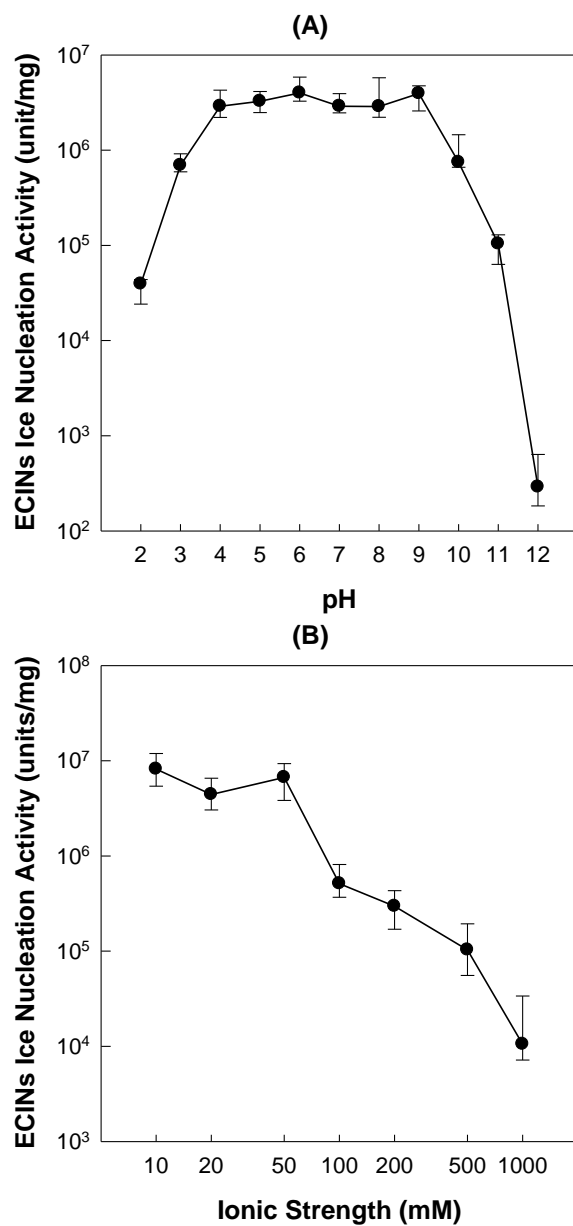


Figure 23 Effects of (A) pH and (B) ionic strength on INA of ECINs\*.

\* Ionic strength for (A) was 0.02M, and pH for (B) was 7.0.

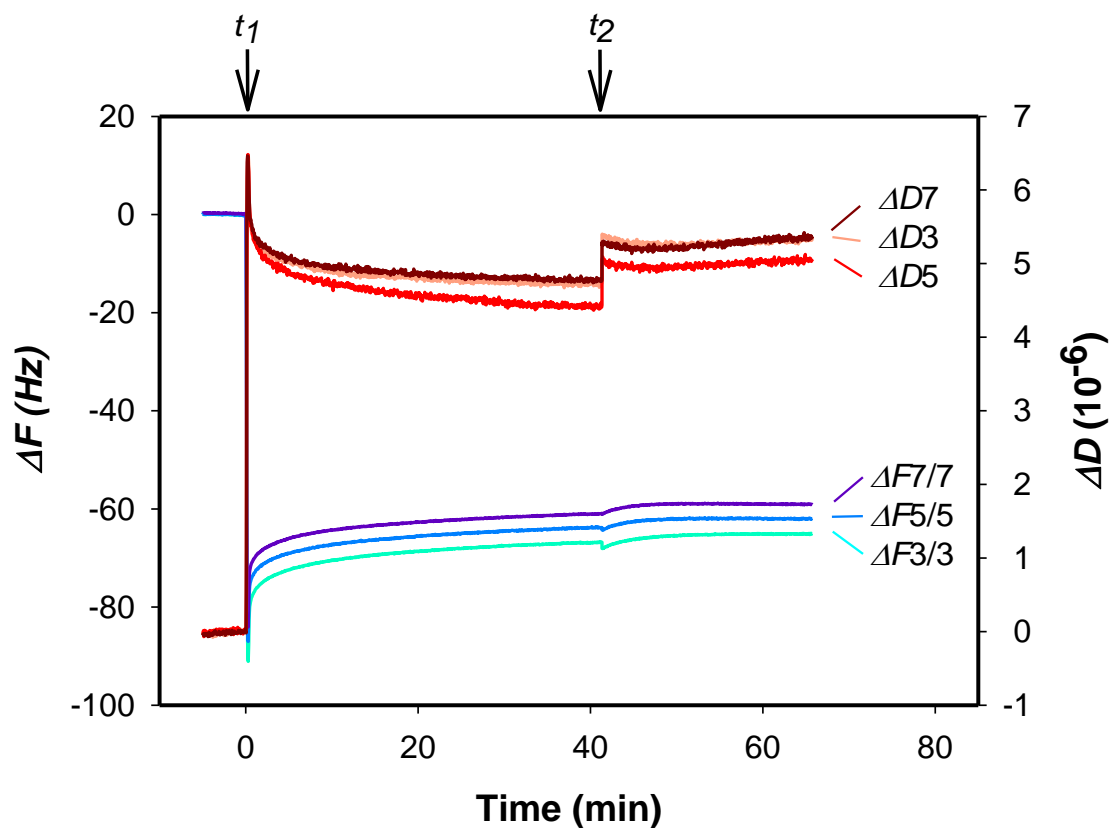


Figure 24 Frequency shift ( $\Delta F$ ) and energy dissipation shift ( $\Delta D$ ) induced by the adsorption of 0.1% ECINs solution at pH = 5.0 and I = 0.05 M on the zein-coated gold crystal surface\*.

\*  $\Delta F$  and  $\Delta D$  are measured simultaneously at three overtones ( $n = 3, 5, 7$ ) and normalized by their overtone number. The arrows indicate the time for the injection of sample ( $t_1$ ) and rinsing with buffer ( $t_2$ ).

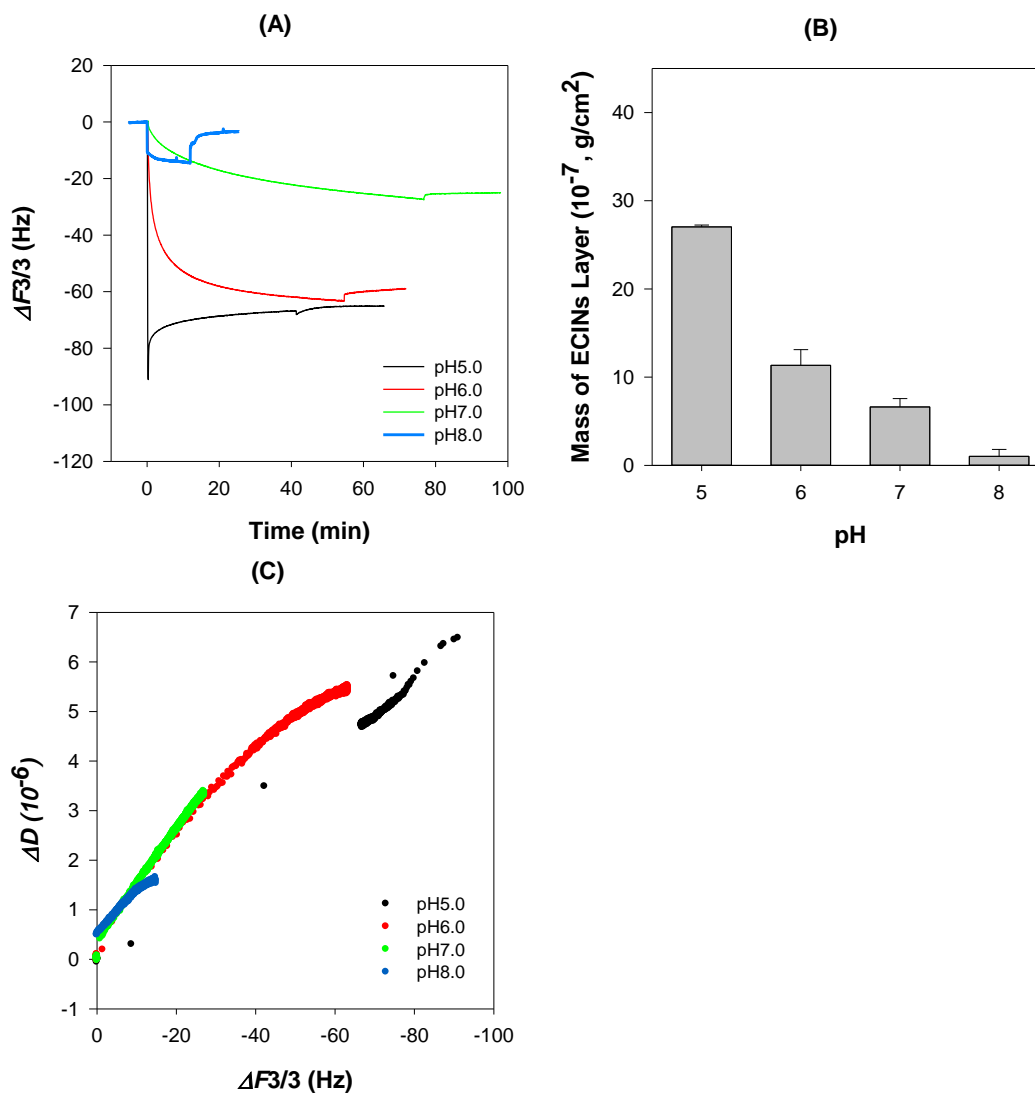


Figure 25 Effect of pH on the (A) Change of frequency ( $\Delta F_{3/3}$ ), (B) mass of adsorbed ECINs layer on hydrophobic zein surface and (C)  $\Delta D$ -  $\Delta F$  plots\*.

\* The ECINs solutions were prepared to the concentration of 0.1% w/v in 0.02M buffers at various pH, 10.05M.



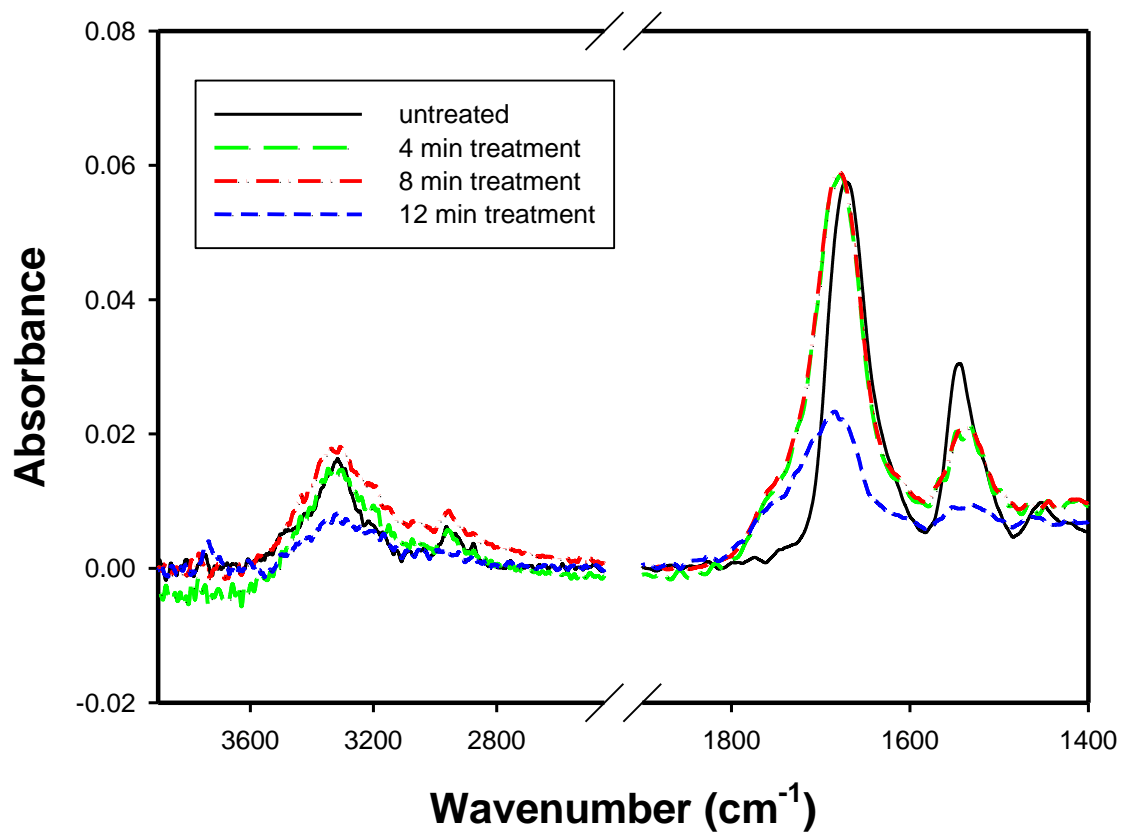


Figure 26 Modification to the surface of zein film by UV/ozone treatment, monitored by FT-IR. IR spectra of zein surface before and after 4-, 8-, and 12-min UVO-treatments were compared.

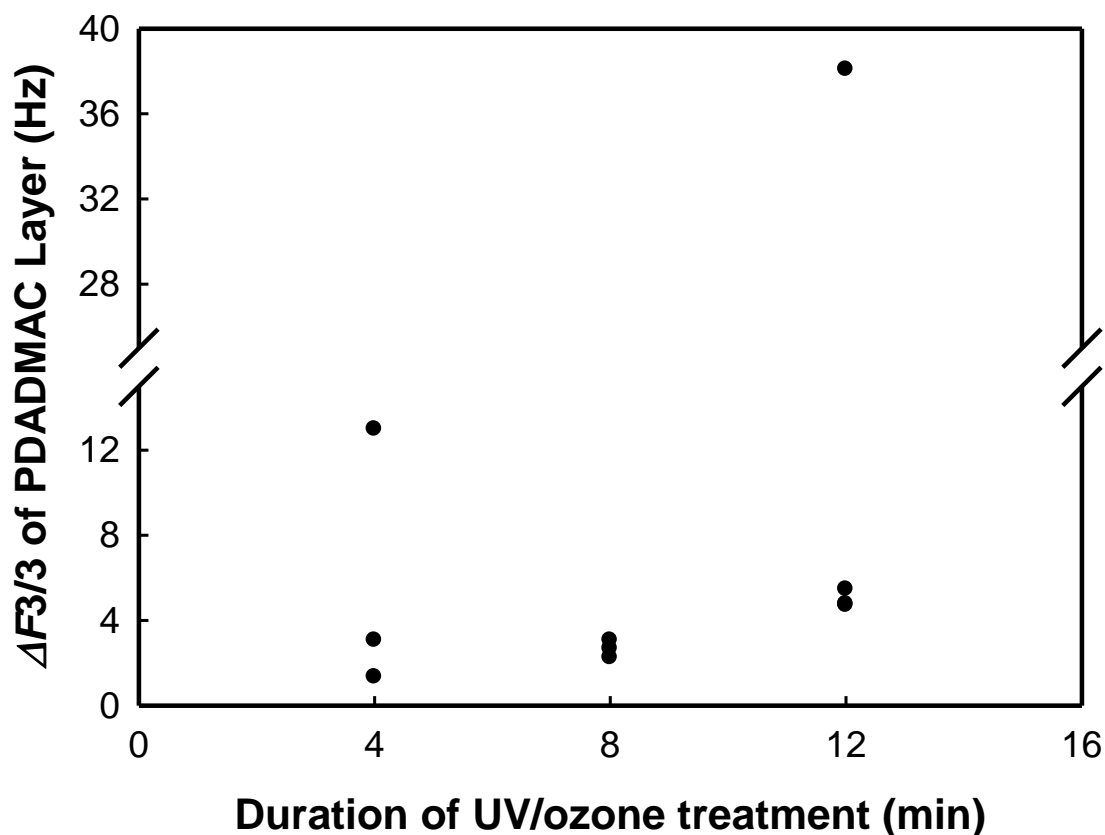


Figure 27 Adsorption of PDADMAC on UVO-treated zein surface monitored by QCM-D\*.

\* After 4-, 8- and 12-minute UVO treatment of zein surface, the adsorption of PDADMA was compared with aspect of  $\Delta F3/3$  from QCM-D monitoring. Three repeats for each condition were plotted individually to reveal the difference in repeatability. Adsorption on films with 8-min UVO treatment was most reproducible and was chosen for further ECINs adsorption. The PDADMAC solution was prepared to the concentration of 0.1% in 0.02M Tris buffers at pH 7.0 and 10.05M.

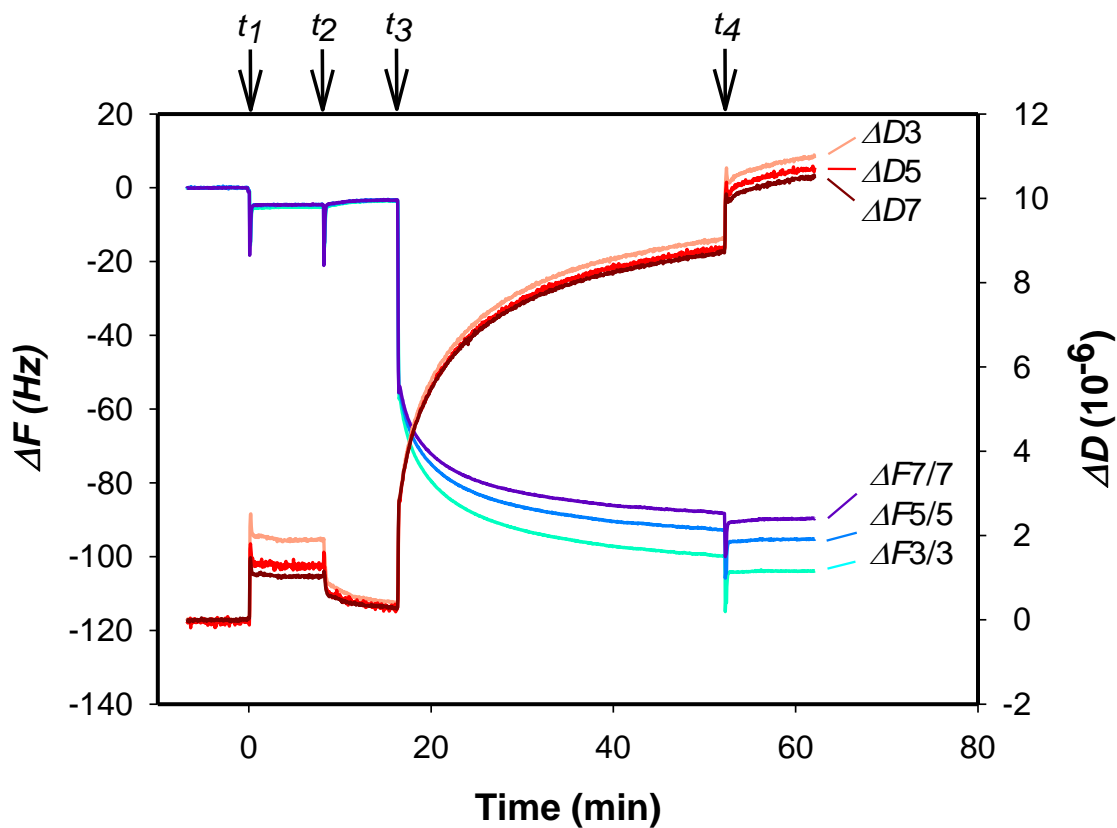


Figure 28 Frequency shift ( $\Delta F$ ) and energy dissipation shift ( $\Delta D$ ) induced by the adsorption of 0.1% PDADMAC and then 0.1% ECINs solution at pH = 7.0 and 10.05 M on the 8min-UVO-treated zein-coated gold crystal surface\*.

\*  $\Delta F$  and  $\Delta D$  are measured simultaneously at three overtones ( $n = 3, 5, 7$ ) and normalized by their overtone number. The arrows indicate the time for the injection of PDADMAC ( $t_1$ ), rinsing with buffer ( $t_2$ ), injection of ECINs ( $t_3$ ) and rinsing with buffer ( $t_4$ ).

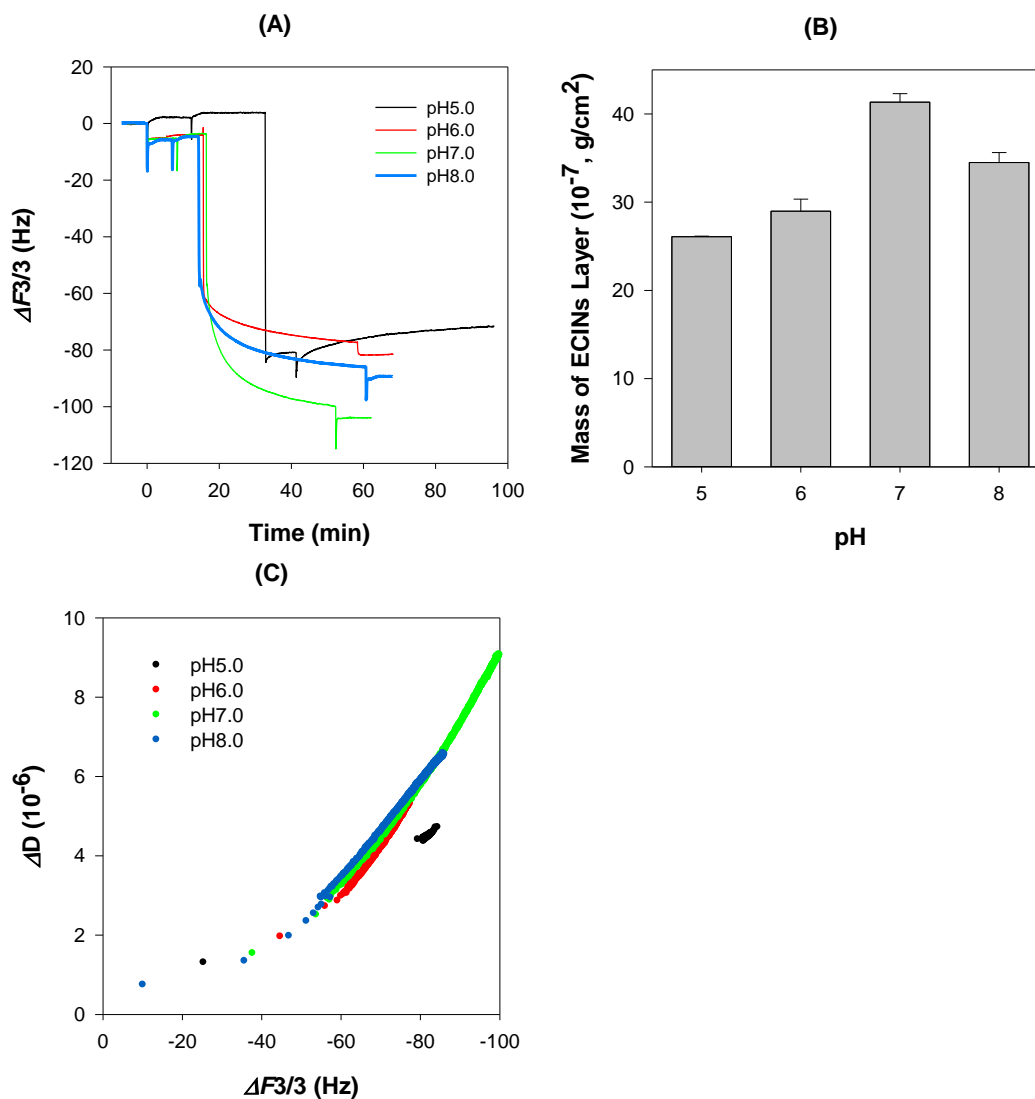


Figure 29 Effect of pH on the (A) Change of frequency ( $\Delta F_{3/3}$ ) and (B) mass of adsorbed ECINs layer on the surface of UVO-treated-zein/PDADMAC bilayer and (C)  $\Delta D$ -  $\Delta F$  plots\*.

\* The ECINs and PDADMAC solutions were prepared to the concentration of 0.1% in 0.02M buffers at various pH, and I 0.05M.

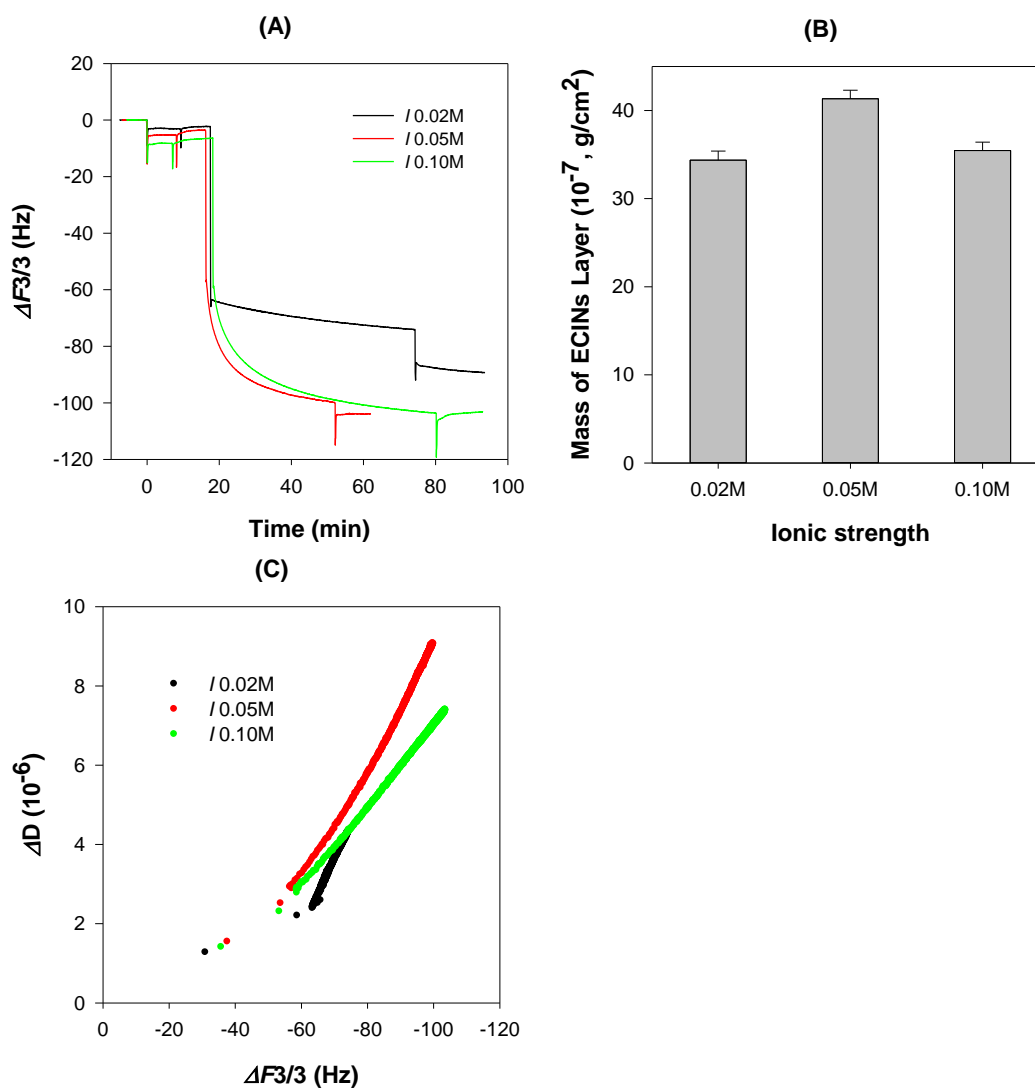


Figure 30 Effect of IS on the (A) Change of frequency ( $\Delta F3/3$ ) and (B) mass of adsorbed ECINs layer on the surface of UVO-treated-zein/PDADMAC bilayer and (C)  $\Delta D$ -  $\Delta F$  plots\*.

\* The ECINs and PDADMAC solutions were prepared to the concentration of 0.1% in 0.02M pH7.0 Tris buffers adjusted to various ionic strengths.

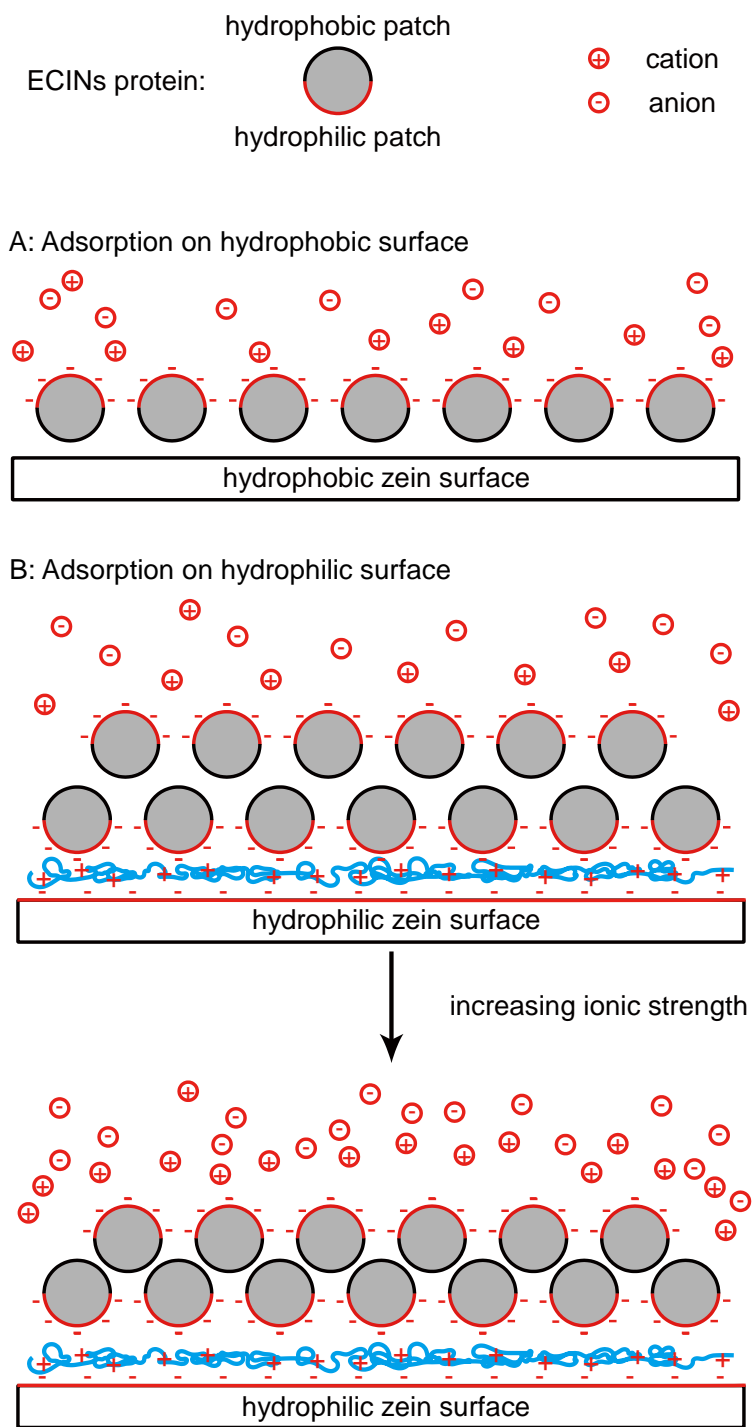


Figure 31 Overview of the adsorption of ECINs on the A: hydrophobic zein surface and B: UVO-treated hydrophilic zein surface.

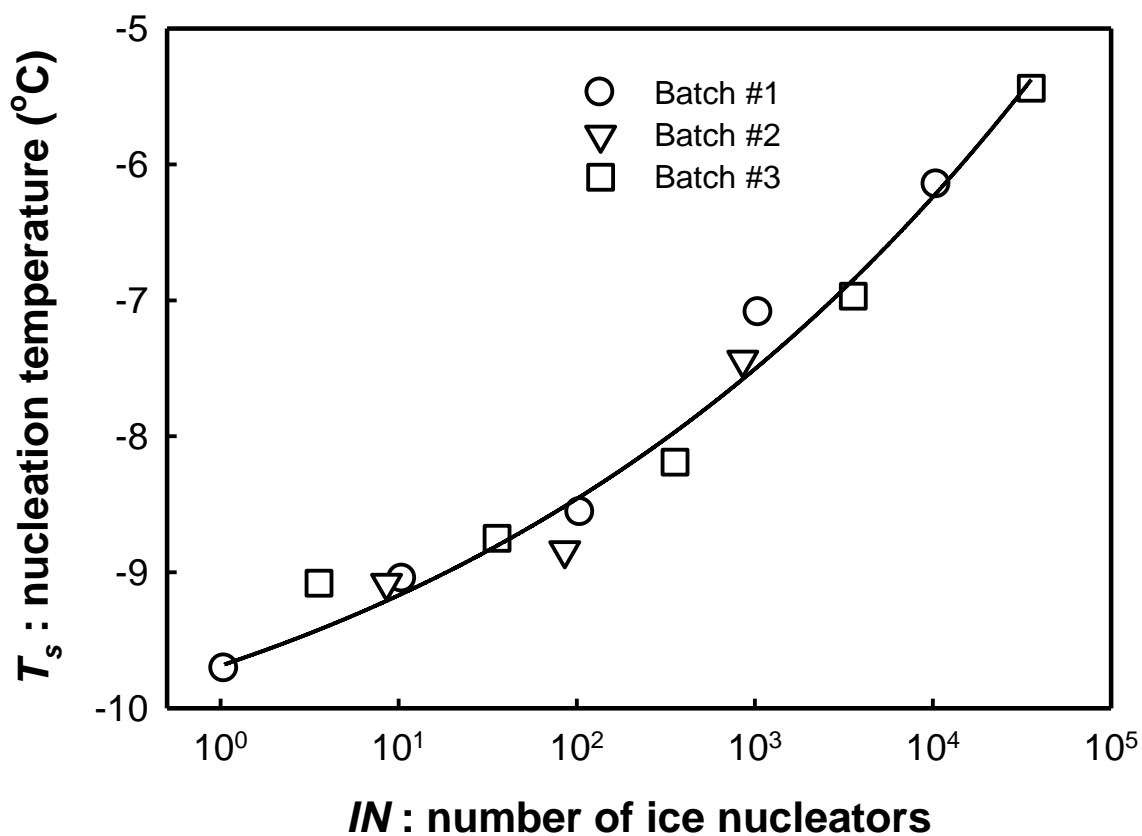


Figure 32 Modeling to the nucleation temperature of water containing various amount of ice nucleators. ECINs isolated from three batches of bacteria were used for test as described in the materials and methods, shown as data in three different symbols. The curve represents modeling result.\*

\* Three batches of ECINs were individual prepared with their INA determined as  $1.05 \times 10^6$ ,  $8.56 \times 10^5$  and  $3.57 \times 10^6$  units/mg, respectively, as described in the methods.

## CHAPTER FIVE. IMPROVEMENT TO BAKING QUALITY OF FROZEN DOUGH BY NOVEL ZEIN-BASED ICE NUCLEATION FILMS

*As of submission of this dissertation, the work in this chapter has been submitted in the title of “Improvement to Baking Quality of Frozen Dough by Novel Zein-Based Ice Nucleation Films” to the Journal of Cereal Science for consideration of publication.*

### **Abstract**

In the present study, the protective effects of zein-based ice nucleation films (INFs) on both improvement of baking quality of frozen dough and energy saving were investigated. Using quantification method developed in chapter four, the zein-based ice nucleation films (INFs) prepared from 0.1, 0.5 and 1.0 mg/mL ECINs solution exhibited activity ranging from 89 to 175 units/mm<sup>2</sup>. By using INFs to wrap frozen dough during five freeze/thaw cycles, the specific volume of bread loaf was increased by up to 25% and the total volume portion of gas cells was 1.2 times larger compared to the control frozen dough. The reason was attributed to 40% more viable yeast cells preserved by INFs. In addition, INFs also reduced the water loss by frozen dough resulting in higher water content in bread crumb. Combining the protective effects on both specific volume and water content from zein-based INFs, the obtained bread showed 68% lower firmness and fracturability and 2.4 times higher resilience compared to the control. In addition to improving the baking properties of frozen dough, INFs also cut down the time of dough freezing by 6%, which potentially saved 357 kJ energy in the current case. The INFs was



also superior in that on zein-based INFs, ECINs showed desirable affinity with the surface to sustain at least fifteen repetitive uses on freezing water.

## Introduction

In recent years, using biodegradable biopolymer and improving functionality are two emerging trends in the research on food packaging. The increased interest in using biopolymer is due to their abundant and renewable resources and their environmental-friendly nature. A good example of biopolymer is zein, a major storage protein in corn, and also the main co-product of bio-fuel industry. Zein can conveniently form hydrophobic films and coatings that are suitable in making packaging for fatty foods and sandwiches (Andersson, 2008). Furthermore, zein was made into functional food packaging bearing antimicrobial and antioxidant activities by incorporating lysozyme, albumin proteins, disodium EDTA and phenolic compounds into zein films (Gucbilmez et al., 2007; Mecitoglu et al., 2006; Arcan and Yemenicioglu).

Biogenic ice nucleators, such as extracellular ice nucleators (ECINs) isolated from *Erwinia herbicola*, are a group of lipoglycoproteins that have been demonstrated to minimize the supercooling by initiating ice nucleation at elevated subzero temperatures. Li and Lee comprehensively reviewed the applications of biogenic ice nucleators in food processing such as freeze-drying, freeze-concentration, freeze-texturing and improving efficiency of freezing (Li and Lee, 1995). In addition, ECINs also revealed its protective functions on frozen foods. Earlier, ECINs were demonstrated to protect quality of fish fillet by inhibiting denaturation of fish actomyosin during freeze/thaw cycles (Zhu and Lee, 2007). In Chapter Three, the baking quality of frozen bread dough was improved by

mixing with ECINs. The major mechanism was revealed as the cryopreservation of ECINs on yeast viability and gluten network in frozen dough.

As previously discussed, due to lack of food safety approval, mixing ECINs into food is currently not allowed. In addition, mixing ECINs into solid food, such as meat, vegetable and fruits, is sometimes unfeasible. Yet mixing ECINs into processed food is inconvenient and unfriendly to formulation and labeling. In Chapter Four, the optimum physicochemical conditions to immobilizing ECINs on the zein films were determined. Also, a novel method was developed to quantify the activity of ice nucleation films (INFs). The activity of developed INFs using optimal conditions (pH 7, IS 0.05 M) has reached 64.1 units/mm<sup>2</sup>.

This part of study used the TBC-plasticized zein-based ice nucleation films (INFs) to wrap frozen bread dough during five freeze/thaw cycles. The baking quality and yeast viability of frozen dough wrapped zein-based INFs were investigated and compared to the controls, i.e. fresh dough and frozen dough, as well as the frozen dough mixed with ECINs, and frozen dough wrapped by polyethylene-based INF. The prospect of INFs on energy saving was investigated from the effects of INFs on freezing process of dough. At last, the repetitive usage of INFs on water freezing was evaluated.

## **Materials and Methods**

### *Materials*

The  $\alpha$ -zein of biochemical-grade purity was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Glacial acetic acid was purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Poly (diallyldimethylammonium chloride) (PDADMAC, also abbreviated as PDDA) solution, average Mw 200,000-350,000 (medium molecular

weight), 20 wt. % in H<sub>2</sub>O was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Low-density polyethylene (LGA 105) film was obtained from ExxonMobil Cooperation. Deionized water was used throughout the experiment. Bakery materials including all purpose flour (10% protein, 73% carbohydrate, 14% moisture), Fleischmann's® ActiveDry™ bakery yeast, and shortening (92% fat, GreatValue®) were purchased from local stores.

#### *Preparation of extracellular ice nucleators*

The *Erwinia herbicola* subsp. *ananas* was obtained from the American Type Culture Collection (ATCC Cat. No. 11530) and routinely cultured in yeast extract (YE) medium consisting of 2.0% yeast extract, 1.0% sucrose, 0.2% L-serine, 0.2% L-alanine, 0.086% K<sub>2</sub>SO<sub>4</sub>, and 0.14% MgSO<sub>4</sub> under 18°C at shaking speed of 200 rpm.

Extracellular ice nucleators (ECINs) were isolated from *E. herbicola* Cells which were harvested by centrifugation at 10,000 xg for 20 min and then re-suspended in 20 mM Tris/HCl pH 8.0. Sonication of cells was done under output of 50 watts on ice, 10 seconds with 10 seconds interval for 3 times. Cells and debris were removed by centrifugation at 10,000 xg for 20 min and supernatant was subjected to filtration through 0.45 µm filters. The filtrate was put on ultracentrifugation at 160,000 xg for 2 hour and pellet was re-suspended in 20 mM Tris/HCl with 20 mM MgCl<sub>2</sub>, pH 8.0. Lyophilized ECINs in the form of powder were stored at -20°C.

### *Preparation of ice nucleation films*

Zein solutions were prepared by dissolving zein and tributyl citrate (TBC) in acetic acid to 16% and 4% respectively. Two hundred milliliter of zein solution was poured into 28×36 cm Teflon pans and dried in 40 °C oven to cast films.

TBC-plasticized zein films or PE films were treated in a UV/Ozone chamber (model 42, Jelight Co. Inc., Irvine, CA) for 8 minutes, then immersed in 0.1% PDADMAC solution (pH 7.0, IS 0.05 M) for 30 min and washed with 20 mM pH 7.0, IS 0.05M Tris buffer. The films were then immersed in 0.1% ECINs solution (pH7.0, I 0.05 M) for 30 min, then washed with the same buffer and dried under ambient condition.

### *Differential scanning calorimetry (DSC)*

DSC analyses were performed on a DSC 823E thermal analyzer (Mettler-Toledo Inc., Columbus, OH) supplied with liquid nitrogen and compressed nitrogen gas. To measure the ice nucleation temperature of water on films, the film was cut exactly to cover the bottom of crucible, and  $10 \pm 0.1$   $\mu\text{L}$  dH<sub>2</sub>O was transferred carefully onto films and spread to cover the whole film surface. The samples were then subjected to a temperature decrease from 4 °C to -30 °C at a rate of -1 °C/min. Crucible with films but without water were used as reference.

### *Dough preparation, yeast viability and bread baking*

A basic dough recipe was adopted from AACC method 10-10B (AACC, 2000). Each dough contained 450 g flour, 252 g water, 7 g Fleischmann's® ActiveDry™ yeast, 27 g sucrose, 6.75 g salt, 27 g shortening was mixed. The ECINs-blended dough was prepared by dispersing ECINs (ice nucleation activity:  $6.9 \times 10^6$  units/mg) in water and

then mixing to 0.35mg per gram of dough. After mixing, dough was sheeted and rolled into cylindrical shape with length of about 20 cm and diameter of 6~7 cm. Some dough pieces were then tightly wrapped with zein-based or PE-based ice nucleation films and any void was rolled out to ensure close contact between films and dough. All dough pieces were then frozen at -20 °C for 48 h and then thawed in 4 °C refrigerator for 24 h as one freeze/thaw cycle. After five freeze/thaw cycles, yeasts in the frozen dough were then extracted and the viable cells were enumerated as previously described in Chapter Three. Dough pieces were fermented and baked as previously described in Chapter Three. Breads were cooled to room temperature before characterization.

#### *Characterization of bread*

Specific volume of bread, water content, texture profile analysis (TPA), color and gas cell structure in crumb were characterized by AACC method 74-9 (AACC, 2000) and as previously described in Chapter 4.

#### *Reusability test*

Ice nucleation films in crucible were prepared as previously described and subjected to repetitive DSC test to examine their reusability. In each cycle,  $10 \pm 0.1$   $\mu\text{L}$   $\text{dH}_2\text{O}$  was transferred onto the films, and the ice nucleation temperature of water was determined as previously described. Then the water was carefully removed before the next cycle. Each film was subjected to fifteen test cycles.

### *Statistical analysis*

One-way ANOVA or two-way ANOVA was performed using Sigma-Plot v.12.0 with integrated SigmaStat module. Values with no common letter were significant different ( $P < 0.05$ ).

## **Results and Discussion**

### **Preparation of zein films with ice nucleation activity**

The zein-based ice nucleation films (INFs) were prepared from ECINs solutions at three concentration levels (0.1, 0.5, and 1.0 mg/mL) as described in the methods. The ice nucleation temperatures of water on these films were measured by DSC to examine the ice nucleation activities (Figure 33). The ice nucleation temperature is defined as the lowest temperature that supercooled water reaches, where the latent heat is released and water starts to form ice. On the surface of zein films without ECINs, water supercooled to the temperature as low as  $-15.5\text{ }^{\circ}\text{C}$  before nucleation. In contrast, the zein-based INFs allowed supercooled water to nucleate at elevated temperatures ranged from  $-7.1$  to  $-6.8\text{ }^{\circ}\text{C}$ . Comparatively, PE-based INFs prepared using 1.0 mg/mL ECINs solution also made water nucleate at about  $-6.7 \pm 0.1\text{ }^{\circ}\text{C}$ . Using Equation (9), the ice nucleation activities of zein-based INFs were calculated, which increased from 89 units/ $\text{mm}^2$  on zein-E0.1 to 175 units/ $\text{mm}^2$  on zein-E1.0 (Figure 34). The activity of PE-E1.0 was calculated as 197 units/ $\text{mm}^2$ .

Zein forms freeze/thaw stable films, which can be used in packages for frozen foods (Padua et al., 2000). However, pure zein films are rigid and brittle, and plasticizers are needed to improve their mechanical properties. The research in Chapter Two revealed

that, with plasticization by 20% tributyl citrate, zein films showed acceptable flexibility and toughness for food wrappings, more importantly, less water absorption and lower water vapor permeability, which allowed the TBC-plasticized zein films to successfully wrap dough pieces through freeze/thaw cycles in this part of work.

In order to introduce the ice nucleation function to films, extracellular ice nucleators (ECINs) isolated from *Erwinia herbicola* were immobilized on the surface of films. The research in Chapter One demonstrated a successful method, where the hydrophobic surface of zein films were modified by UV/Ozone treatment and subsequently bore negative charges (Shi et al., 2009). As revealed by the work in Chapter Four, ECINs adsorbed onto UV/ozone-treated-zein/PDADMAC bilayer at pH 7.0 and I 0.05 M, introducing the ice nucleation properties to the films.

The work in Chapter Four has also developed a novel method to quantify the ice nucleation activity of INFs, providing empirical Equation (9) for calculation. In the present work, more concentrated ECINs solutions prepared INFs exhibiting higher ice nucleation temperature of water, due to more ice nucleators have adsorbed on film surface. This was confirmed by the calculation of ice nucleation activity of INFs. Given that ECINs can be dissolved in buffer up to about 1 mg/mL, the activity of Zein-E1.0 at  $175 \text{ unit/mm}^2$  has probably reached the maximum level that could be prepared with the current materials and conditions. The relationship between INA of films and the concentration of ECINs solutions revealed in Figure 34 can serve as a reference when preparing ice nucleation films with controllable activity.

### **Qualities of fresh- and frozen-dough breads**

Dough pieces were wrapped with zein films (Fz-Zein) and zein-based INFs prepared using 0.1, 0.5 and 1.0 mg/mL ECINs solutions (Fz-Zein-E0.1, Fz-Zein-E0.5 and Fz-Zein-E1.0). Other dough pieces were wrapped with PE-based INFs prepared from 1.0 mg/mL ECINs solutions (Fz-PE-E1.0). A negative control was dough pieces without ECINs (Fz). Additionally, ECINs were blended to 0.35 mg per gram of dough (Fz-E). All these seven types of dough were subjected to five freeze/thaw cycles before baked to breads, whose properties were compared to the bread baked from fresh dough (Fresh).

According to AACC method, specific volume was calculated by dividing the volume by the mass of a bread loaf. Bread baked from fresh dough had the highest specific volume at 4.1 cm<sup>3</sup>/g (Figure 35). The control frozen dough and those wrapped by zein films, only had half of the specific volume as the fresh-dough bread. Among the frozen-dough breads, those mixed with ECINs showed increased specific volumes of 2.88±0.04 cm<sup>3</sup>/g. The two types of bread baked from dough wrapped by zein-based and PE-based INFs (Fz-Zein-E1.0 and Fz-PE-E1.0) also revealed 25% increase in specific volume compared to the breads from control frozen dough.

Results of water content were summarized in Figure 36, which were clearly divided to two groups. The breads baked from unwrapped frozen dough contained about 36.8%~37.7% water which was significantly lower than the breads baked from film-wrapped frozen dough containing more than 39% water.

The texture profile analysis to breads included three aspects: the firmness, the fracturability and the resilience (Figure 37). The fresh-dough bread (Fresh) showed



lowest firmness and fracturability, in contrast to the control frozen-dough bread (Fz) showed about six-fold higher values in both firmness and fracturability. In the other frozen-dough breads, either blended with ECINs or wrapped by INFs, the firmness and fracturability were increased compared to fresh-dough breads, but significantly lower than the frozen-dough bread without ECINs ( $P<0.05$ ). Wrapping by INFs was significantly more effective in decreasing the firmness and fracturability than blending with ECINs. And the higher the activity of the films, the more effectively the INFs decreased the crumb firmness ( $P<0.05$ ). According to AACC method, the resilience was calculated as a ratio of the work spent on withdrawing to that spent on penetration. Analysis to the frozen-dough bread wrapped by INFs (Fz-Zein-E0.1/0.5/1.0 and Fz-PE-E1.0) revealed quite good resilience of about 0.36~0.38, which was even higher than the fresh-dough bread (Fresh) with resilience of 0.29. The control frozen-dough bread (Fz) and the ECINs-blended frozen-dough bread (Fz-E) showed the lowest levels of resilience.

Cross-sectional images of bread crumb were taken in which the differences in the cross-sectional area of each bread followed the same trends as the specific volume of each bread (Figure 38). The gas cell structures in bread crumb were analyzed which calculated the size of the cell (mean cell area and mean perimeter), cell density and the area ratio of total cell (Table 12). From both the cross-sectional image and the analysis on gas cell structures, it can be observed that, in the crumb of bread baked from fresh dough, there was a large amount of fine gas cells (about  $0.42 \text{ mm}^2$ ) homogeneously distributed in the bread crumb, which accumulatively added to about 50% of the area in crumb. In the control frozen-dough bread, the cell density was dramatically reduced to only 15% of the fresh one. Consequently, even with gas cells enlarged by four times to about  $1.8 \text{ mm}^2$ , the

total area ratio of gas cells in control frozen-dough bread was significantly lowered ( $P < 0.05$ ). The treatment on frozen dough, either blending with ECINs or wrapping by INPs, significantly prevented reduction on the total area ratio of gas cells, by detaining the reduction in cell density while enlarging the gas cells. In addition to the differences in gas cell structure, the crumb of fresh-dough bread looked whiter suggested by smaller  $dE$  (Table 13). In contrast, the frozen-dough bread, except those blended with ECINs, looked significantly darker (larger  $dE$ ,  $P < 0.05$ ).

### **Yeast survival in frozen dough**

It was previously demonstrated that deteriorated properties of frozen-dough bread were largely attributed to the lost in yeast viability (Rosell and Gomez, 2007). Thus, the viable yeasts were extracted from dough after freeze/thaw cycles, enumerated and compared to that in fresh dough (Figure 39). There were originally  $62 \pm 3$  million viable yeast cells per gram of fresh dough (Fresh), more than half of which lost their viability after five freeze/thaw cycles as the concentration of viable yeast in control frozen dough (Fz) decreasing to  $26 \pm 2$  million cells per gram of dough. Wrapping by zein films and low-activity INFs, i.e. Zein-E0.1 were ineffective in preserving the yeast viability. Wrapping by moderate-activity INFs, i.e. Zein-E0.5, was only effective on the surface but did not improve the yeast survival in the center of the dough. With high-activity INFs, i.e. Zein-E1.0 and PE-E1.0, the cryoprotective effects from the INFs on yeast viability persisted throughout the whole dough, retaining about 35 million viable yeasts per gram of dough.

### **Reusability of zein-based ice nucleation films**

Zein films prepared from 1.0 mg/mL ECINs solutions were subjected to repetitive uses for freezing water droplets, with the nucleation temperature at each run monitored by DSC. After fifteen repetitive tests, the nucleation temperature of water on the films decreased from -6.7 to -7.0 °C. However, statistical analysis suggested that there was no significant change in the nucleation temperature along the fifteen tests.

### **Potential of zein-based ice nucleation films on energy saving**

The effects of blended ECIN, wrapping by zein films and wrapping by high-activity ice nucleation films (Zein-E1.0) on freezing processes of frozen was recorded as in Figure 40. As discussed in Chapter Three, blended ECINs did not affect the initial cooling rate of the dough, however, reduced the time of phase transition and consequently reduced the time of freezing by 20%, where the time of phase transition was defined as the duration of temperature changes between  $-4.9 \pm 0.5$  °C, and the time of freezing was defined as the duration of temperature decreased from 10 °C to -15.9 °C. Wrapping by films was beneficial in preventing dehydration (Figure 36). However, the films (Zein and Zein-E1.0) slightly interfered the heat transfer resulting in a slower rate of temperature decrease initially at about 13 °C/hr compared to 16 °C/hr in the control frozen dough (Fz and Fz-E). Due to slower heat transfer, the time of phase transition and time of freezing in dough wrapped with zein films were respectively 0.2 and 0.8 hours longer than the control (Fz). Wrapped by Zein-E1.0 accelerated the phase transition of dough. Thus, even though it took longer time for dough to decrease temperature, the total time of freezing in the dough wrapped by high-activity INFs (Fz-Zein-E1.0) was still reduced by 0.4 hours, which was about 6% reduction. With the freezer operated at

averagely 248 Watts, the energy potentially saved for freezing of dough reached about 357 kJ. The calculation suggested that zein-based ice nucleation films can potentially reduce energy consumption of freezing process. However, it may not be as effective as ECINs used alone.

## **Discussions**

### *Effects of INPs on the baking quality of frozen dough*

Bread from freshly prepared and baked dough should have a soft and chewy crumb (low firmness, low fracturability, high resilience) that showed a white color (small dE value). In contrast, breads baked from frozen dough usually show coarse texture and darker colors, especially after repetitive temperature abuses. The main reason of the deterioration in bread quality of frozen dough is attributed to the freezing damage to yeast cells (Rosell and Gomez, 2007), same as the observation in present study that less than half of the yeast cells retained the viability after five successive freeze/thaw cycles (Figure 39). In Chapter Three, the mechanism of cryoprotective effects by ECINs was explained as their ability to increase nucleation temperature and to modulate a moderately fast freezing rate in dough, under which conditions the viability of yeast cells was improved. The results in this part of work indicated that high-activity INFs (Zein-E1.0 and PE-E1.0) was as effective as blending ECINs on protecting yeast viability and the effects of INFs can transfer from surface to the center of dough, though the mechanism of the transfer is unknown (Figure 39). Moderate-activity INFs (Zein-E0.5) was only effective on the surface, whereas low-activity INFs and zein film reveals no cryoprotective effects statistically.

A direct consequence of the lost in yeast cell viability is the reduction on the total area ratio and density of gas cells (Table 12). In addition, injured and dead yeast cells release reducing agents that weaken the gluten network in dough as observed in Chapter Three and other research works (Rosell and Gomez, 2007). During fermentation, the weaker gluten network was less capable to hold gas cell expansion, resulting in enlargement of gas cells as suggested by the larger mean cell area and perimeter. These changes in gas cells structure affected the apparent color of the bread crumb. The evenly distributed fine gas cells with thinner cell wall like in the fresh-dough bread crumb tended to reflect light rather than absorbing light and give the crumb a whiter appearance, in contrast to the few large gas cells gave a darker appearance to the crumb in control frozen-dough bread (higher dE values, Table 13) (Pomeranz, 1960). The moderate- and high-activity INFs (Zein-E0.5, Zein-E1.0 and PE-E1.0) were equally effective as blending ECINs in increasing the total area ratio of gas cells in frozen-dough bread, but less effective in improving the apparent color of bread crumb. Zein film and low-activity INFs was ineffective on preserving the above qualities.

The reduced total volume of gas cells, suggested by the smaller total area ratio, was a direct reason of the smaller specific volume of the whole bread with a harder texture of the crumb (Figure 35 and Figure 37). Due to improved yeast survival rates, both blending with ECINs, and wrapped by INFs increased the specific volume of bread and thus reduced the hardening of the bread crumb compared to the control frozen dough. On the other hand, crumb texture is also largely affected by the water content. It seems that wrapping by films, significantly prevented the water loss during freezing storage and thus kept higher water contents in the bread (Figure 36). Therefore the crumb of breads

baked from film-wrapped frozen dough showed significantly decreased firmness and fracturability and increased springiness (resilience) ( $P < 0.05$ ).

#### *Advantage of zein-based INFs*

Blending ECINs and wrapping by ice nucleation films both revealed protective effects on improving the baking quality of frozen dough. However, the efficacy of the two methods varied. Blending of ECINs was especially effective on preserving the yeast viability both on the surface and inside the dough. For ice nucleation films, only those with high activity, i.e. Zein-E1.0 and PE-E1.0, showed equal preservation to yeast viability on both surface and center of the dough. The film with moderate-activity (Zein-E0.5) only preserve yeast viability at the surface but not the center, whereas the low-activity film (Zein-E0.1) was ineffective on this aspect. Therefore, blending ECINs and wrapping with high-activity INFs provided comparable preservation on the gas cell structures, crumb color and specific volume of bread. On the other hand, zein films served as a good barrier to prevent dehydration of frozen dough, resulting in higher water content in breads. Consequently, wrapping by INFs at all activity levels exceeded blending ECINs on reducing the hardening and improving springiness of bread crumb from frozen dough, and thus improved the texture properties of bread. Here, it's worth to note that the dough was made into a cylindrical shape with 20 cm long and diameter of 6~7 cm. The dough became flattened after freeze/thaw cycles. Obviously, the efficacy of INFs was dependent on the geometrical shape and size of the dough and the contact area between the dough and films, whereas the efficacy of blending ECINs was independent on the shape and size of the dough. Among the ice nucleation films, the activity level was the determinate factor on their efficacy on improving the baking quality of frozen dough.

Films with higher activity were more effective, while films with same activity results in same quality of breads. Zein-based INFs and PE-based INFs prepared from the same procedures were shown to be equally effective on preventing dehydration and improving the baking quality of frozen dough. PE is widely used in food packaging to provide a good barrier to water vapor. The water vapor permeability of the low-density PE was reported as about 0.001 g.mm/(h.m<sup>2</sup>.mmHg), compared to 0.0086 g.mm/(h.m<sup>2</sup>.mmHg) of 20%-TBC-plasticized zein film. The observed comparable effects of zein-based and PE-based INFs on frozen dough suggested that zein-based films provide comparable protections to frozen food. In addition, the successfully development of PE-based INFs indicated the universality and flexibility of the current used method on preparing functional food packaging.

In contrast to their beneficial influence on the baking quality of frozen dough, zein films now become a detrimental factor on energy saving. The films slowed down the heat transfer and prolonged the freezing process. Although ECINs alone led to 20% reduction on the time of freezing, after immobilized on films, the reduction was decreased to 6%. Therefore, in the current case, high-activity INFs (Zein-E1.0) only potential saved 357 kJ energy, compared to 1250 kJ energy when ECINs was used alone.

However, in addition to better improving the texture properties of frozen-dough bread, ice nucleation films offered additional benefits in lowering the usage of ECINs compared to blending ECINs. In the current study, the activity introduced to one dough pieces by blending ECINs was  $1.7 \times 10^9$  units, compared to  $7.7 \times 10^6$  units by wrapping with Zein-E1.0 film. It suggested that by blending method about 200 times more ECINs would be used to acquire similar baking quality of frozen dough by wrapped with high-

activity INFs. Additionally, the ECINs immobilized on INFs is reusable as revealed in the experiment, although the reusability is largely dependent on types of frozen foods. If applied on liquid and semi-solid foods, such as juice, ice cream and so on, the reusability of the INFs could be extended to more cycles. Otherwise, on solid foods, the reusability of the INFs could be quite limited.

In summary on this part of research, the effect of the zein-based INFs on yeast viability and the bakery quality of frozen dough upon freeze/thaw cycles was studied and compared to the effects by blended ECINs and by PE-based INFs. Both zein-based and PE-based INFs prepared from 1.0 mg/mL ECINs solutions were comparatively effective on preserving the yeast viability as was blended ECINs, resulting in better retention on the bread qualities including gas cell structures and specific volumes. Nevertheless, wrapping by INFs, especially the zein-based INFs, prevented water loss by dough during freezing storage, and thus further reduced the hardening of bread crumb compared to blended ECINs. In addition, on zein-based INFs, ECINs were showed desirable affinity with the surface to sustain at least fifteen repetitive uses on freezing water. The present study demonstrated the potentials in the zein-based INFs as a functional packaging for frozen food applications.



Table 12 Analysis on gas cell structure of bread crumb baked from fresh dough and frozen dough.

	Image analysis			
	Area ratio of total cell (%)	Cell density (cells/cm <sup>2</sup> )	Mean cell area (mm <sup>2</sup> )	Mean perimeter (cm)
Fresh	50.9±2.5a	122±12a	0.42±0.04a	0.19±0.01a
Frozen	33.3±2.0b	19±1c	1.8±0.2c	0.87±0.06c
Frozen, blended with 0.1% ECINs	38.9±1.5a	33±2bc	1.2±0.1b	0.60±0.03b
Frozen, wrapped by films				
Zein	36.4±2.9b	26±1bc	1.4±0.2bc	0.63±0.08b
Zein,0.1mg/mL ECINs	35.6±8.9b	27±1bc	1.4±0.3bc	0.64±0.13b
Zein,0.5mg/mL ECINs	40.4±3.6a	25±1bc	1.6±0.2bc	0.70±0.06b
Zein,1.0mg/mL ECINs	40.5±2.6a	35±5bc	1.2±0.1b	0.54±0.07b
PE,1.0mg/mL ECINs	40.5±0.4a	38±1b	1.1±0.1b	0.52±0.02b

\* Values with on common letters are significantly different ( $p < 0.05$ ).

Table 13 Color analysis of bread crumb baked from fresh dough and frozen dough.

	Color analysis			
	L	a	b	dE
Fresh	74.0 $\pm$ 0.9a	-0.6 $\pm$ 0.4a	15.1 $\pm$ 0.2ab	22.2 $\pm$ 0.7a
Frozen	60.0 $\pm$ 1.3e	-0.2 $\pm$ 0.4a	15.9 $\pm$ 0.1b	35.4 $\pm$ 1.2e
Frozen, blended with 0.1% ECINs	70.6 $\pm$ 0.3b	-0.5 $\pm$ 0.3a	15.7 $\pm$ 0.9b	25.5 $\pm$ 0.2b
Frozen, wrapped by films				
Zein	59.2 $\pm$ 0.7e	-0.1 $\pm$ 0.3a	14.5 $\pm$ 0.5ab	35.7 $\pm$ 0.5e
Zein,0.1mg/mL ECINs	59.0 $\pm$ 2.2e	0.2 $\pm$ 0.2a	13.9 $\pm$ 0.5ab	35.7 $\pm$ 2.1e
Zein,0.5mg/mL ECINs	60.6 $\pm$ 0.7d	-0.6 $\pm$ 0.4a	13.2 $\pm$ 1.0a	34.0 $\pm$ 0.4de
Zein,1.0mg/mL ECINs	63.2 $\pm$ 1.3d	-0.8 $\pm$ 0.2a	14.0 $\pm$ 0.6ab	31.7 $\pm$ 1.1cd
PE,1.0mg/mL ECINs	66.0 $\pm$ 0.5c	0.2 $\pm$ 0.6a	14.8 $\pm$ 0.2ab	29.4 $\pm$ 0.4c

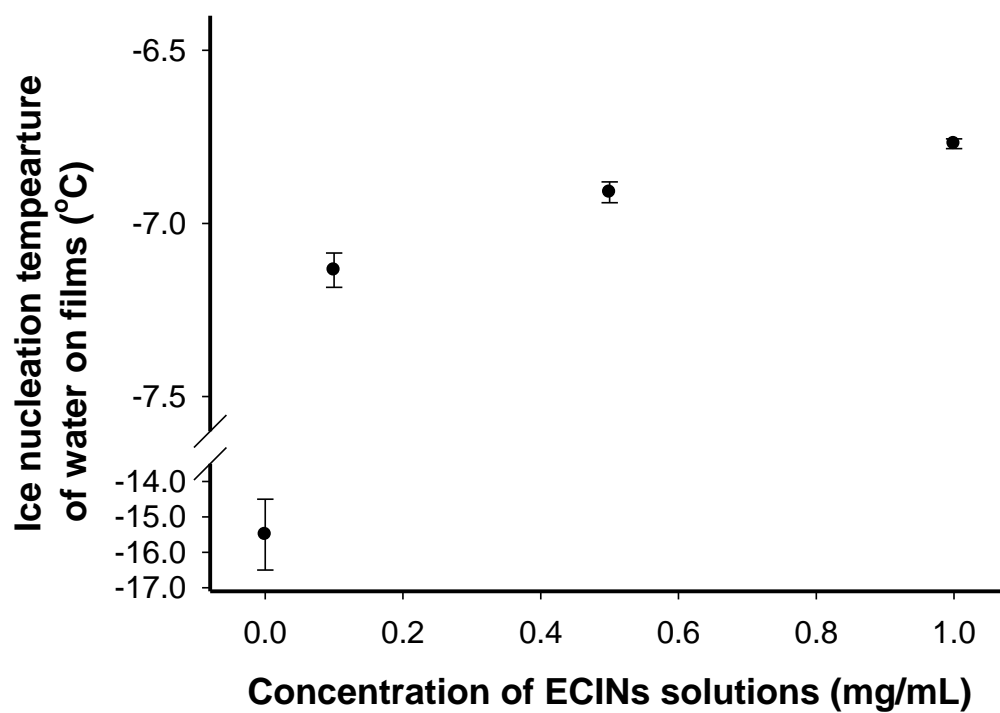


Figure 33 Nucleation temperature of water on the surface of zein-based INFs prepared from ECINs solutions at various concentrations.

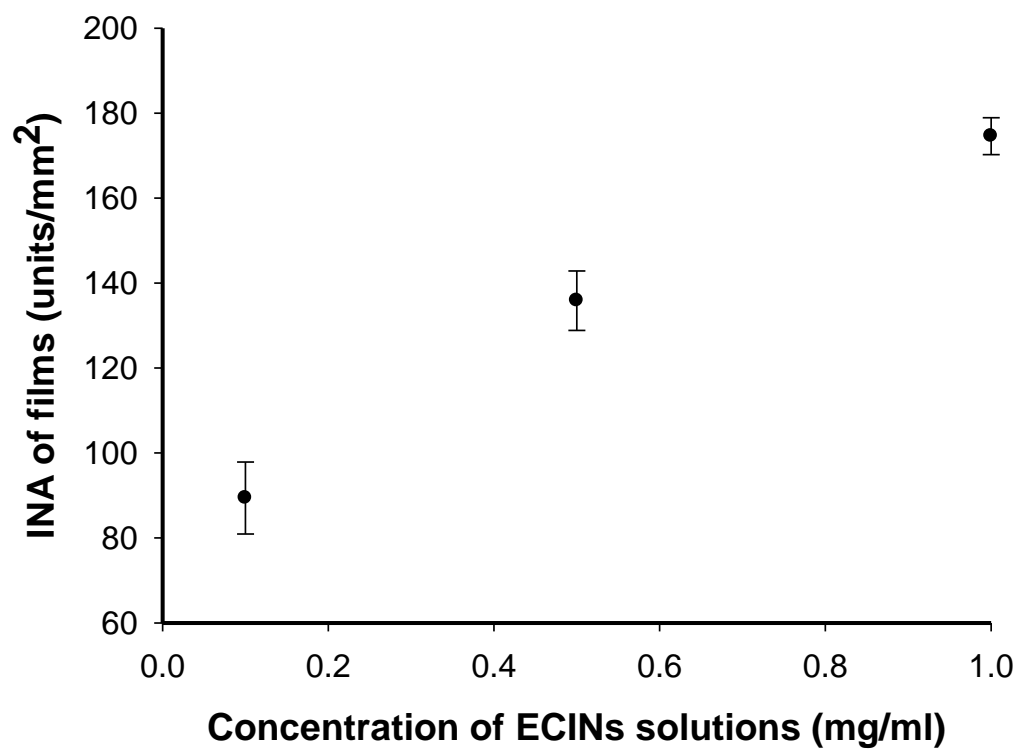


Figure 34 Ice nucleation activity of zein-based INFs prepared from ECINs solutions at various concentrations.

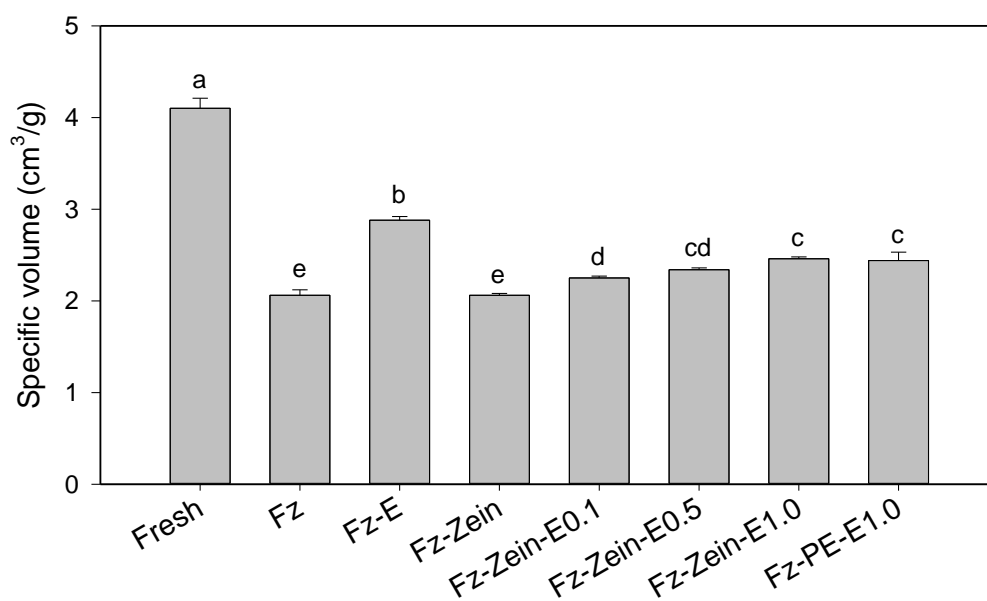


Figure 35 Specific volume of breads baked from fresh and frozen dough\*.

\* The letters on top of error bars indicated the result of statistical analysis. Values with no common letters are significantly different ( $p < 0.05$ ).

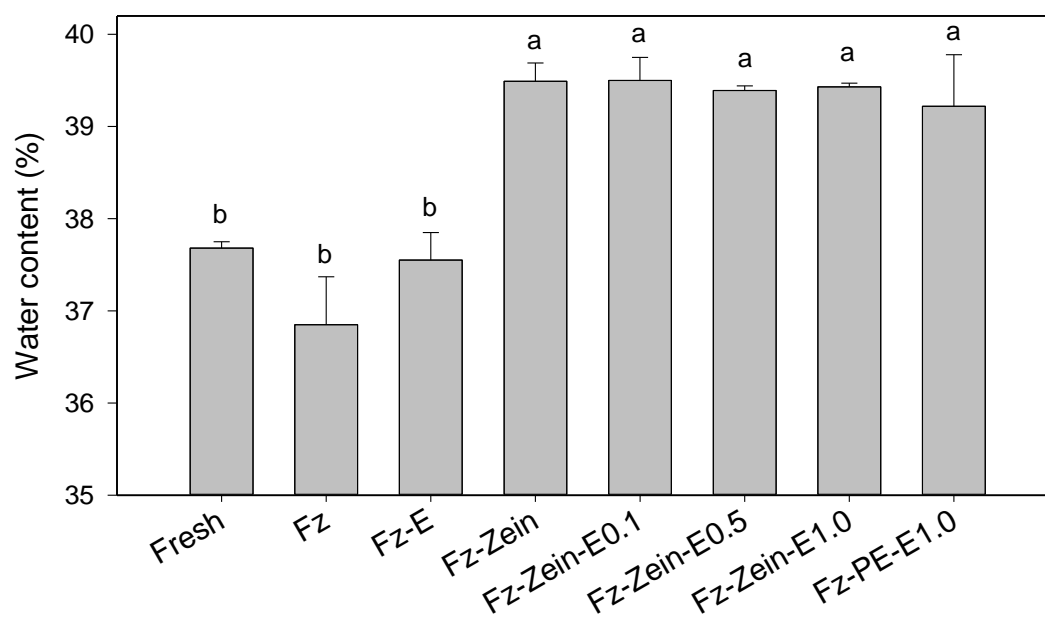
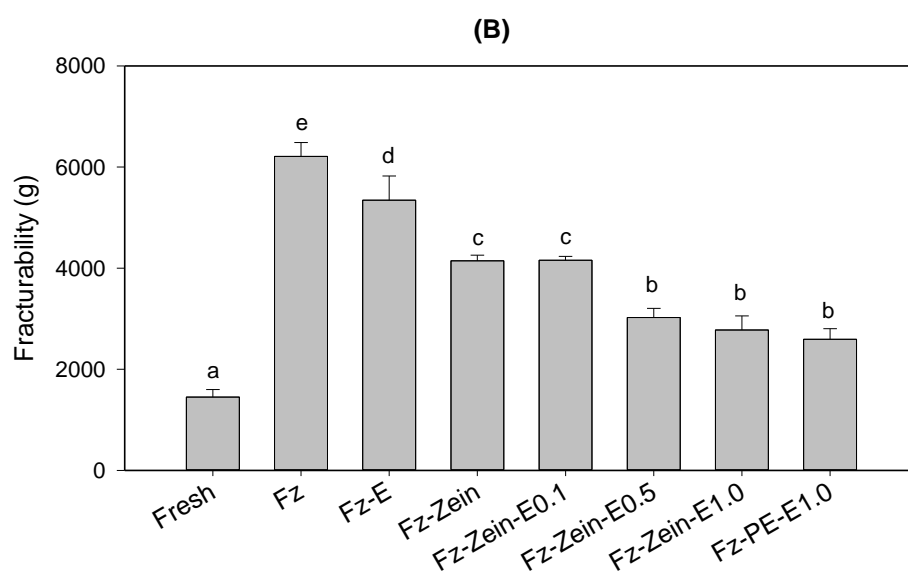
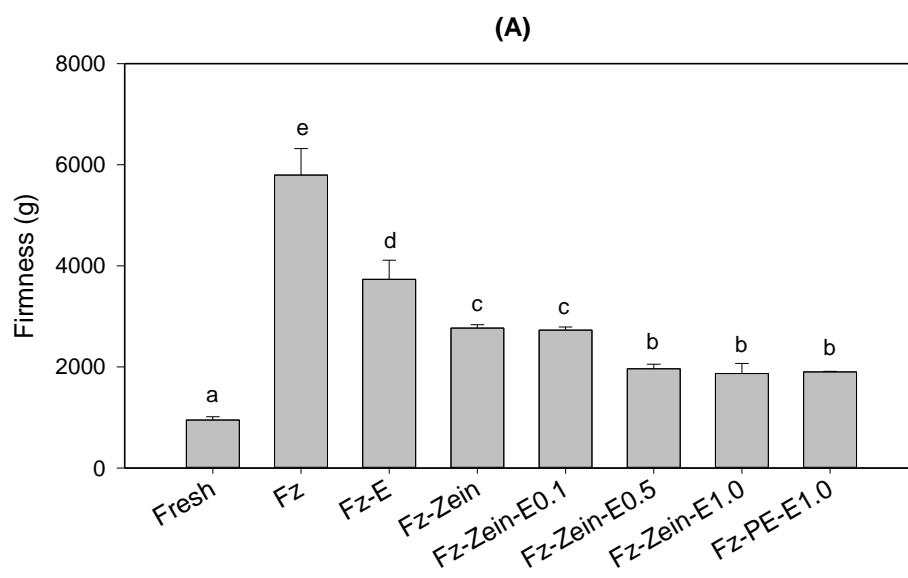


Figure 36 Water contents of breads baked from fresh and frozen dough\*.

\* The letters on top of error bars indicated the result of statistical analysis. Values with no common letters are significantly different ( $p < 0.05$ ).



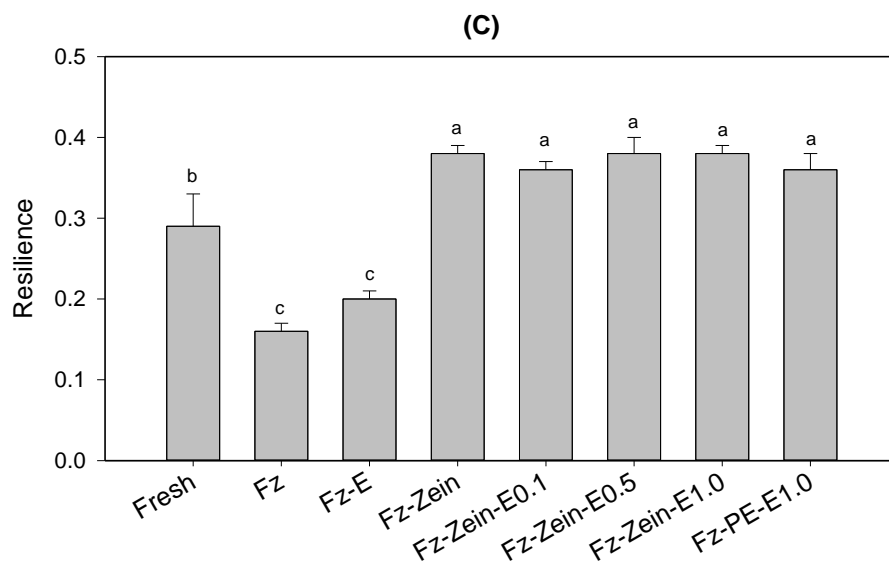


Figure 37 Texture profile analysis on (A) Firmness, (B) Fracturability and (C) Resilience, to the breads baked from fresh and frozen dough\*.

\* The letters on top of error bars indicated the result of statistical analysis. Values with no common letters are significantly different ( $p < 0.05$ ).



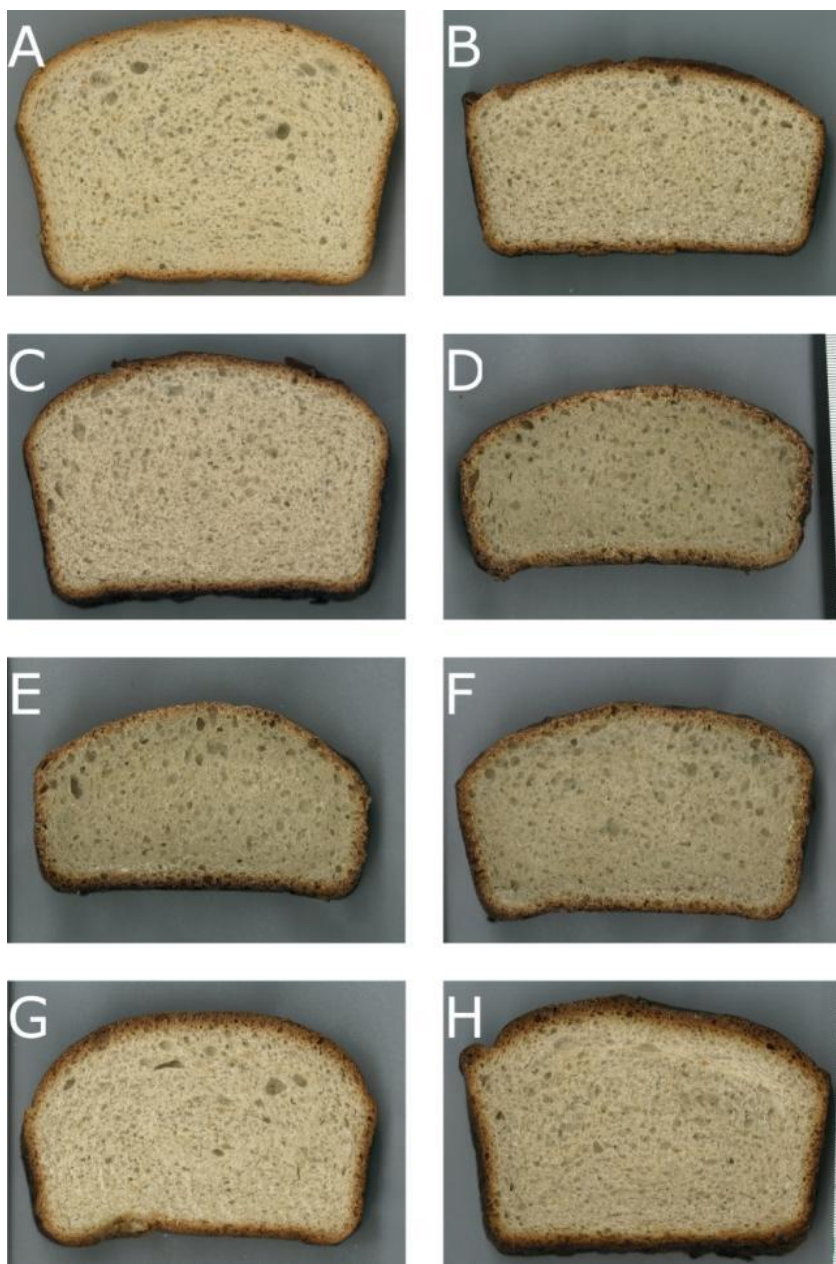


Figure 38 Cross section of bread baked from (A) fresh dough, (B) frozen dough, (C) frozen dough blended with ECINs, and frozen dough wrapped by (D) zein, (E) zein-E0.1, (F) zein-E0.5, (G) zein-E1.0 and (H) PE-E1.0 films\*.

\* Each image was 13.3 cm  $\times$  9.6 cm.

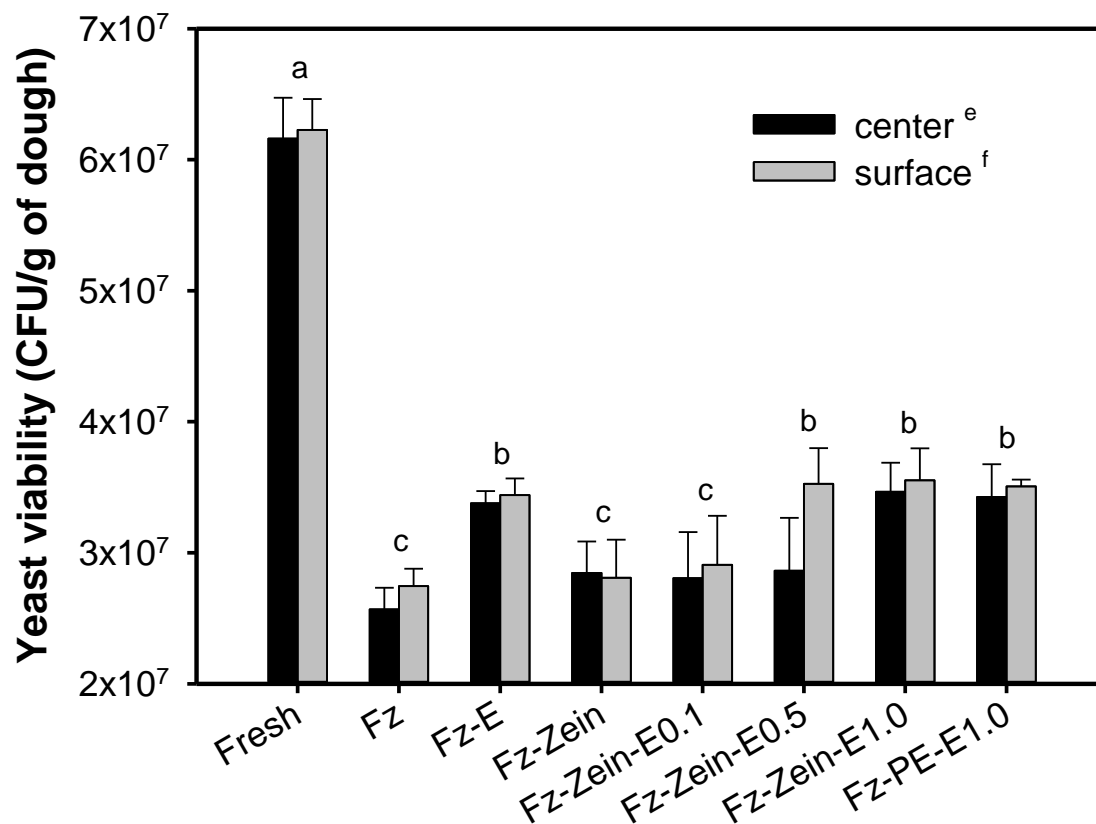


Figure 39 Yeast viability in the fresh dough and frozen dough\*.

\* The letters on top of error bars and in the legend indicated the result of statistical analysis. Values with no common letters are significantly different ( $p < 0.05$ ).

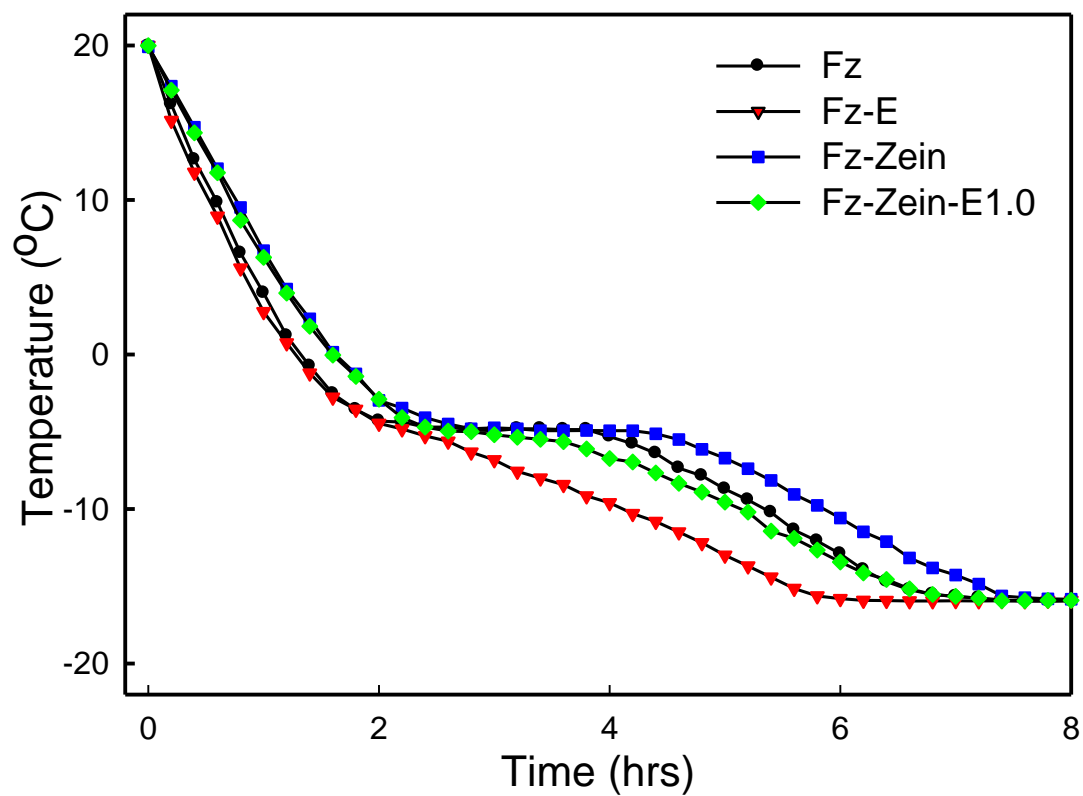


Figure 40 Effects of blended ECINs and wrapping films on the freezing process of dough

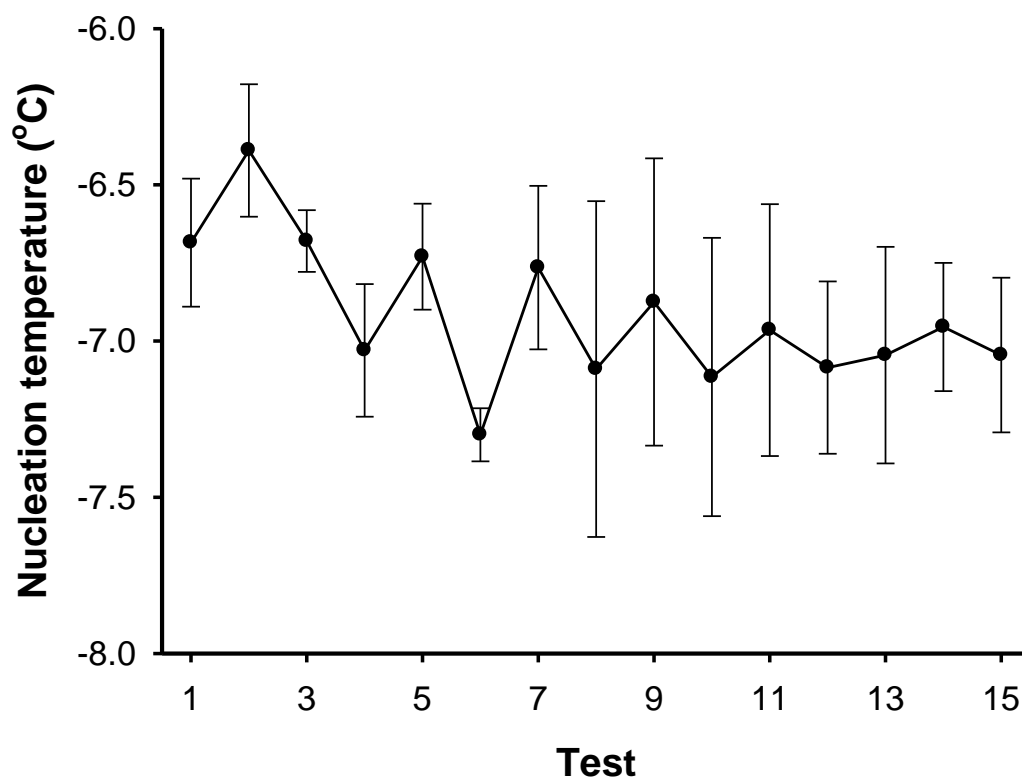


Figure 41 Nucleation temperature of water on the surface of zein-based INFs after repetitive usage

## SUMMARY AND FUTURE WORK

This research aimed at developing zein-based ice nucleation films for the applications of frozen foods. For this purpose, the work was subsequently divided into three steps, i.e. preparing zein films with appropriate properties, developing ice nucleation films and verifying the efficacy of ice nucleation films on frozen food applications.

In order to prepare zein films, three aspects of zein film properties were considered, including surface properties (morphology and hydrophilicity), mechanical properties and water resistance. Acetic acid was chosen as the solvent to prepare zein films with smooth and flawless surface which is preferable for development of functional packaging. UV/ozone treatment was applied on zein films to convert the intrinsically inert surface to a negative-charged hydrophilic surface by forming carboxyl groups on surface, but not changing the bulk hydrophobic nature of zein films. In order to reduce the brittleness of cast films, zein protein was physically plasticized by tributyl citrate. The later method turned out to be quite effective in increasing the flexibility of zein films. It was revealed that 10~20% tributyl citrate provided brilliant plasticization to zein films forming a matrix which was stable even at low temperature. Furthermore, plasticization with tributyl citrate allowed zein films to sustain at high humidity and water activity of food, eventually making zein film applicable on frozen foods.

To introduce ice nucleation properties to zein films, the adsorption of ECINs on films surface were monitored, which indicated the maximum ECINs adsorption was achieved on the UV/ozone-treated hydrophilic zein surface at pH 7.0 and ionic strength of 0.05 M. On the other hand, a novel and convenient method was developed and verified

to quantify ice nucleation activity of develop films. The method correlated the number of ice nucleators with the water nucleation temperature measured by DSC, providing an empirical equation and parameters based on experimental data. Using this method, the INA of zein films was successfully assessed and the highest INA on the TBC-plasticized zein films was 175 units/mm<sup>2</sup>, which has possibly reached the upper limits with current materials and conditions.

Both blending ECINs and wrapping with zein-based ice nucleation films (INFs) protected frozen bread dough through freeze/thaw treatments. Blending ECINs and wrapping with high-activity INFs were equally competent in preserving the yeast viability in frozen dough, possibly by modulating the freezing rate of dough. Analysis to gluten network revealed less degradation and depolymerization with the protection from ECINs, probably due to higher yeast survival which released less reducing agents. Therefore, both blending ECINs and wrapping with high-activity INFs preserved the crumb gas cell structures resulting in apparently white bread crumb. Blending ECINs was slight more effective in increasing the specific volume of bread. However, wrapping with INFs at all activity levels significantly prevented dehydration of frozen dough, resulting in higher water content in bread. This allowed all INFs to significantly decrease the crumb hardness and to increase the crumb springiness. Among the zein-based INFs with different ice nucleation activities, the higher activity on the film, the better protection that the INF provided to the frozen dough.

On the aspect of energy saving, blending ECINs shortened the freezing process of dough by 20%, which potentially saved 1250 kJ under current conditions. In contrast, zein-base INFs was much less effective in decreasing the time of freezing, because the

films slowed down the heat transfer and make temperature decrease at lower rate. Nevertheless, INFs still accelerated the phase transition of dough, the thus still reduced the total time of freezing by 6% compared to the control dough without any protection, which potentially saved 357 kJ energy under current conditions.

In order to further compare the methods of blending ECINs and wrapping with INFs and to reveal the advantage of the later method, the total activity introduced to each dough was calculated for the two methods. Blending ECINs used  $1.7 \times 10^9$  units per dough. Comparatively, wrapped with INFs used  $7.7 \times 10^6$  units per dough. In addition, the ECINs immobilized on INFs exhibited reusability on freezing water.

This research is of major significance because it developed a series of methods to control the surface and bulk properties of zein film, which are versatile and applicable on other biopolymer matrix, given that poor mechanical properties and water sensitivity are two common problems with most biopolymers. The method for immobilizing ECINs on the surface of zein films also initiated an effective and flexible way for the development of functional food packaging. Last but not least, this research provides the first confirmative and direct experimental results demonstrating the cryoprotective effects of ECINs as biogenic ice nucleators on a frozen food (frozen bread dough) and a live organism (yeast).

On the other hand, further research could be done to extend the current study. Firstly, sensory tests could not be done with the breads from frozen dough due to ECINs lacking safety approval. One day, if the safety issue could be solved, sensory test would be necessary assay to evaluate the effects of ECINs on frozen food. Secondly, it was

interesting to see that the cryopreservative effects of ECINs could be transferred from the surface to the center. But it is unclear of the mechanism that how the effects reached the parts of dough other than the area directly contacting films. Further study is needed to clarify this issue. The third question is regarding the application of INFs. It is expected that the INFs would be more effective on liquid and semi-solid foods. But on solid foods, the size and shape of the food items, as well as how to increase the contact areas between food and INFs, would be critical factors determining the effectiveness of INFs. In the future study that would try the INFs on other foods, these factors need to be taken into consideration.

Regarding ECINs, with their demonstrated potential in on cryopreservation, future research could focus on the effects of ECINs on preserving other microorganisms. Cryopreservation of microorganism is conventionally used in labs and industries to store viruses, bacteria, yeast, fungi, algae, and protozoa. Many factors influence the effectiveness of cryopreservation, among which, composition of the medium is of most importance. Manipulation to the storage medium is considered most practicable and addition of suitable cryoprotective additives (CPAs), such as glycerol and  $\text{Me}_2\text{SO}$ , usually show protective effects on microorganisms from freeze damaging. However, very few CPAs are successful and even less CPAs are universal (Hubalek, 2003). In the present research, ECINs revealed protective effect on yeast cell in saline at concentration as low as 0.001 mg/mL. Therefore, it is absolutely worthwhile to investigate the versatility of ECINs as a novel CPA in cryopreservation of microorganisms. However, ECINs are not easy to work with, mainly due to unclear composition and properties. This study provides some basic knowledge about the effects of physicochemical conditions on



ice nucleation activity of ECINs. However, further study is necessary to clarify the composition of ECINs and how ECINs function in cryopreservation.

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## **APPENDIX A: REDUCING THE BRITTLINESS OF ZEIN FILMS THROUGH CHEMICAL MODIFICATION**

*The work in this chapter has been published in the title of “Reducing the Brittleness of Zein Films through Chemical Modification” in Journal of Agricultural and Food Science (Volume 59, Issue 1, Pages from 56 to 61, 2011).*

The initial purpose was to address the issue on poor mechanical properties of cast zein films. However, the final zein films for preparing ice nucleation films did not use chemically modified zein, mainly because of the concerns that chemical modification with lauryl chloride may disable zein films being used for food-contact materials. Another reason was that it was later found that plasticization by tributyl citrate fulfilled the required improvement to zein films for current application.

### **Abstract**

To reduce the brittleness of zein films, a new type of zein-based biomaterial was synthesized by chemical modification of zein with lauryl chloride through an acylation reaction. The final products were confirmed by  $^1\text{H}$  NMR, FT-IR analysis, and SDS-PAGE. Thermal analysis detected no micro-phase separation in the synthesized polymer matrix. As the content of lauryl moiety increased, the glass transition temperatures of modified zein decreased as large as 22 °C due to the plasticization effect of the lauryl moiety. In addition, mechanical and surface properties of cast films from acylated zein were also investigated. The elongation at break of modified zein sheet was increased by about seven-fold at the high modification level with some loss of mechanical strength. The surfaces of modified zein films were as uniform as unmodified zein film but more

hydrophobic, further suggesting that no micro phase separation happened during the film formation process. The final zein films for preparing ice nucleation films did not use chemically modified zein, mainly because of the concerns that chemical modification with lauryl chloride may disable zein films being used for food-contact materials. Another reason was that it was later found that plasticization by tributyl citrate fulfilled the required improvement to zein films for current application.

## **Introduction**

Zein, an alcohol-soluble protein enriched in the endosperm of corn, is a main co-product of the bio-ethanol industry. The commercial  $\alpha$ -zein contains two main components with apparent molecular weights of 19kD (Z19) and 22 kD (Z22). Although zein has a long history of being used as a coating material for candies, medical tablets and paper (Lawton, 2002), other applications of zein are still limited mainly due to the undesirable properties of the zein-based materials (Chen and others 2006; Gao and others 2007; Zhong and others 2008). Like many other proteins, zein, without plasticizers, forms brittle films with poor flexibility that cannot withstand industrial processing. Some low molecular weight molecules, such as some polyols and fatty acids, could be used as plasticizers to improve the mechanical properties of zein films (Lawton, 2002). However, phase separation as a result of immiscibility between zein and these plasticizers is difficult to be avoided, which usually leads to instability of the mechanical properties and often heterogeneous surfaces. Another disadvantage of using polyol plasticizers is that they exacerbate the sensitivity of zein-based materials to moisture (Ghanbarzadeh and others 2007a; Ghanbarzadeh and others 2007b; Park and others 1996). Thus, new efforts

are needed to improve the water resistance and mechanical properties of zein-based materials.

Chemical modification allows an extensive exploration to the novel functionalities and applications of synthetic polymers. The methods could also be used on biopolymers to generate new biomaterials to substitute synthetic polymers (Lesiak-Cyganowska and others 2001; Liu and Li, 2002; Tomasik and Schilling, 2004). Cross-linking agents have often been used to improve the properties of protein films. In many reported studies, the addition of cross-linkers, such as formaldehyde, glutaraldehyde, transglutaminase, multivalent carboxylic acids etc., to zein has been commonly used to increase the mechanical strength and reduce the water permeability. However, such approach often showed little or even negative effects on the flexibility (Kim and others 2004; Oh and others 2004; Parris and Coffin, 1997; Selling and Sessa, 2007; Sessa and others 2007; Yamada and others 1995; Yang and others 1996). Wu et al. (Wu and others 2003b) used polycaprolactone (PCL) to modify zein, where the hydrophobic chain of PCL not only cross-linked the zein molecule, but also served as plasticizers and thus significantly increased the elasticity of zein sheets. On the other hand, palmitic chloride was used to acylate the zein in order to improve the thermo-processibility and water resistance. However, the study did not clarify whether the modification improved the intrinsic mechanical properties of zein or the by-products such as palmitic acid played a role as plasticizer (Brauer and others 2007).

In this part of work, it was aimed to obtain zein derivatives with improved film properties through chemical modification by lauryl chloride, which would introduce hydrophobic alkyl chains onto zein molecules. The synthesis was examined by combined

$^1\text{H}$  NMR, FT-IR analysis, and SDS-PAGE, and the flexibility and hydrophobicity of zein derivative films were characterized.

## Materials and Methods

### *Materials*

The  $\alpha$ -zein of biochemical-grade purity was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Deuterated dimethyl sulfoxide (DMSO- $d_6$ ) was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Lauryl chloride (99%, Acros Organics), triethylamine (TEA), dimethyl sulfoxide (DMSO), hydrochloric acid, ethanol (95%), and glycerol were obtained from Fisher Scientific, Inc. (Pittsburgh, PA). Pluronic® F127 was from BASF Corporation (New Jersey, US). Milli-Q water was used throughout the experiment.

### *Modification by acylation reactions*

Acylation of zein was performed as previously described (Brauer et al., 2007) with some modifications. TEA (3, 9 or 15 mmol) and lauryl chloride (1, 3, or 5 mmol) per gram zein were added to a 5% w/v solution of zein in DMSO drop-wise at ambient temperature while stirring. The resultant solution was heated to 65 °C under stirring for 3 h. Subsequently, the reaction mixture was poured into water, acidified to pH 5-6 using HCl in order to neutralize the extra TEA and to facilitate its removal through dialysis. The product, acylated zein, was separated by centrifugation, and washed with water for three times before lyophilization. The powder obtained was dissolved in 80% ethanol and dialyzed against 80% ethanol for one week, and then dialyzed against water for 24 hours, followed by lyophilization. The lauryl-substituted zein molecules were named zein-C12-1, zein-C12-3 and zein-C12-5, respectively, according to the amount of lauryl chloride

per gram of zein. If the average molecular weight of zein was assumed to be 20 kD, the initial molar ratio of lauryl chloride versus zein was 20:1, 60:1, and 100:1, respectively.

#### *<sup>1</sup>H NMR spectroscopy*

The <sup>1</sup>H NMR spectra were acquired using a Varian VNMRS 500 MHz spectrometer. The <sup>1</sup>H solution spectra of zein and modified zein were recorded in DMSO-d<sub>6</sub> at 25 °C.

#### *ATR-FTIR spectroscopy*

The attenuated total reflection fourier transform infrared spectra were collected under ambient conditions, using a Thermal Nicolet Nexus 670 FT-IR spectrometer (Thermo Electron Corp., Madison, WI) with a Smart MIRacle™ horizontal attenuated total reflectance Ge crystal accessory. Each spectrum was averaged by 128 scans with 4 cm<sup>-1</sup> resolution.

#### *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

Each sample was dissolved in sodium dodecyl sulfate/non-reducing sample buffer to 1mg/ml solution, and then heated at 95 °C for 5 min. each sample solution (5 µL) or protein marker was loaded onto sodium dodecyl sulfate polyacrylamide gel with 5% stacking gel and 15% separating gel. The gel was run in BioRad Mini-Protean® II system under 90 V for about 2 hours. Gel was then stained with Coomassie blue (Ausubel and others 1987) and photographed by Monochrome gel documentation system.

#### *Differential scanning calorimetry (DSC)*

DSC analyses were performed on a DSC 823E thermal analyzer (Mettler-Toledo Inc., Columbus, OH) supplied with liquid nitrogen and compressed nitrogen gas.

Approximately 5 mg of each sample were packed into aluminum crucibles with lid and heated from 32 to 140 °C at a rate of 10 °C/min, followed by cooling down to -120 °C at a rate of 30 °C/min, in order to remove any prior thermal history within the samples. Subsequently, the thermograms were obtained by heating the samples to 210 °C at a rate of 10 °C/min. Glass transition temperatures ( $T_g$ , the midpoint of the glass transition) and the melting temperatures ( $T_m$ , the lowest point of each melting peak) were automatically analyzed from the thermograms by the STARE software accompanied with the instrument. Triplicate readings were taken.

#### *Film surface properties*

Zein films were prepared by a spin casting method as described previously with some modifications (Shi and others 2009). Sample solutions were prepared by dissolving zein or modified zein into glacial acetic acid to the concentration of 0.1% (w/v) and 1% (w/v), and then heated at 70 °C for 30 min. Zein films were prepared by spin coating the solutions onto pre-cleaned silicon (Si) wafers, using a Laurell model WS-400A-6NPP/LITE spin coater (Laurell Technologies Corp., North Wales, PA) at 4000 rpm for 80 seconds. Clear and homogeneous zein films were obtained and dried at 50 °C in a oven overnight to remove the residual solvent. The obtained films were denoted in the format as “ZF5-0.1”, where ZF was abbreviation of “zein film”, the number “5” indicated the level of added lauryl chloride in the reaction, and the number “0.1” indicated the concentration of solution used for film preparation. For example, the film ZF5-0.1 was prepared from 0.1% (w/v) zein-C12-5 in acetic acid solution.



The surface morphology images of zein films were collected by tapping mode atomic force microscopy (TP-AFM) on a NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA) under ambient conditions.

The surface hydrophilicity was represented by the static water contact angle measured on a VCA Optima XE Dynamic Contact Angle Analyzer (AST Products Inc., Billerica, MA) at ambient condition. The images were recorded by a CCD camera immediately after the water droplets were deposited onto the zein film surface. The water contact angles were determined using the associated software provided by the manufacturer. At least six measurements were averaged for each sample.

#### *Mechanical properties measurement*

Zein sheets were prepared by solvent casting in Teflon Petri dishes using solutions consisted of 20% zein, zein-C12-1, zein-C12-3, or zein-C12-5 in acetic acid plasticized by 25% Pluronic® F127 and 12.5% Glycerol based on the weight of zein or modified zein and then dried in vacuum oven at 50 °C for 48 hours. Yellow and transparent sheets were obtained from each sample. The zein sheets were denoted as ZS, ZS1, ZS3 or ZS5, where ZS stands for zein sheet, and ZS1, 3 and 5 stands for sheets made from different amount of acylated zein. Dumbbell-shaped specimens with a dimensions of  $40 \times 10 \times (0.05 - 0.30)$  mm<sup>3</sup> were cut from the central region of the sheets and stored in 50% relative humidity desiccators under ambient temperature for more than 30 days before conducting tensile test. Mechanical properties were measured on a TA.XT2 Texture Analyzer (Texture Technologies Corp., New York, US) with an initial grip distance of 20 mm and a test speed of 6 mm/min.

## Results and Discussion

### *Preparation and Structure of Acylated zein*

According to Gianazza et al. (Gianazza and others 1977), about 8.2% of the amino acids were either Serine or Threonine and 26.6% amino acids were any of Glutamic acid/Glutamine/Aspartic acid/Asparagine. The hydroxyl group and the amino group could potentially serve as the sites to form ester or amide bonds in the acylation reaction. However, due to the limitation of protein conformation, some of the hydroxyl and amino groups may be buried inside the protein, thus are not accessible to reactions. In the present study, acylated zein molecules were synthesized with three different amount of lauryl chloride. To ensure complete removal of residue lauryl chloride (or its hydrate lauryl acid), the products underwent extensive dialysis.

SDS-PAGE analysis of the modified zein was used to reveal the modification extent upon adding different amounts of lauryl chloride (Appendix figure 1). The two major bands at 19kD (Z19) and 22kD (Z22) represented the two main components in  $\alpha$ -zein (Lee and others 1976). Because the samples were run in a non-reducing condition, the bands at around 39kD should be the respective dimeric forms of Z19 and Z22 (Paulis and Bietz, 1988). The bands at around 11kD and 14kD were also suggested to be two  $\beta$ -zein components (Shukla and Cheryan, 2001). On the SDS-PAGE gel, modified zein would shift to higher molecular weights. Interestingly, after modification at the two higher levels of lauryl chloride, three bands appeared at the position between 19kD and 22kD. Without further analysis, it is not sure what forms of zein the three aggregates comprised. In spite of the sequence homology, the reactivity and surface hydrophobicity of the two components were found to be different based on reversed-phase HPLC

analysis (Paulis and Bietz, 1988). And their molecular conformation was also thought different (Tatham and others 1993). Thus, it is possible that the lowest band came from the modification of the Z19 component. According to our previous experience, the Z19 component was more accessible to chemical modification than the Z22. The addition of 12-carbon alkyl chains would lead the denatured protein to adopt different thermodynamic conformation in SDS-PAGE gel compared to the pure protein. In addition, other studies suggested that the hydrophobic interactions, exceeding the down effects from the lost positive charges, enhanced the binding of SDS onto protein attached with long-alkyl-chain (Gudiksen and others 2006; Shirai and others 2008). This could result in higher density of positive charge on acylated protein molecules which would migrate faster than a pure protein with the same molecular weight in electrophoresis. Thus, low-degree modified Z19 aggregate may appear to separate from original Z19 bands, even though the molecular weight did not change significantly.

Modified zein would bind less staining dye per unit mass, therefore it is not appropriate to use densitometry method to calculate the unmodified zein contents in the samples, which is based on analyzing the relative intensity of each band in the image (Wu et al., 2003b). The zein contents can only be roughly estimated: there was minimal 12% modified zein in zein-C12-1, while this value increased to more than 24% in both zein-C12-3 and zein-C12-5, regardless of the different amounts of added lauryl chloride.

$^1\text{H}$  NMR spectra of zein and its derivatives also confirmed the successful grafting of lauryl groups onto zein molecule. In comparison with the  $^1\text{H}$  NMR spectrum of zein (Appendix figure 2a), the ratio of the number of H in  $\text{CH}_2$  ( $\delta$  1.21) to those in  $\text{CH}_3$  ( $\delta$  0.83) significantly increased from 0.26:1 to 0.32:1, 0.47:1 and 0.51:1, respectively

(Appendix figure 2b-d), which suggested more alkyl chains in the molecule of modified zein and further indicated that the lauryl groups were grafted to the molecule of zein as expected.

By combining the results of SDS-PAGE and  $^1\text{H}$  NMR analysis, we found that at the initial lauryl chloride to zein molar ratio of 20:1, zein was only slightly acylated, and at the ratio of 60:1 and 100:1, acylation occurred to a similar extent, suggesting the possible saturation of the reaction groups on zein surface.

Moreover, the FTIR spectra of the samples also revealed the difference in molecular structures between modified and pure zein. The spectrum of lauryl chloride (Appendix figure 3a) contained a sharp peak at  $1798\text{ cm}^{-1}$  which is associated with the  $\text{C}=\text{O}$  stretching in  $\text{CO-Cl}$  group (Grant and others 2008). Another sharp peak at  $720\text{ cm}^{-1}$  represented the vibrations of alkyl chain with at least four methylene groups (Grandtner and others 2005). The three adjacent peaks at  $2958$ ,  $2923$  and  $2854\text{ cm}^{-1}$  were assigned to the vibration of the methyl group, the symmetric and asymmetric vibrations of the methylene group, respectively (Song and others 2008). After modification, the newly formed ester and amide groups intensified the absorption of the amide I band (stretching vibrations of the  $\text{C}=\text{O}$  bond of the amide) at around  $1650\text{ cm}^{-1}$  and the amide II band (bending vibrations of the  $\text{N-H}$  bond) at  $1544\text{ cm}^{-1}$ . The vibrations of attached hydrocarbon chains gave rise to those bands in the  $2800 - 3000\text{ cm}^{-1}$  range and the bands at  $1447\text{ cm}^{-1}$  and  $720\text{ cm}^{-1}$ .

Besides acylating the protein, lauryl chloride could also react with water and form fatty acid. The fatty acid could function as the plasticizer and interfere with other

characterizations. The free fatty acid could be detected from the FTIR spectrum as a shoulder peak at 1700-1800  $\text{cm}^{-1}$ , as seen in the previous study on modifying soy proteins with palmitic acid chloride (Brauer et al., 2007). In Appendix figure 3, the absence of absorption bands at around 1798  $\text{cm}^{-1}$  confirmed the effective removal of the fatty acid by the dialysis procedure (Stuart, 2004).

### *Thermal Properties*

DSC is conventionally used to study the plasticizer compatibility with biopolymer and the effectiveness of plasticization. The DSC thermograms of lauryl chloride, zein and modified zein were shown in Appendix figure 4 with respective glass transition temperatures and specific heat summarized in Appendix table 1. Previously, Sessa et al. reported that the Japanese white zein had a lower  $T_g$  than Freeman zein, at around 158 °C (Sessa and others 2008; Sessa et al., 2007). Whereas, Wu et al. also performed DSC to Showa zein and found two  $T_g$  at 180.1 and 200.1 °C, potentially attributed to the two zein sub-fractions (Wu et al., 2003b). The zein used in this study could be from the same manufacturer as their zein. However, the thermograms of zein obtained here were quite different. Two distinct glass transition processes were observed, with  $T_g$  at 92.0 and 173.5 °C, which may be attributed to the two components in  $\alpha$ -zein, Z19 and Z22, respectively. The differences in glass transition temperatures among zein may be due to the different origins of zein.

Appendix table 1 summarized the glass transition temperatures of zein and modified zein samples measured by DSC. After modification, the lower  $T_g$  decreased from 92.0 °C to 69.8 °C, by about 22.0 °C, while the higher  $T_g$  one (i.e., 173.5 °C) only decreased by about 5 °C. This also suggested that the reaction showed certain preference

between various components in zein, and was consistent with the SDS-PAGE and  $^1\text{H}$  NMR studies which suggested that, compared to Z22, Z19 is more reactive in acylation.

Pure lauryl chloride had a melting point at  $10.4^\circ\text{C}$ . Modified zein did not show any endothermic peak at that temperature, indicating that there was no microcrystal in the samples formed by lauryl chains. The FT-IR spectra also confirmed this, because the absorption in  $2800 - 3000\text{ cm}^{-1}$  range derived from the vibration of methylene groups in ordered long hydrocarbon chains didn't shift to a lower wavelength compared to the spectrum obtained for our modified zein (Song et al., 2008).

### *Film Surface Properties*

The surface properties are believed to be important for the barrier properties of films, where homogenous and smooth surface is usually preferred. In our previous study, it was observed that the spin casted zein films from acetic acid solutions bear smooth and featureless surfaces (Shi et al., 2009). However, a heterogeneous surface is often observed on biopolymer films that are simply plasticized with fatty acids, due to the instability of the emulsions formed between fatty acids and biopolymers, resulting in phase separation afterwards. For this reason, cast zein films plasticized by fatty acids often show granules on the top of film surface (Park et al., 1996; Lai and Padua, 1997; Ghanbarzadeh et al., 2007b). AFM tapping mode images shown in Appendix figure 5 revealed that chemical modification by lauryl chloride did not create any visible aggregates or pattern, and the film surfaces maintained as uniform as the untreated zein.

Water permeability and moisture sensitivity of protein-based materials were also directly affected by surface and overall hydrophobicity (Wu and others 2003a). Water

surface contact angle, representing the surface hydrophobicity, was also measured on the zein and modified zein films. Appendix table 2 contained static water contact angles of films prepared by zein and its derivatives. Films casted from unmodified zein showed a relatively large water surface contact angles, due to its hydrophobic nature. Meanwhile, water contact angles of modified zein films were further slightly increased, due to the increased surface hydrophobicity through the acylation reaction, implying possible decrease in water permeability and moisture sensitivity.

### *Mechanical Properties*

Plasticized with Pluronic<sup>®</sup> F127 and glycerol, zein and acylated zein were cast into thin sheets and their mechanical properties were measured. As shown in Appendix table 3, the elongation at break of zein-C12-5 was about 7 times of the control. Given that the tensile strength was slightly lowered after modification, the modified zein sheets containing higher portions of lauryl moiety revealed higher toughness, which was calculated as the energy required for breaking divided by the volume of specimen tested. At the same time, the Young's modulus was decreased from 213.48 MPa of pure zein films to 70 ~ 90 MPa of modified zein sheets but no obvious difference among each modification level.

Unplasticized zein cast films are usually brittle and exhibit no yielding point before rupture at low strain. Plasticized zein films containing 15% (w/w) free fatty acids, such as stearic acid or palmitic acid slightly decreased the tensile strength, but showed little effects on the elongation at break and the Young's modulus, whereas the same plasticization level of oleic acid could bring down both tensile strength and Young's modulus (Parris and others 2002; Santosa and Padua, 1999). Additionally some

processing during film formation, such as kneading, blowing and/or extrusion could strengthen the plasticization effect of free fatty acids in zein films (Santosa and Padua, 1999; Lai and others 1997). This may suggest that processing may be necessary to input energy to promote the interactions between fatty acid plasticizers and zein molecules. In contrast, covalently attaching acyl chain will allow the plasticizers to evenly distribute in the zein film matrix, as suggested by the previous DSC and AFM results, to allow maximum interactions. Thus, this chemical modification was able to significantly increase the extensibility of zein sheets.

In summary on this part of work, new zein-based biomaterials were synthesized through a single step acylation reaction with lauryl chloride. By chemically attaching plasticizer molecules on the protein, modified zein showed lower glass transition temperatures. Also, solvent cast sheets from modified zein became more flexible and tougher, which suggested that potential improved processibility of acylated zein compared to pure zein. In addition, the solvent cast films from modified zein maintained the uniformity and smooth surface as pure zein films, indicating that the attachment of lauryl chains did not introduce any macro- or micro-scale phase separation. Furthermore, the hydrophobicity on the surface has been further increased because of the attached alkyl chains, which could potential improve the water barrier properties of these zein-based biomaterials. This study suggests a good potential of acylated zein as food packaging and coating materials.

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Appendix table 1 Glass transition temperatures of zein and modified zein samples  
measured by DSC

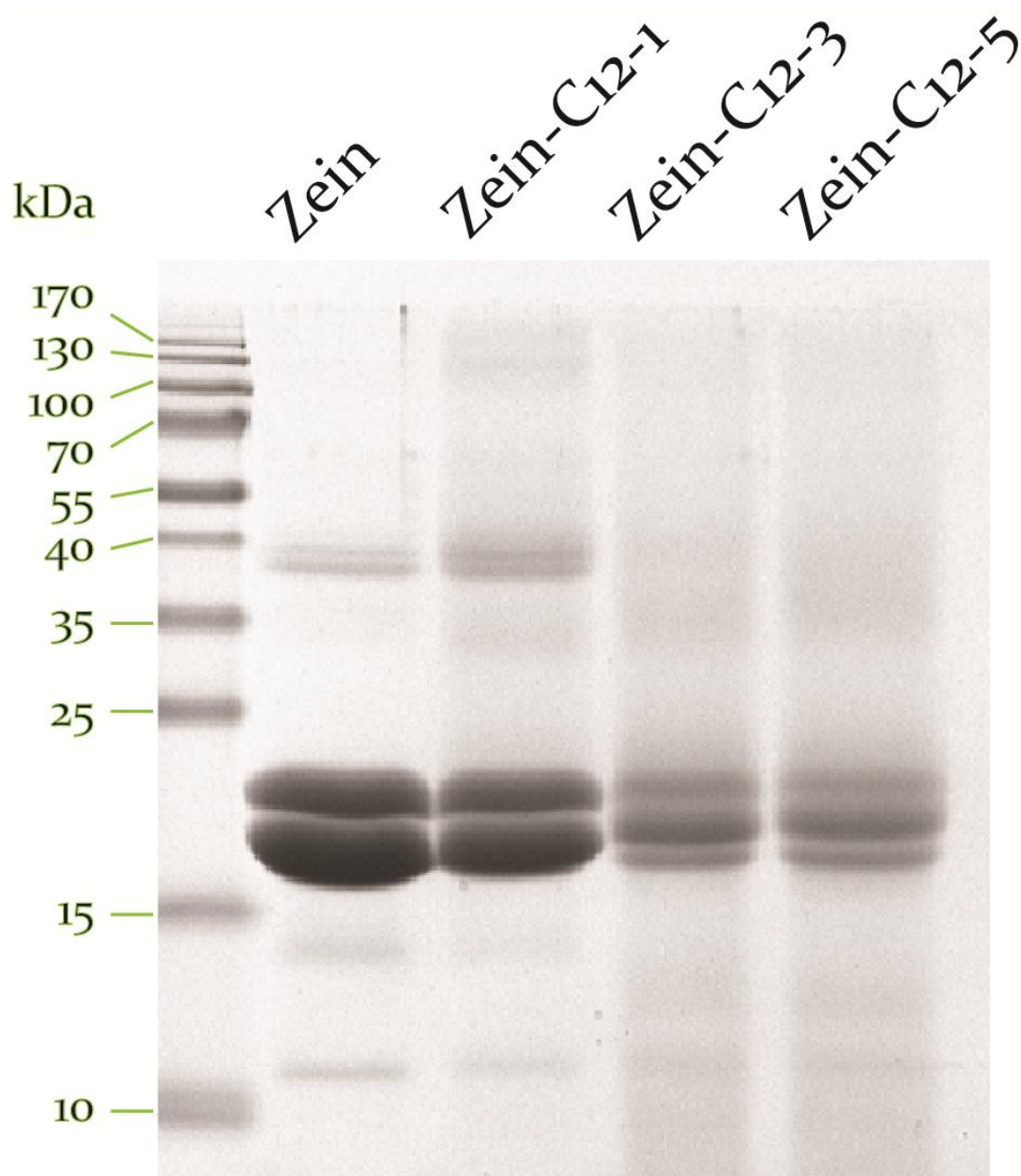
Samples	Glass Transition Temperature	
	$T_g^1$ (°C)	$T_g^2$ (°C)
zein	$94.4 \pm 2.1$	$173.6 \pm 0.3$
zein-C12-1	$81.1 \pm 2.2$	$177.2 \pm 1.3$
zein-C12-3	$74.7 \pm 1.6$	$169.8 \pm 0.6$
zein-C12-5	$68.8 \pm 1.0$	$167.7 \pm 0.3$

Appendix table 2 Surface water contact angles of zein and modified zein

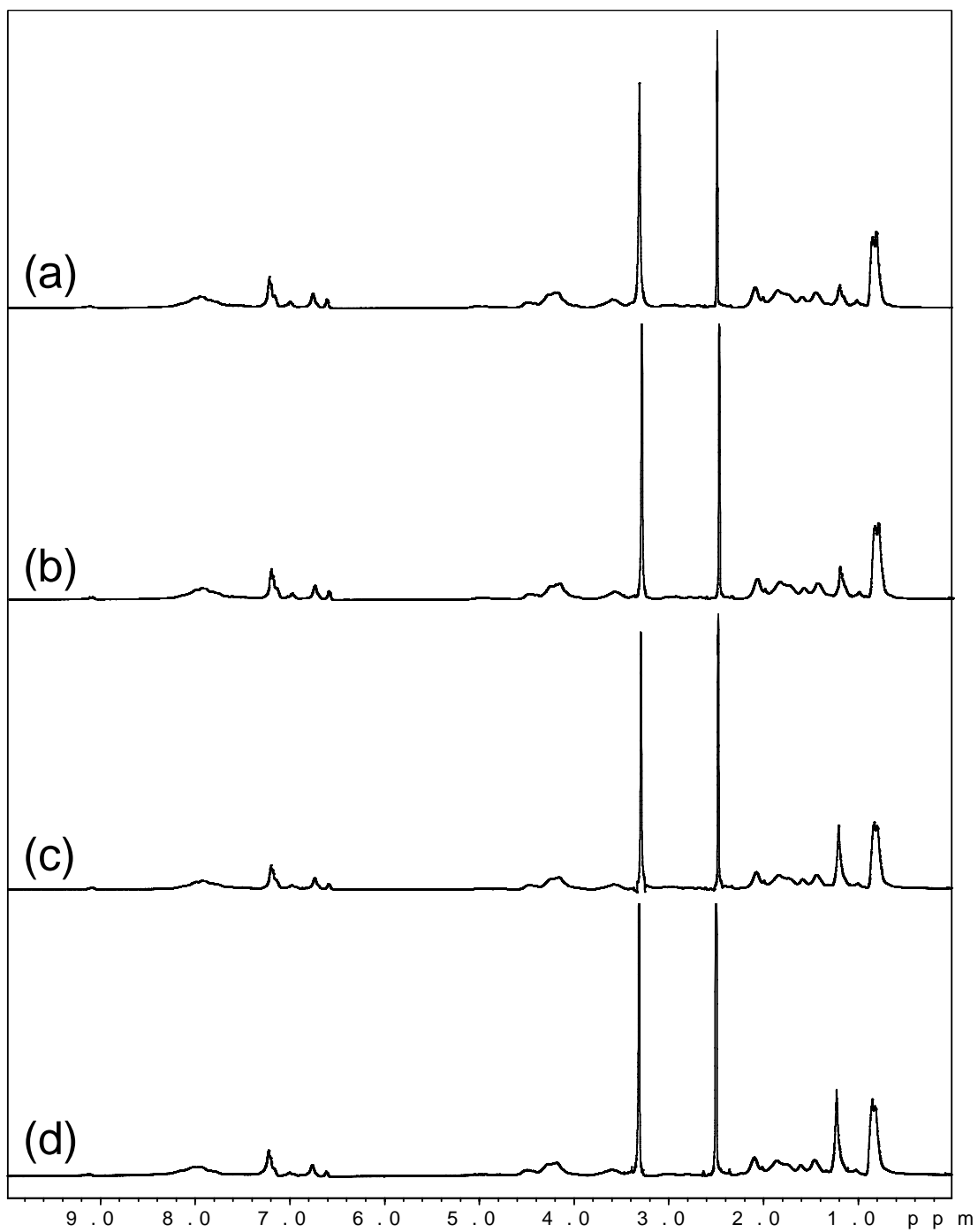
Samples	Water Contact Angle (degree)
ZF	$75.6 \pm 1.1$
ZF1	$78.5 \pm 0.5$
ZF3	$86.4 \pm 0.3$
ZF5	$86.0 \pm 1.0$

Appendix table 3 Mechanical properties of cast films made from pure zein and modified zein

	<b>Young's Modulus (MPa)</b>	<b>TS (MPa)</b>	<b>ETB (%)</b>
<b>ZS</b>	215.48 $\pm$ 27.54	3.53 $\pm$ 0.62	46.78 $\pm$ 20.53
<b>ZS1</b>	96.29 $\pm$ 36.04	2.82 $\pm$ 0.35	101.92 $\pm$ 56.61
<b>ZS3</b>	89.31 $\pm$ 41.50	3.00 $\pm$ 0.30	163.10 $\pm$ 69.29
<b>ZS5</b>	78.66 $\pm$ 26.51	3.66 $\pm$ 0.49	302.25 $\pm$ 23.68

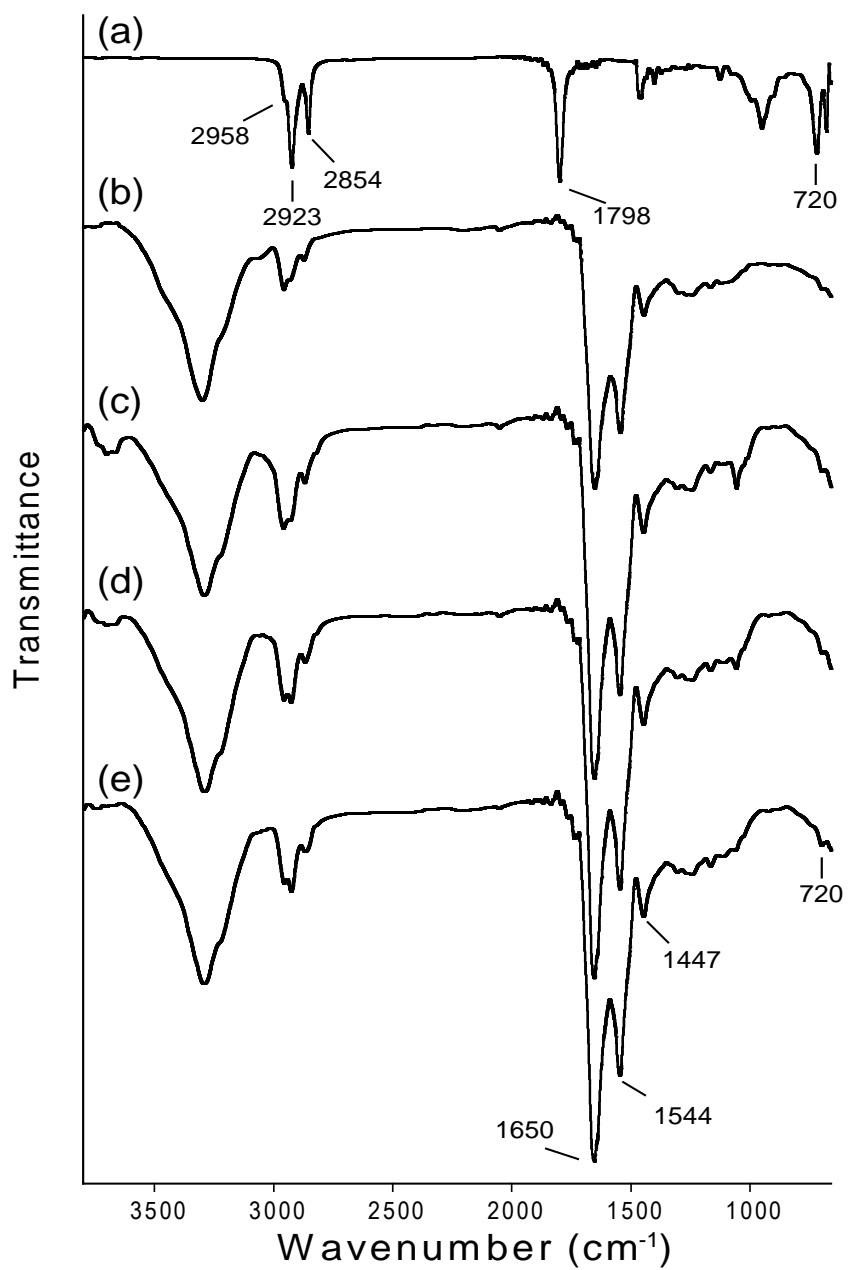


Appendix figure 1 SDS-PAGE of pure zein and modified zein. The protein marker was run in the first lane, with the molecular weights labeled.

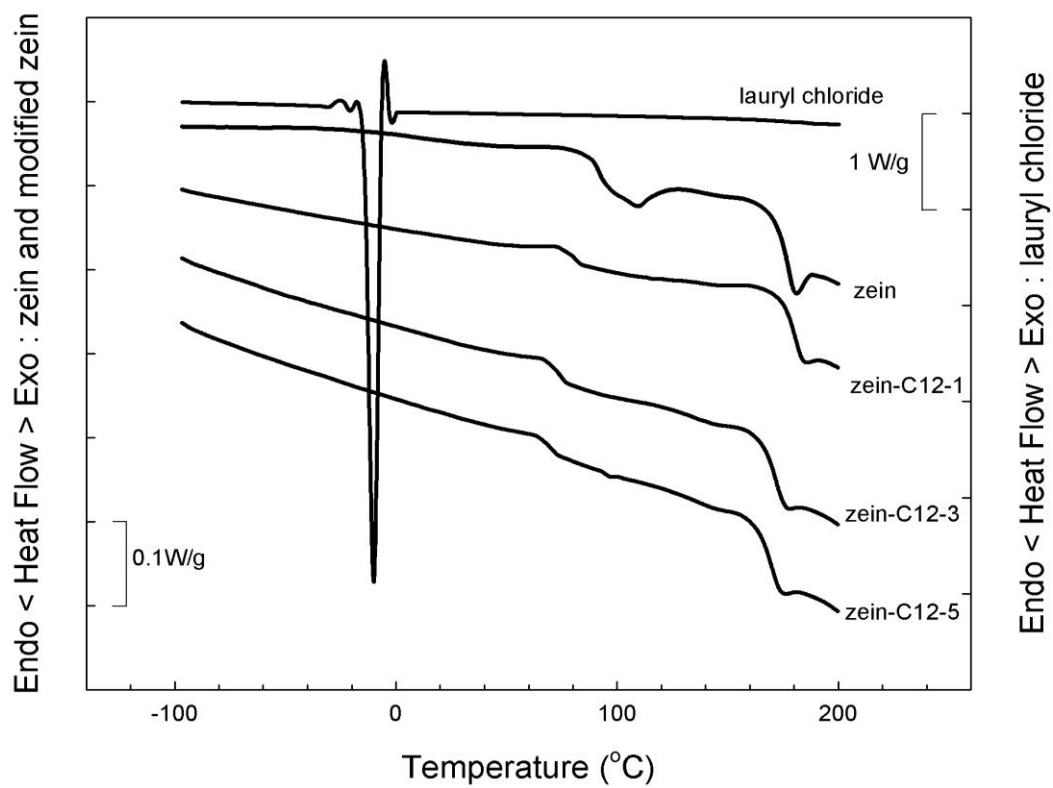


Appendix figure 2  $^1\text{H}$  NMR spectra of (a) zein, (b) zein-C12-1, (c) zein-C12-3 and (d) zein-C12-5

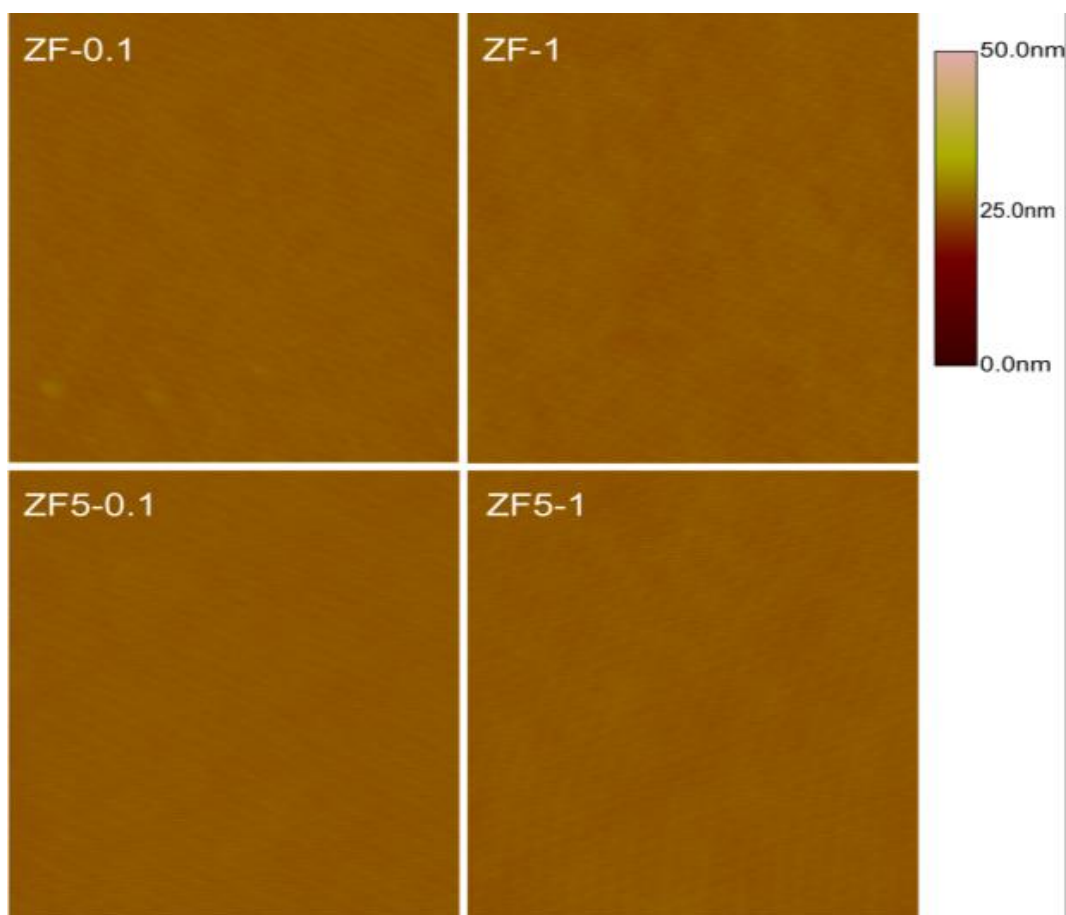




Appendix figure 3 FT-IR spectra of (a) lauryl chloride, (b) zein, (c) zein-C12-1, (d) zein-C12-3 and (e) zein-C12-5



Appendix figure 4 DSC thermograms for lauryl chloride, zein, and modified zein



Appendix figure 5 Tapping mode atomic force microscopy (TP-AFM) images of films prepared from zein 0.1% and 1% solutions (ZF-0.1, ZF-1) and from zein-C12-5 0.1% and 1% solutions (ZF5-0.1, ZF5-1). Each image size is 500 nm  $\times$  500 nm, and the z scale is 50 nm

## **APPENDIX B: PRODUCTION AND CHARACTERIZATION OF ICE NUCLEATORS FROM *XANTHOMONAS* *TRANSLUCENS***

The initial purpose of the work was to look for alternative resources to isolate biogenic ice nucleators which might avoid the safety issue of ECINs, given that *Xanthomonas* strains are currently used for commercial production of xanthan gum. However, this work revealed that the intrinsic ice nucleation activity of *Xanthomonas* ice nucleators was too low which was not significantly improved with the current trials on optimizing the conditions of production and isolation. Some experimental data in this work, as noted in the legend, were collected by Jing Deng, a visiting scholar in laboratory of Dr. Tung-Ching Lee.

### **Abstract**

The ice nucleation properties of bacterial strains in the genera of *Xanthomonas* have been recognized and investigated since 1970s. However, the environmental factors for the production of ice nucleators by *Xanthomonas* are still not clear, and the cell-free ice nucleators have not been isolated or characterized. In order to obtain ice nucleators from *X. translucens*, the effect of culture conditions on the expression of ice nucleation activity (INA) by the bacteria was investigated. The cells began expressing INA at late log phase and continued accumulating the activity until early stationary phase. The optimum temperature for INA expression by *X. translucens* was 18 °C, but requiring 80-hour cultivation. On the other hand, cold shock at 4 °C after growth at 23 °C was able to

induce the INA expression and save cultivation time. Sucrose was identified as the best carbon source and 2% peptone in the medium generated highest INA. Meanwhile, it was found that most of INA were enriched in the outer membrane rather than extruded into the environmental medium via surface vesicles. The cell-free *Xanthomonas* ice nucleators (XINs) were isolated by sonication and were composed of 29% protein, 12% carbohydrate and 10% lipids. Compared with the extracellular ice nucleators (ECINs) from *Erwinia herbicola*, the INA of XINs was about 1000 times lower at all the tested temperature. Moreover, atom force microscopy suggested more aggregated granules present in the XINs samples than in ECINs. This part of study provides fundamental understanding on the cultivation condition and properties of ice nucleators by *X. translucens*.

## Introduction

Ice-nucleating (IN) active strains of bacteria are able to express strong protein-based heterogeneous ice nuclei which introduce the formation of ice and thus minimize the supercooling step in water freezing (Lundheim, 2002). IN active bacteria have been found from the genera of *Pseudomonas*, *Erwinia* and *Xanthomonas*. The environmental factors for the expression of ice nuclei by *Pseudomonas* and *Erwinia* with the isolation and purification of ice nuclei have been investigated for decades (Muryoi and others 2003; Hwang and others 2001; Obata and others 1998; Li and Lee, 1998a; Watabe and others 1993; Obata and others 1990).

Bacterial ice nuclei are believed to be protein-based complexes containing polysaccharide and lipid moieties (Kozloff and others 1991). Three major chemically

distinct but related types of ice nuclei were identified based on the highest temperatures at which they are able to initiate ice nucleation: Type I, nucleating supercooled water at temperatures above -5 °C; Type II, nucleating supercooled water between -5 and -7 °C; and Type III, effective at temperature in the range of -7 to -10 °C (Phelps and others 1986). It was previously proposed that the ice nucleation proteins (INP) synthesized in cytoplasm of *Pseudomonas* cells were transported onto outer membrane forming the class C ice nucleators. Then through a series of modification, the glycosylated INPs in membrane formed class B ice nucleators, which were finally extruded and anchored on the outer membrane via the phosphatidylinositol (PI), forming class A ice nucleators (Kozloff et al., 1991).

*Xanthomonas* has a long history of being used in food, oil recovery, cosmetics, water-based paints and other industries for the production of xanthan gum (Rosalam and England, 2006). And the effect of cultivation conditions of *Xanthomonas* on the production of Xanthan gum has been extensively examined. Nevertheless, few efforts have been devoted to the studies on the cultivation conditions for ice nucleating activity (INA) production, although some *Xanthomonas* strains were found to have ice nucleation activity as early as 1990's (Watanabe and others 1996). Among the few studies, Kawahara and Obata carried out a qualitative evaluation on the production of ice nucleators and xanthan gum by *Xanthomonas campestris* pv. *translucens* in various medium composition and cultivation conditions. It showed that with galactose, xylose, mannose, arabinose, maltose or lactose as carbon source, INA could be expressed within 2-day growth at 18°C while the production of xanthan gum was slow (Kawahara and Obata, 1998). The effect of additives in medium on the its growth profile of *X. campestris*

INXC-1 was measured (Watanabe and others 1993a). It was also identified that some food-originated compounds, such as 4-hydroxy-3-nitrophenylacetic acid, could slightly enhance its expression of INA (Watanabe and others 1994; Watanabe and Watanabe, 1994). It was further proved that the *Xanthomonas* cells could be sterilized by high pressure treatment at lower temperature while still retaining the INA (Honma and others 1993).

Given the limited study on the effect of cultivation condition on the production of ice nucleators by *Xanthomonas* strains, in the present study, the INA of *Xanthomonas* was systematically and quantitatively examined under different growing environment. The effect of cultivation temperature, induction by cold shock, type of carbon source and concentration of peptone on the INA was investigated. Meanwhile, the subcellular location of ice nucleators was identified and furthermore the cell-free ice nucleators was isolated and compared with those from *Erwinia herbicola*. This work provided fundamental information about production and isolation of ice nucleators from *Xanthomonas*, which will benefit its utilization in a variety of applications.

## Materials and Methods

### *Bacterial strain and cultivation conditions*

The *Xanthomonas translucens* pv. *Translucens* NBRC 13559, previously classified as *X. campestris* pv. *Translucens* (Vauterin and others 1995) was obtained from NITE Biological Resource Center, Chiba, Japan. Half milliliter frozen stock was used to inoculate into 6 mL *pseudomonas* medium (2% Bacto-peptone, 1.05% lactose•H<sub>2</sub>O, 0.86% K<sub>2</sub>SO<sub>4</sub>, 0.14% KCl and 0.14% MgSO<sub>4</sub>•7H<sub>2</sub>O, pH 7.0) and shaken under 18 °C at

speed of 200 rpm. When the optical density (OD) at the wavelength of 600 nm (OD<sub>600</sub>) reached 1.0, 3 mL culture was inoculated into 150 mL *pseudomonas* medium with various carbon or nitrogen sources and cultured at designated temperatures (18 °C, 23 °C, 30 °C) and at shaking speed of 200 rpm. OD<sub>600</sub> and INA were measured at regular intervals during cultivation.

The *Erwinia herbicola* subsp. *ananas* was obtained from the American Type Culture Collection (ATCC Cat. No. 11530) and routinely cultured in yeast extract (YE) medium consisting under 18°C as previously described (Li, 1998).

#### Effect of Cold Induction Time and Temperature on INA

Aliquots from stationary-phased *X. translucens* cultures which were originally grown at 23 °C or 30 °C were incubated at 4 °C, 12°C or 18 °C for 24 h. OD<sub>600</sub> and INA were measured at one-hour interval afterwards.

#### *Effect of Different Carbon Sources and Nitrogen concentration on INA*

The effects of various carbon sources on INA were examined by addition of various saccharides to the *pseudomonas* medium as substitutions to lactose. The effect of the concentration of Bacto-peptone on INA was also tested. For each medium tested, cells were grown to the stationary phase and the INA was recorded as the highest level of activity that the culture reached. Three repeats were carried out for each test.

#### *INA assay*



Ice nucleation activity was determined by the droplet-freezing assay (Vali, 1971; Li and Lee, 1998a). Solid samples were dispersed into sodium phosphate buffer (pH 7.0) before series dilution with dH<sub>2</sub>O. Liquid samples were directly used for series dilution.

### *Subcellular fractionation*

The method was adopted from (Sidhu and others 2008; Wu and others 2006) with some modifications. Bacteria at early stationary phase were collected by centrifugation at 10,000  $\times g$  for 30 min and supernatant was collected as medium fraction. Cells were washed by suspending the cells in 20 volumes of distilled water and then collected by centrifugation at 10,000  $\times g$  for 30 min. Spheroplasts were prepared by treating cells with 1.5 mg/mL lysozyme in 30% (w/v) sucrose, 45 mM Tris/HCl, 1.5 mM EDTA, pH 8.0, on ice for one hour. The periplasmic fraction was collected as the supernatant after centrifugation at 10,000  $\times g$  for 30 min. Spheroplasts were washed once with 20% (w/v) sucrose, 30 mM Tris/HCl, 1 mM EDTA, pH 8.0 followed by centrifugation at 10,000  $\times g$  for 30 min. Pellets were suspended in 10 mM Tris/HCl, pH 8.0 and then sonicated on ice, for 6 seconds with 30 seconds interval for 10 times. The suspension was treated with *DNase* I to decrease the viscosity. The supernatant collected after centrifugation at 17000  $\times g$  for 30 min was put into ultracentrifugation at 138,000  $\times g$  for 1 hour with the obtained supernatant as the cytoplasmic fraction and pellets as the total membrane fraction. For further fractionation, total membrane fraction was re-suspended with 1% Triton X-100, 10 mM MgCl<sub>2</sub>, 50 mM Tris/HCl, pH 8.0 and incubated at room temperature for 30 min followed by centrifugation at 156,000  $\times g$  for 30 minutes. The supernatant was inner membrane fraction and the pellet re-suspended in 20 mM MgCl<sub>2</sub>, 20mM Tris/HCl, and

pH 8.0 was outer membrane fraction. Fractions were dialyzed against 20 mM MgCl<sub>2</sub>, 20 mM Tris/HCl, pH 8.0 buffer before protein and INA assays.

#### *Isolation of ice nucleators*

Generally, *Xanthomonas* ice nucleators (XINs) and extracellular ice nucleators (ECINs) were isolated from *X. translucens* and *E. herbicola*, respectively. Cells were harvested by centrifugation at 10,000 ×g for 20 min and then re-suspended in 20 mM Tris/HCl pH 8.0. Sonication of cells was done on ice at output of 50 watts, 10 seconds with 10 seconds interval for 3 times. Cells and debris were removed by centrifugation at 10,000 ×g for 20 min and supernatant was subjected to filtration through 0.45 µm filters. The filtrate was ultracentrifuged at 160,000 ×g for 2 hour and pellet was re-suspended in 20 mM Tris/HCl with 20 mM MgCl<sub>2</sub>, pH 8.0. After lyophilization, the samples in the form of flakes were stored at -20 °C.

#### *Determination of XINs composition*

Protein concentration was measured by Bradford method (Bradford, 1976) using bovine serum albumin as standard with Bio-Rad Protein Assay Reagent (Hercules, CA, United States). After development of color, absorbance at 595 nm was measured.

Carbohydrate concentration was measured using Phenol-Sulfuric acid Assay (Frederick, 2004) using D-glucose as standard. After development of color, absorbance at 490 nm was measured

Lipid was extracted with twenty volumes of acidified chloroform/methanol (2/1 v/v) as previously described (Findlay and Evans, 1987). After extraction, centrifugation was applied to remove denatured protein, then one-fifth volume of 50 mM CaCl<sub>2</sub> was

added and mixed to form an emulsion. After phase separation, the upper phase (chloroform/methanol/water, 3/42/47) and layer between phases containing denatured protein was carefully removed. And solvents in lower phase were evaporated with nitrogen gas. The glass tubes were weighted before adding extraction liquid and after evaporation of solvents. The weight change was calculated as the lipid components.

#### *Turbidimetric titration*

Twenty milliliter of 1mg/ml ice nucleators in DI water solution at the ionic strength of 0.01M was prepared and filtered through 0.45  $\mu$ m filter. The solutions were magnetically stirred throughout the titration process at room temperature. The change in transmittance of the solution during change of pH was monitored continuously using a turbidity meter (Brinkmann-910, Brinkmann Instruments, USA) equipped with a 1-cm path length optical probe operating at 490 nm. The turbidity meter was calibrated with filtered DI water as 100% transmittance. Initial pH in solutions was adjusted to 8.7 and 0.1 M HCl was added drop-wise to gradually acidify the solutions. Turbidity reading was recorded one minute after pH was stable.

#### *Transmission electron microscopy*

*X. translucens* cells collected at early stationary phase were re-suspended in 20 mM Tris buffer. One milliliter cell suspensions were placed on carbon- and Formvar-coated nickel grids and then stained with 2% aqueous phosphotungstic acid (pH 7.3) and examined with a JEOL 100CX II transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating under standard conditions.

#### *Tapping Mode Atomic Force Microscopy*

TP-AFM images were collected by NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA) with a Sietched RTESP7 cantilever (Veeco Nanoprobe, Camarillo, CA) under ambient conditions. Images were analyzed using the software provided by the manufacturer.

### *Statistical analysis*

One-way ANOVA was performed using Sigma-Plot v.12.0 with integrated SigmaStat module. Different letters indicate significant difference ( $P < 0.05$ ).

## **Results and Discussion**

### *Effect of temperature on the expression of ice nucleation activity*

Bacterial growth and production of INA are influenced by many factors, the most important of which is growth temperature (Hirano and others 1985). Small temperature shift usually significantly varies the growth profile of bacteria. This study first investigated the effect of cultivation temperature on the growth and INA production of *X. translucens*. As shown in Appendix figure 6, *X. translucens* grew faster at higher temperature, and reached stationary phase in 26 hours at 30 °C (growth curve not shown), compared to 48 hours at 23 °C and 96 hours at 18 °C, respectively. As other environmental microorganisms, the optimal temperature for the growth of *Xanthomonas* was determined as 30 °C (Hirano et al., 1985). However, this temperature was not favored for INA expression. No INA was detected in the cultures grown at temperature above 25 °C, suggesting that low temperature was a critical condition for expressing the activity. In both cultures grown at 18 °C and 23 °C, bacteria began to show significant activity at late exponential phase and continued to accumulate the activity until reaching the maximum at early stationary phase. However, the maximum activity of culture grown

at 18 °C reached  $6 \times 10^7$  units/mL, which was 1000 times higher than that at 23 °C. Therefore, 18 °C was determined as the optimum temperature for INA expression.

Although grown at 18 °C, *X. translucens* cells expressed high level of ice nucleation activity; it would need about 80 hours to reach a high level of INA. Previous studies on *Pseudomonas* and *Erwinia* bacteria suggested that low-temperature incubation was able to induce more INA expression (Nemecekmarshall and others 1993; Fall and Fall, 1998; Castrillo and others 2001). In this study, the effect of cold temperature induction on the INA expression was examined, by transferring early-stationary-phase bacterial grown at 23 °C to different low temperatures (18 °C, 12 °C, 4 °C) for various durations. As shown in Appendix figure 7, the lowest cold-shock temperature (4 °C) was most effective in INA induction. After 2-hour incubation at 4 °C, the INA increased by about 100 fold. After 4-hour incubation, the INA increased by 1000 times. During the incubation from 4 hours to 24 hours, the INA did not show significant change, suggesting that 4-hour cold induction at 4 °C was sufficient for INA induction.

As similar phenomenon has been observed for ice-active *Pseudomonas* and *Erwinia* strains, it was found that cold temperature mainly induced the formation of type I ice nuclei while not affected the amount of type II and III ice nuclei. This suggested that the aggregation of ice nucleation protein on the bacterial surface was induced at low temperature and formed more effective nucleation sites and thus higher activity (Burke and Lindow, 1990; Castrillo et al., 2001; Fall and Fall, 1998; Nemecekmarshall et al., 1993). By cold induction, the production of INA was comparable to the cultivation at 18 °C. However, the cultivation time was considerably shortened from 80 hours to 52 hours, which included 48-hour culturing at 23 °C and 4-hour cold induction).

### *Effects of growth medium on the expression of ice nucleation activity*

Compositions in the growth medium, such as carbon source and concentration of peptone, have great impact on the bacterial growth and production of biomolecules. To investigate whether the carbon source in the growth medium affects the level of INA expression, the strain was grown at 18 °C in *pseudomonas* medium with six different carbohydrates as the sole carbon sources and the INA was measured at stationary phase. As shown in Appendix figure 8, the highest INA was obtained from the culture containing sucrose, followed by glucose, lactose, maltose and mannose, whereas sorbitol resulted in lowest INA production. The effect of carbon source reflects the efficiency of utilizing certain sugar in the synthesis pathway. speculated possible pathway was that sucrose provide the bacteria a easy and rich source of fructose which can be later phosphorylated and react with glutamine to form Glucosamine, which was suggested to essential for the synthesis of type II and type I ice nuclei (Kozloff et al., 1991). However, details on synthesis and assembly of ice nuclei are not confirmative.

The effect of different concentrations of Bacto-peptone as sole nitrogen source on INA expression was further examined (Appendix figure 9). It was showed that from 0.1% to 1% of Bacto-peptone, no statistically significant difference in expressed INA was observed. Further increasing Bacto-peptone from 1% to 2% resulted in enhanced INA expression ( $p < 0.05$ ). These results were consistent with the fact that bacterial ice nuclei are protein-based substances (Kozloff et al., 1991).

### *Subcellular location of ice nucleators on X. translucens*

Ice nucleation proteins (INPs) from *Pseudomonas* and *Erwinia* have been demonstrated to locate on the bacterial outer membrane (Kozloff et al., 1991). It is also

observed that about 10% to 30% ice nuclei are shedded into the medium and become extracellular ice nucleators (ECINs) (Li and Lee, 1998a; Phelps et al., 1986). With the aspect of *Xanthomonas*, information on the distribution and subcellular location of ice nucleators is still lacking. Thus in this study, the ratio of INA between fractions of whole cells and cell-free supernatant in *X. translucens* culture was determined, with the results summarized in Appendix table 4. Almost all (99%) of INA was located in/on the cells, and less than 1% of INA found in supernatants. The minimum activity in supernatant could be due to the lysis of cells and release of the ice nucleators into medium (Kawahara and Obata, 1998).

Since most of INA of *X. translucens* remained on/in the cells, the subcellular location of INA was further determined. The whole cells were separated into outer membrane, periplasmic, inner membrane and cytoplasmic fractions and the total INA and specific INA were determined for each fraction. As shown in Appendix table 5, most of the INA was kept in the cytoplasm, while some INPs were transported across the inner membrane into the periplasm before they were inserted into the outer membrane (Wandersman, 1992; Pugsley, 1993). Specific INA data indicated that the percentage of ice nucleation proteins in total proteins was higher in the outer membrane fraction than the in the other fractions, which suggested that the outer membrane was the target location of ice nucleation proteins.

#### *Characterization to Xanthomonas ice nucleators*

The ice nucleators from *X. translucens*, here denoted as XINs, was isolated using sonication to lyse the cells and differential centrifugation to collect the membrane fraction (Hobb and others 2009). The XINs showed a yellowish color tightly associated

with the lipid component during chloroform extraction. The color may come from the yellow pigments, xanthomonadins, which are composed of brominated, aryl-polyene esters similar to those of carotenoids (Starr and others 1977). The chemical composition of XINs was determined (Appendix table 6) and compared to that of ECINs. Previous study revealed that ECINs contain 40% protein, 9% carbohydrate and 3.4% lipid. Compared to ECINs, less protein contents and more carbohydrate and lipid were detected in XINs. *X. translucens* is well known to synthesize xanthan gum which was attached on the surface of cell or released into surrounding environment. This explains the high level of carbohydrate associated with isolated XINs.

Additionally, water solubility of ECINs and XINs showed different dependency on pH. As shown in Appendix figure 10, ECINs and XINs formed transparent solution at pH above 5. When the pH decreased to below 3.8 for ECINs and 4.7 for XINs respectively, the solution became increasingly turbid and precipitation was noticeable. When pH decreased to below isoelectric point (IEP), the protein components in the ice nucleators turned to positively charged and consequently interacted with the carbohydrate components were negatively charged. The inter-molecular associations caused the ice nucleators to precipitate, shown as the decreased transparency of the solutions. Therefore, this result implied that the ECINs and XINs may have an IEP around 4.2 and 4.7, respectively. Similarly, an ice nucleating active protein isolated from *Pseudomonas putida*, was reported to have an IEP at 5.3 (Xu and others 1998).

The activity of type I, II, III ice nuclei in XINs and ECINs were subsequently compared by measuring the ice nucleation activity at temperatures of -4 °C, -6 °C and -8 °C, respectively (Phelps et al., 1986). As shown in Appendix table 7, for each type of ice



nuclei, ice nucleation activity of XINs was found to be 1000-fold lower than ECINs, which was consistent with previous researches showing that *inaX* gene in *Xanthomonas* encodes much weaker ice nuclei (Zhao and Orser, 1990). Specifically, INA of XINs at -4 °C was undetectable, indicating there was minimal amount of type I ice nuclei in isolated XINs, although previous observation suggested that another *X. campestris* strain did produce type I ice nuclei after cultivation under 25 °C for 72 hours (Watanabe and others 1993b).

In the TEM image of *E. herbicola*, many surface vesicles were detected on the bacteria (Li and Lee, 1998b), whereas no surface vesicles were seen in case of *X. translucens* (Appendix figure 11). Surface vesicles can be shed into the medium, also readily dissociated by sonication. The lack of surface vesicles on *X. translucens* could partially explain the previous results that supernatant of *Xanthomonas* culture media revealed little INA, and that XINs prepared by sonication had much less INA than ECINs.

The size and morphology of ECINs and XINs were further compared using atomic force microscopy. Globular structures were dominant in both XINs and ECINs, with some rod-shaped structures, as shown in Appendix figure 12. It seemed that the rod-shaped structures were actually chain-shaped aggregates of the globular structures. The primary globular structures in both ice nucleators had similar size with diameter of 10 nm, whereas the XINs were prone to form larger aggregates possibly due to the high content of polysaccharides, which could entrap the individual ice nucleators and subsequently decreased the apparent ice nucleation activity.

*Xanthomonas* ice nucleators (XINs) were unique among other bacterial ice nucleators, in that majority of the ice nucleators were tightly bound in the outer membrane of *Xanthomonas* cells, not like ECINs which were extruded and loosely bound to the surface of the *Erwinia herbicola*, confirmed by the TEM image. This explained why the sonication was not as effective for isolation of XINs as for ECINs.

In a summary on this part of study, the effect of cultivation conditions on the INA expression from *Xanthomonas* was examined systematically. 18°C was the optimum growing temperature and cold shock at 4°C could induce the INA expression and shorten the total cultivation time. Sucrose was found as the best carbon source and high peptone concentration in the medium favored high INA. Meanwhile, XINs were characterized and compared with ECINs. XINs were enriched in outer membrane of the bacteria and the isoelectric points may be close to pH4.7, lower than that of ECINs. The chemical composition of XINs were determined, and showed lower protein but higher carbohydrate contents than ECINs. XINs revealed 1000-fold less INA than ECINs, which may be partially due to the lack of surface vesicle on the outer membrane.

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Appendix table 4 Distribution of ice nucleation activity between fractions of whole cells and supernatant in the culture of *X. translucens*\*

	Volume (ml)	INA (units/ml)	Total INA (units)	Ratio (%)
culture	81.8	$2.23 \times 10^8$	$1.82 \times 10^{10}$	100
supernatant	78.2	$2.30 \times 10^6$	$1.80 \times 10^8$	0.99
cell	3.6	$5.02 \times 10^9$	$1.81 \times 10^{10}$	99.01

\* Data collected by Jing Deng

Appendix table 5 Distributions of ice nucleation activity in subcellular fractions of *X.**translucens* cells

Fractions	Volume (ml)	INA (units/ml)	Protein concentration (mg/ml)	Total INA (units)	specific INA (units/mg protein)
periplasmic	400	$3.3 \times 10^2$	0.38	$1.3 \times 10^5$	$8.6 \times 10^2$
cytoplasmic	40	$9.1 \times 10^3$	10.9	$3.7 \times 10^5$	$8.4 \times 10^2$
inner membrane	10	$1.0 \times 10^2$	5.02	$1.0 \times 10^3$	$2.1 \times 10^1$
outer membrane	10	$9.2 \times 10^3$	4.36	$9.1 \times 10^4$	$2.1 \times 10^3$

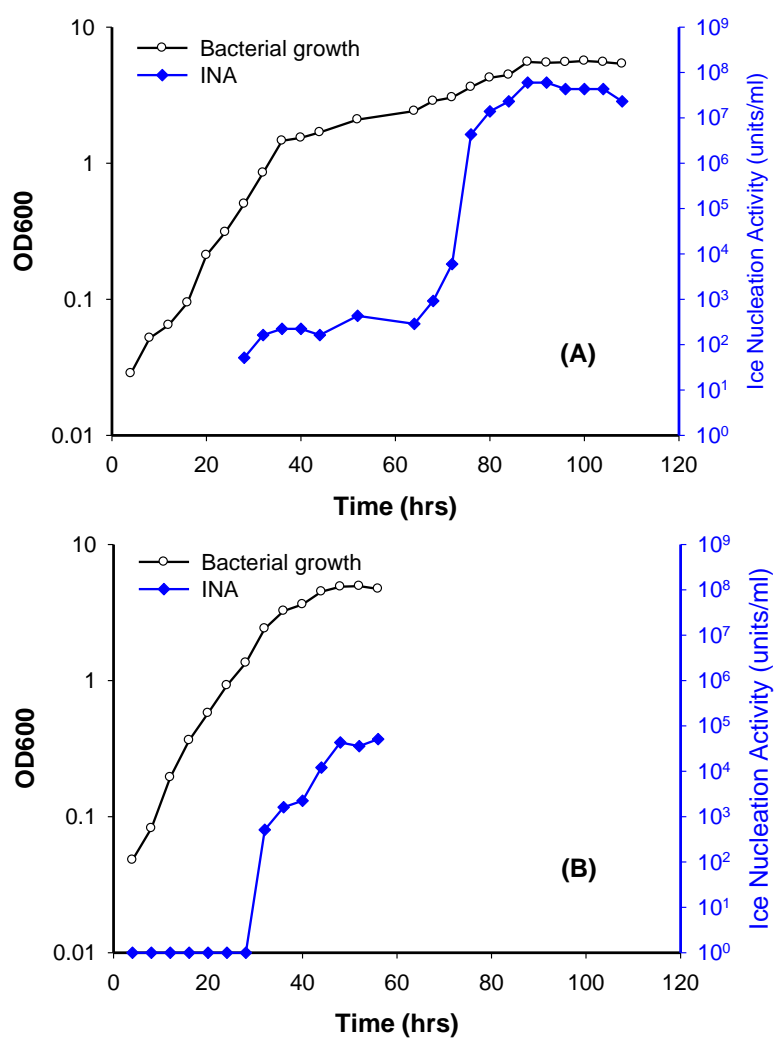
Appendix table 6 Compositions of cell-free *Xanthomonas* ice nucleators (XINs)

	<i>XINs</i>
<b>Protein</b>	28.99 $\pm$ 2.73%
<b>Carbohydrate</b>	11.76 $\pm$ 3.23%
<b>Lipid</b>	10.30 $\pm$ 1.80%



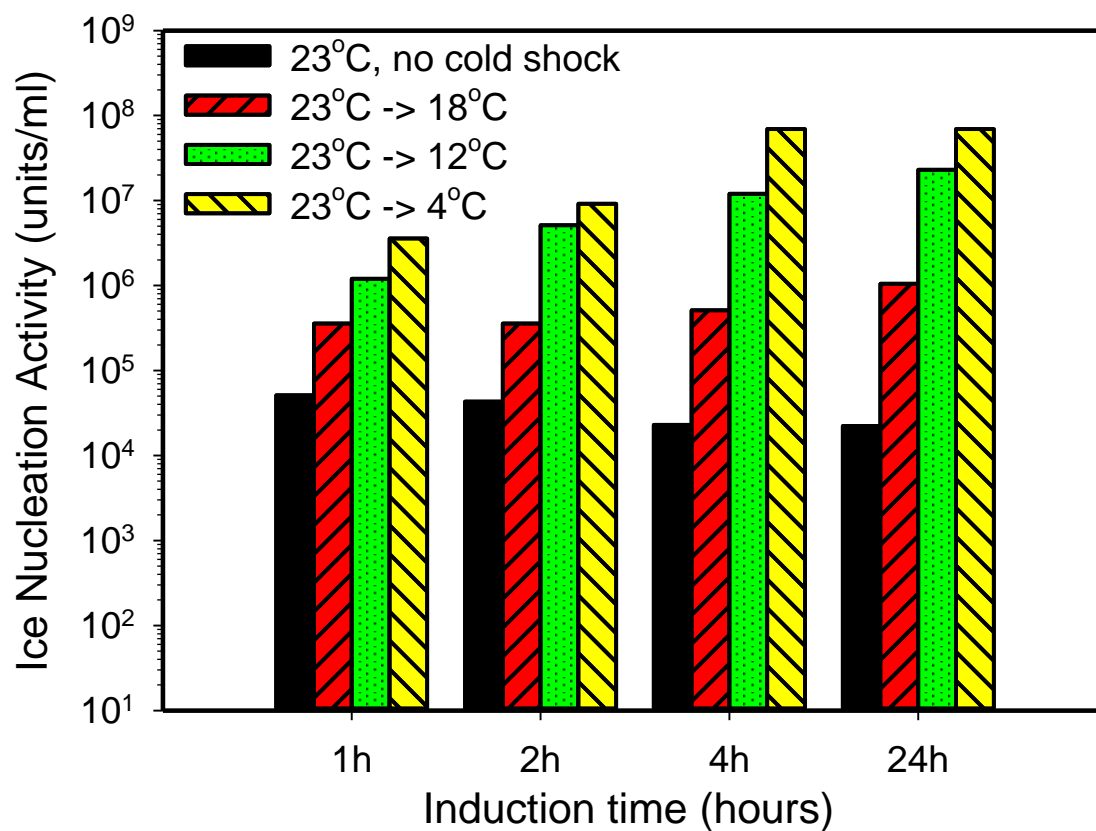
Appendix table 7 Comparison of ice nucleation activity in ECINs and XINS at various functional temperatures

	<i>INA (units/mg)</i>		
	-4 °C	-6 °C	-8 °C
XINS	<1	32 ± 5	(1.0 ± 0.3) × 10 <sup>4</sup>
ECINs	645 ± 67	(2.2 ± 0.9) × 10 <sup>4</sup>	(7.8 ± 3.8) × 10 <sup>7</sup>



Appendix figure 6 Bacterial growth and Expression of INA by *X. translucens* at different temperatures: (A) 18 °C; (B) 23 °C\*

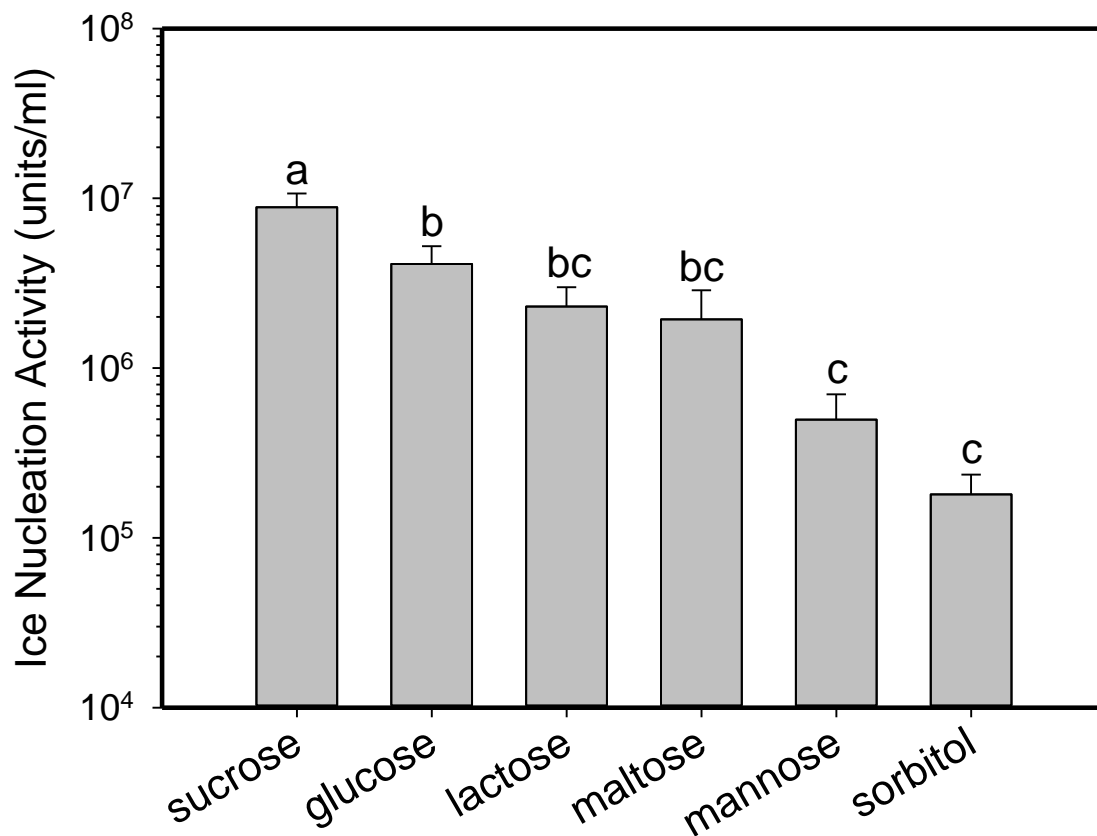
\* Data collected by Jing Deng



Appendix figure 7 Effect of temperature and duration in cold induction on the INA of X.

translucens grown at 23 °C\*

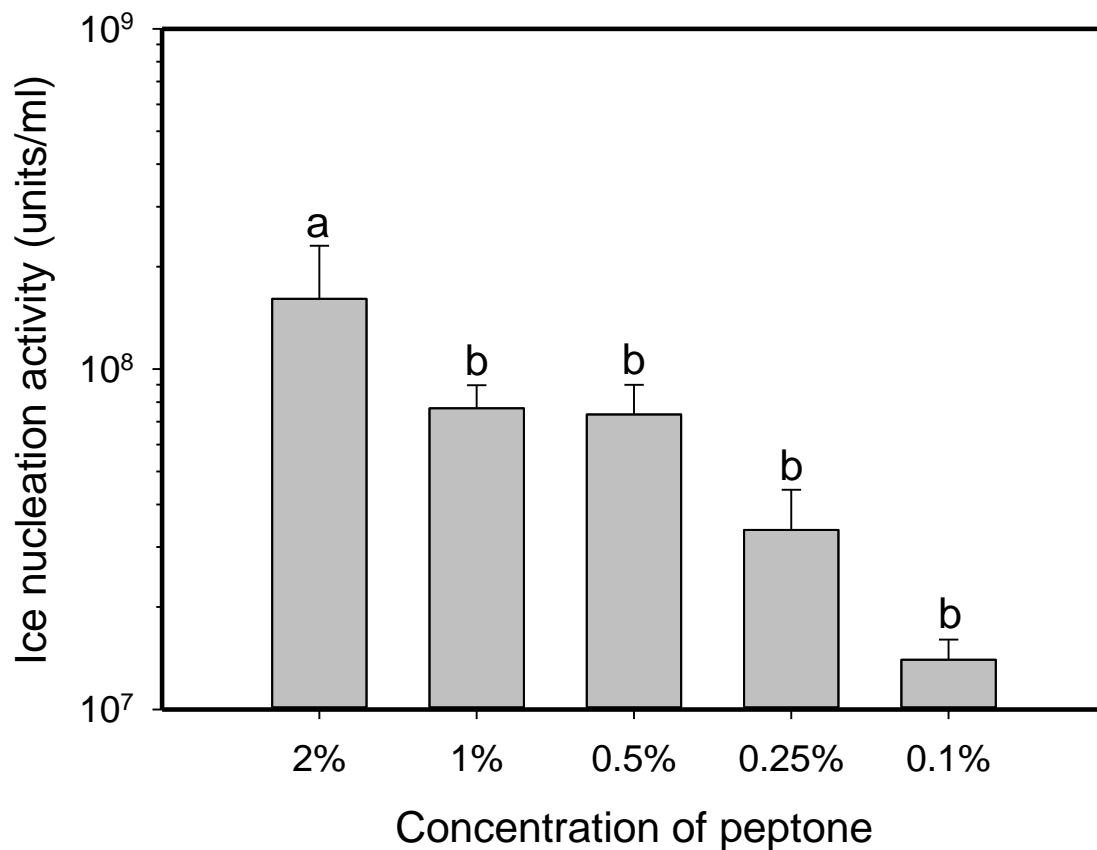
\* Data collected by Jing Deng



Appendix figure 8 Effect of carbon source on the expression of ice nucleation activity by *X. translucens*\*

\*The letters on top of error bars indicated the result of statistical analysis. Different letter indicate significant difference ( $p < 0.05$ ).

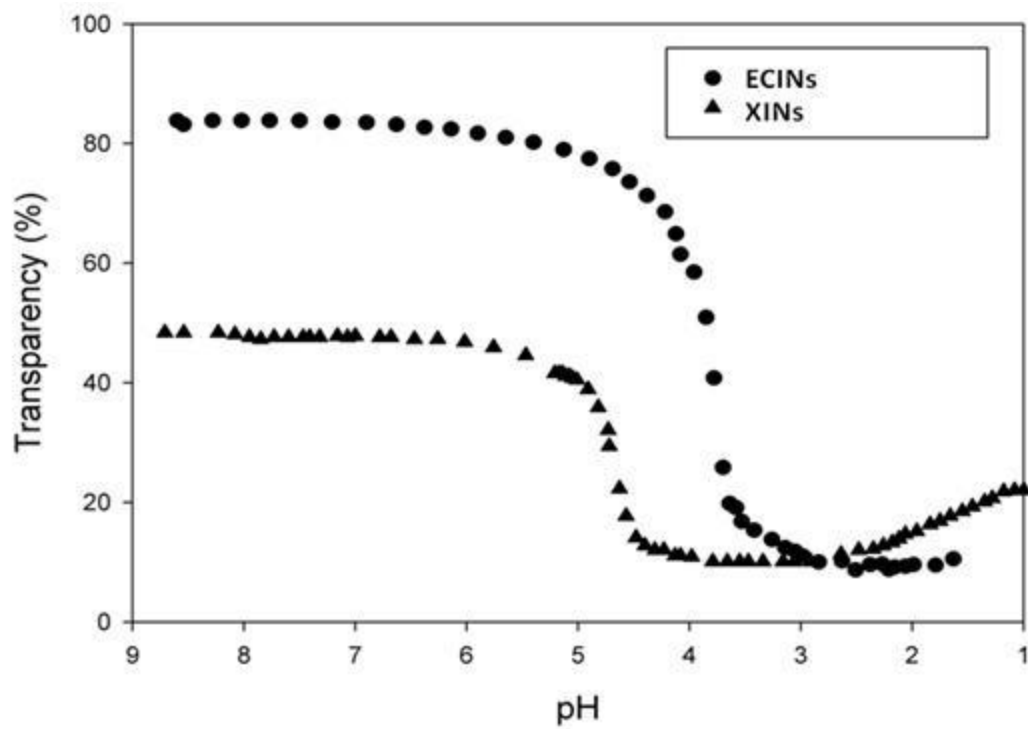
\* Data collected by Jing Deng



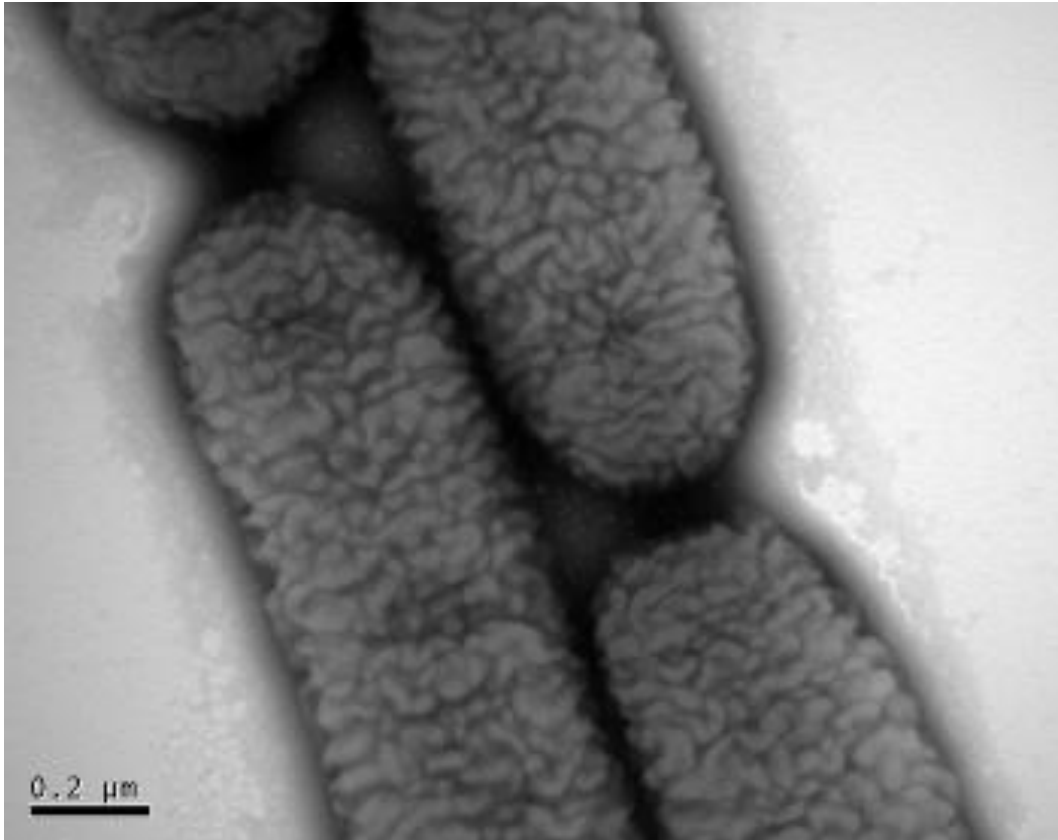
Appendix figure 9 Effect of the concentration of peptone on the expression of ice nucleation activity by *X. translucens*

\*The letters on top of error bars indicated the result of statistical analysis. Different letter indicate significant difference ( $p < 0.05$ ).

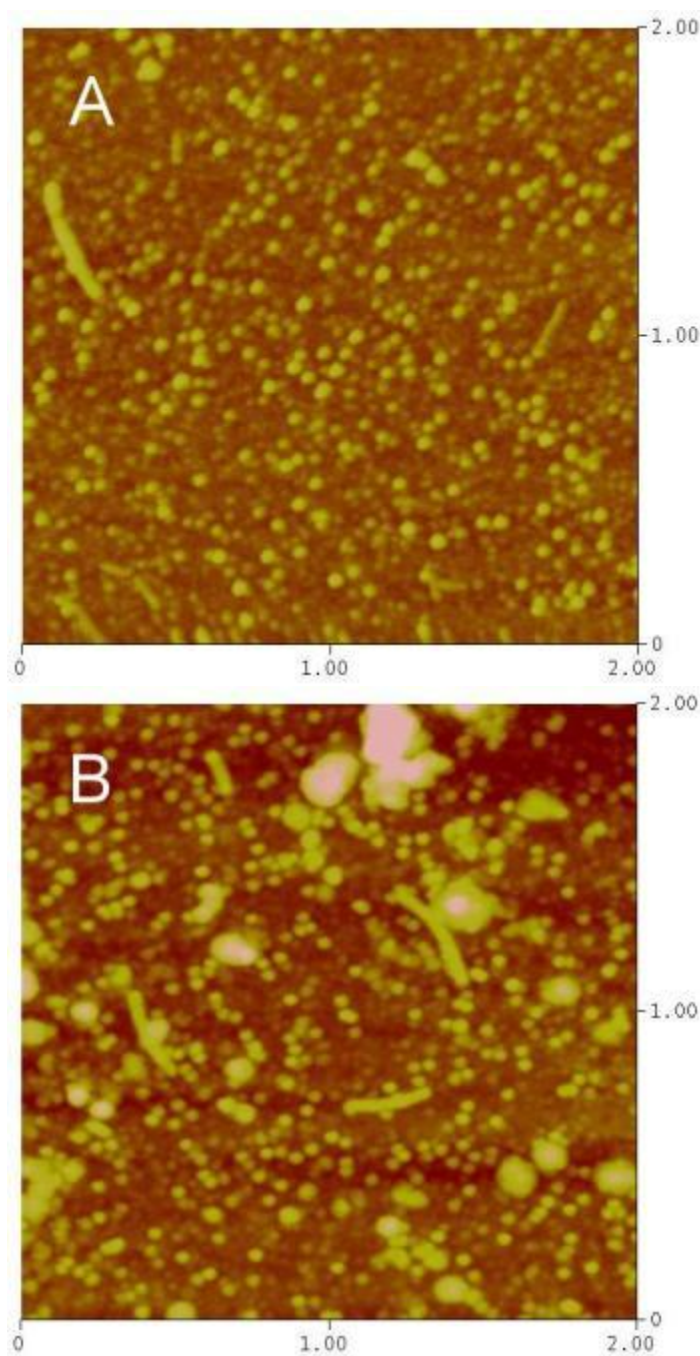
\* Data collected by Jing Deng



Appendix figure 10 Effect of pH on the water solubility of XINs and ECINs



Appendix figure 11 Transmission electron microscopic image of *X. translucens* at early stationary phase



Appendix figure 12 Atomic force microscopic images of (A) ECINs and (B) XINs. Scan size of (A) and (B)



## **APPENDIX C: PURIFICATION OF ECINS USING ICE AFFINITY METHOD**

### **Abstract**

This part of work tried to purify the extracellular ice nucleators (ECINs) from *Erwinia herbicola* using its potential ice affinity properties. The experiment did not increase the specific ice nucleation activity of ECINs but resulted in about 90% loss of the total activity of ECINs. The defects and potential improvement of the method were discussed as a reference for further purification of ECINs.

### **Introduction**

Three models that describe the repeating units forming into repetitive templates with lattice-matching surfaces of ice nucleation proteins are illustrated in Figure 4 in the previous introduction section. Two of models have been proposed by Warren and coworker and the third one by Mizuno (Warren and others 1986; Mizuno, 1989). All these models suggested certain affinity between ice nucleation proteins with ice as previously reviewed.

Anti-freeze protein (AFP) has also been suggested to have high affinity to ice lattice. The ice affinity method was firstly described on purification of AFP by Kuiper et al. (Kuiper and others 2003). The method enrich the content of type III recombinant AFP by 50-fold after one round and obtained pure AFP after second round. Many attempts have been spent on studying the binding of antifreeze proteins to ice since 1990s. And the ice affinity method was based on this unique property. Compare to antifreeze proteins,

there was no experimental data supporting the ice binding properties of ice nucleation proteins (INPs), though it was commonly believed that INPs should also consist of surface that lattice-matching with ice (Hew and Yang, 1992). Besides, the template mechanism is most widely accepted to explain the ice nucleation properties of INPs, which also involves the binding between the proteins and water molecules. So ice affinity method may be a potential way to purify the ice nucleation proteins.

## **Materials and Methods**

The experiment was set up as illustrated in Appendix figure 13. It was adopted from Kuiper's methods with significant modification to accommodate the available instruments in our lab (Kuiper et al., 2003). In Kuiper's method, the hollow cold finger was constructed from brass tubing and silver solder. The temperature of the cold finger was controlled by cycling the water/ethylene glycol 3:1 v/v mixture through the insulated plastic tubes with a temperature programmable water bath (model RTE-111M, NESLAB, Portsmouth, NH). In the current study, a thin-wall glass cold finger was used instead of the brass cold finger and the temperature was initially set to  $-1.0^{\circ}\text{C}$ . Pre-cooled cold finger was lowered into pre-chilled 1 mg/mL ECINs solution. Several pieces of ice from DI water were added into the solution to nucleate the freezing. The temperature in bath was manually lowered gradually while the magnetic stir bar stirring the solutions, instead of using programmable water bath. The growth of ice on the cold finger was monitored and finally stopped when approximately 8 mm thick ice shell had formed. The unfrozen solution was denoted as liquid fraction. The ice fraction was washed with pre-chilled DI water to remove any residual liquid fraction, and then placed in a clean beaker to thaw under  $4^{\circ}\text{C}$ .

## Results and Discussion

Purification of ECINs for the purpose of higher activity usually involved a series of chromatographic experiments. However, the purity was improved always with significant sacrifice of activity recovery (Wolber and others 1986; Obata and others 1998; Duman and others 1984; Muryoi and others 2003). For application purpose, methods which enable large-scale purification with high recovery need to be developed.

Ice affinity method was adopted from purification of AFP to purify ice nucleation proteins. However, the result was not as successful as the case of AFP (Appendix table 8). After 48 hours period, about 25 mL of ice was obtained. Protein concentration and INA were both decreased in the ice fraction compared to original solution, resulting in slightly decreased specific INA which indicated no purification. Around 1/10 of the total INA has been collected into ice fraction.

The ice affinity method was suggested to be efficient and effective on purification of AFP. However, the procedure required several critical parameters to be strictly controlled. Most importantly, temperature controlling needs to make sure the decrease of temperature being as slow as possible, such as a linear decreasing from - 0.5 to - 2 °C over 16 hours, so that the growth of ice could be kept very slow and transparent ice crystal was obtained. This requirement was found to be much more difficult to achieve on INPs than on AFPs, because INPs intrinsically facilitate the ice formation. However, the ice affinity method would still worth another trial in future work on purification of INP if following experimental parameters were met:

- A water bath with programmable temperature control.

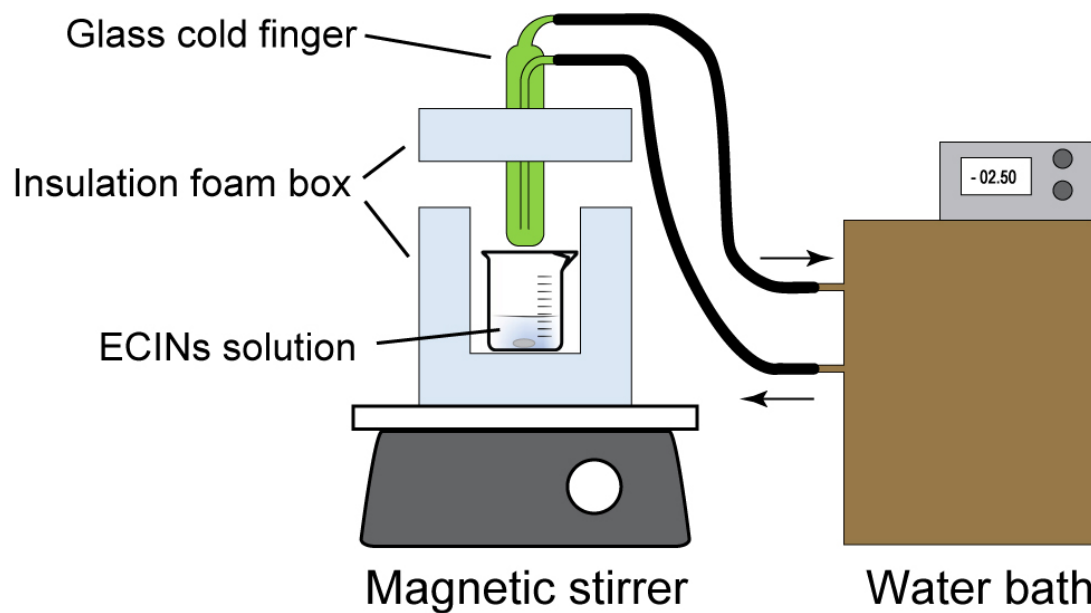
- Tubing, connectors needs good insulation to make sure the low and stable temperature of the cold finger.
- Transparent insulation box instead of foam box for monitoring the growth of ice without opening the box and interrupting the inner temperature.
- Durable metal cold finger, preferably made of brass.

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Appendix table 8 Specific INA of fractions after ice affinity purification

	<b>Specific INA (<math>\times 10^6</math>, units/mg protein)</b>	<b>Total INA recovery</b>
<b>original ECINs solution</b>	6.48	100%
<b>ice fraction</b>	$5.13 \pm 2.41$	8.8%



Appendix figure 13 Cold finger apparatus

## CURRICULUM VITA

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### Education

- Ph.D.** Department of Food Science, Rutgers, the State University of New Jersey, United States, 2012
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### Publication

1. **Shi, K.**; Yu, H. and Lee, T-C, “A novel approach for improving yeast viability and baking quality of frozen dough by adding biogenic ice nucleators from *Erwinia herbicola*”, submitted to *J Cereal Sci*.
2. **Shi, K.**; Yu, H.; Lee, T-C and Huang, Q. R., “Promoting ice nucleation on zein surface through layer-by-layer deposition of extracellular ice nucleators”, submitted to *J Agric Food Chem*.
3. **Shi, K.**; Yu, H.; Lakshmana Rao, S and Lee, T-C, “Improved Mechanical Property and Water Resistance of Zein Films by Plasticization with Tributyl Citrate”, *J Agric Food Chem*, 2012, 60(23), pp. 5988-5993.
4. Yu, H. L.; **Shi, K.**; Liu, D.; Huang, Q. R; “Development of a food-grade organogel with high bioaccessibility and loading of curcuminoids”, *Food Chemistry*, 2012,131(1), pp. 48-54.
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6. Meng, L. M.; Liang, R.; **Shi, K.**; Huang, Q. R.; Shi, T. F.; An, L. J. Synthesis of (PAA-b-F127-b-PAA)- $\beta$ -lactoglobulin Conjugates with Improved Emulsifying Properties. *Chemical Journal of Chinese University*, 2011, 32(11), pp. 2680-2684.
7. Yu, H.; Li, J.; **Shi, K.** and Huang, Q. R., "Structure of modified epsilon-polylysine micelles and their application in improving cellular antioxidant activity of curcuminoids", *Food and Function*, 2011, 2(7), pp. 373-380.
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9. Liu, G., Li, J., **Shi, K.**, Wang, S., Chen, J., Liu, Y. and Huang, Q., “Composition, Secondary Structure, and Self-Assembly of Oat Protein Isolates”, *J Agric Food Chem*, 2009, 57(11), p. 4552-4558
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