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Molecular Mechanisms Involved in Anhydrobiosis of Insect Cells

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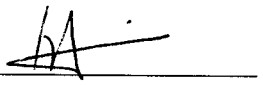

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Molecular Mechanisms Involved in Anhydrobiosis of Insect Cells

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BY

Diyagama Arachchi Ralalage Dilini Sewwandi Samarajeewa

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Molecular Mechanisms Involved in Anhydrobiosis of Insect Cells

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Eastern Illinois University

A Thesis Presented to the Faculty of the Graduate School of Eastern Illinois University in Partial Fulfillment of the Requirements for the Degree of

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Abstract

Animals possessing tolerance to extreme water stress are termed anhydrobiotes. Many desiccation tolerant organisms respond to water stress by intracellular accumulation of selected sugars such as trehalose and larger macromolecules such as Late Embryogenesis Abundant (LEA) proteins and thereby maintain the cell viability. Evidence indicates that the presence of trehalose and Late Embryogenesis Abundant (LEA) proteins may work synergistically to confer cellular protection during drying in eukaryotic cells. We evaluated any increase in cellular desiccation tolerance by expressing different LEA proteins in a non-desiccation tolerant insect cell line *Drosophila melanogaster (*fruit fly) (Kc167 cells) in the presence of trehalose.

Transgenic group I LEA protein expressing cells (Kc167- LEA 1.1 and Kc167- LEA 1.3) and group V LEA protein expressing cells (Kc167- LEA 5) were used for convective droplet drying experiments. Experiments were carried out with and without 200 mM extracellular trehalose. Statistical analysis demonstrated that there was no significant difference (ANCOVA: F1, 171.13 = 37.99, p =0.5217, r^2 = 0.76) between the amount of viable cells after desiccation of Kc167 control and LEA 1.3 expressing cells in the absence of extracellular trehalose. Interestingly, After adding extracellular trehalose to the culture medium, Kc167-LEA 1.3 cells showed significant increase in viability after desiccation compared to the control cells (ANCOVA: F1, 182.06 = 39.20, $p < 0.0001$, $r^2 = 0.79$). Similarly, Kc167-LEA 1.1 showed a significant increase in the viable cells compared to the control Kc167 cells after desiccation. (ANCOVA: F1, 125.03= 41.04, $p < 0.0001$, $r^2 = 0.80$). Transgenic expression of LEA 5 protein also showed significantly increased cell viability of

Kc167 cells during droplet drying in presence of 200 mM trehalose compared to control Kc167 cells (ANCOVA: F1, 153.10 = 39.24, $p < 0.0001$, $r^2 = 0.77$).

To investigate the properties of LEA protein on protection of macromolecular proteins such as enzymes at sub-freezing temperatures, lactate dehydrogenase enzyme assay was carried out. Purified LEA 1.1 protein (0.050 mg/ml) was able to protect lactate dehydrogenase enzyme activity 10 h after freezing at -20°C ($n = 3$, ±SE, $P < 0.05$). Surprisingly, cryomicroscope results have showed that addition of 0.050 mg/ml LEA 1.1 protein into 40 mM HEPES buffer have reduced the freezing temperature of 40 mM HEPES from -14.4°C to -17.3°C changing the ice-crystal shape from rectangular to pentagonal shape. Moreover, protein foldIndex of Kyte-Doolittle plot was performed for group I LEA proteins (LEA 1.1 and LEA 1.3) and showed that both these proteins are unfolded at its native stage while the LEA 5 protein is likely folded protein in aqueous solution.

These results suggest that trehalose and late embryogenesis abundant (LEA) proteins work synergistically to confer cellular protection during insect cell desiccation enlightening the potential of insect cell storage without cryopreservation. Moreover, LEA proteins can be utilized as a potential cryoprotecting agent to minimize the freezing damage of biomolecules that has to be cryopreserved.

Dedication

To My Parents, My Brother and My Cat (Bo)

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I would like to express my deep gratitude to my advisor, Dr. Michael Menze for allowing me the opportunity to work in his lab. His efforts of teaching and training me to become a better scientist are invaluable and will be forever grateful for his endless encouragement and support given me to be strong to get through the long days of my life. I would like extend my gratitude to my committee members Dr. Gary Bulla and Dr. Janice Coons for their support, advisement and service given throughout this study. I would also like to offer my sincere thanks to our collaborator, Dr. Nilay Chakraborty, for his contributions, encouragement and for allowing me the opportunity to work in his lab. Thanks are also extended to Dr. Mary Konkle for the great support given throughout the protein purification studies. Finally, I extend my appreciation to the Eastern Illinois Graduate School and Biological Sciences faculty, for providing me a productive learning environment as well as the support given throughout this study.

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I. Introduction

A. Hyperosmotic Stress

In a typical cell, water is distributed between the cytoplasm and intracellular compartments. Major portions of the free water in the cell can be removed by air drying of cells. In anhydrobiotic animals such as cysts from the brine shrimp *Artemia franciscana,* and in some plant seeds, the cellular water content can be as low as 0.02g H_2O · g dw (dry weight)$^{-1}$ under extreme conditions of desiccation (Vertucci and Leopold 1986; Clegg 1978). The process of water removal can occur at a rapid rate or at a slow rate. Cells can undergo moderate or extreme water deficiencies depending on the quantity of water that has been removed. During desiccation, cells lose their intracellular water to the extracellular fluid due to the movement of water from low solute concentration to high solute concentration. This net flux of water leads to increase solute concentration inside the cell. Under severe conditions of hyperosmotic stress, damages to the cell can occur. With the removal of water cells start to shrink and macromolecules such as proteins may become more 'twisted' in the cytoplasm (Garner and Burg 1994). Ultimately, mitochondrial dysfunction leading to low ATP production, disorganization of macromolecules, and degradative enzymatic reactions take place in the cell generating reactive oxygen species. These oxidizing agents are highly reactive in nature and can cause damage to DNA, protein, and lipids. If protective mechanisms to control the generation of reactive oxygen species are lacking, the cell will be severely damaged (Greenberg 2004; França et al., 2007).

Cellular damages and dysfunctions can lead to the activation of cell death pathways or can cause necrosis (Michea et al., 2002; Copp et al., 2005). Apoptosis is an ATP dependent form of a cellular death pathway which is initiated by cell signaling cascades that are governed by specific enzymes (Elmore 2007). Cell

shrinkage is the initial step of apoptosis, followed by chromatin condensation, DNA fragmentation, and packaging of cellular components into small vesicles (Jin and El Diery 2005). Moreover, necrosis can be caused by extreme stressful conditions and is an 'accidental' type of cell death. Thus, necrosis normally occurs within a short period time and the degree of damage depends on the strength of the stress or insult. Necrosis is not energy dependent and does not result in the formation and distribution of small vesicles with intracellular contents (Elmore 2007).

In anhydrobiotic organisms, accumulation of trehalose or sucrose in excess of 20% of dry weight was recorded during desiccation (Crowe et al., 1992). Furthermore, it has been found that trehalose contains some antioxidant properties and it is capable of direct scavenging reactive oxygen species such as hydrogen peroxide (Jamieson 1998; Béranger et al., 2008; Luo et al., 2008). Moreover, upregulation of antioxidant enzymes such as glutathione peroxidase and reductase, superoxide dismutase, catalase, and thioredoxin is prominent during dehydration in anhydrobiotic organisms and these antioxidant enzymes degrade the reactive oxygen species into less reactive forms, or convert them into substrates for other enzymes in order to protect the cell from degradation (Pereira et al., 2003; Browne et al., 2004; França et al., 2007; Cornette et al., 2010; Rizzo et al., 2010).

B. Anhydrobiosis

Water is a vital component to maintain metabolically active life. In invertebrates it can make up to 90-95% of the molecules present in the organism (Edney 1977; Hadley 1994). Desiccation is a process of water loss which may lead to severe damage of cellular structures and organelles resulting in cell death. Many organisms representing a wide variety of taxonomical groups have the ability to

survive conditions of extreme desiccation and are known as anhydrobiotic organisms (Crowe and Crowe 1992). The most common environmental forces that can create severe water stress are sub-freezing temperatures, xeric climates, and osmotic variations in aqueous habitats (Yancey 2005; Yancey et al., 1982). In order to withstand cell dehydration under these extreme environmental conditions, anhydrobiotic organisms tend to develop protective mechanisms at cellular and molecular levels. However, when cells of non-anhydrobiotic organisms are subjected to dehydration, massive damage to their organelles and membranes due to the negative effects of water deprivation can be observed (Franca et al., 2007; Simonin et al., 2007; Hengherr et al., 2008). The residual water content of anhydrobiotic animals after desiccation for long periods of time is the amount of tightly bound water and can approach extremely low values (Clegg et al., 1978). In nature, anhydrobiotic organisms tolerate extreme desiccation and possess the ability to survive at cellular water contents around 0.02 – 0.05 g $H_2O \cdot g^{-1}$dry mass by entering into a reversible state that approaches a suspended metabolism (Clegg 1973; Crowe and Madin, 1974, 1975; Hinton 1968).

Most anhydrobiotic organisms can withstand almost complete dehydration with undetectably low metabolic activity levels. Therefore, once these organisms undergo anhydrobiosis, they do not show any signs of life. Upon rehydration, they resume their metabolic activity and maintain the competency of reproduction and development (Wright 2001; Clegg 2005; Cornette and Kikawada 2011). Therefore, it is reasonable to assume that water loss during dehydration might trigger the activation of cellular and molecular survival strategies in these anhydrobiotic organisms. The rate of water loss during dehydration is determined by environmental conditions which the organism is exposed to. However, without accumulation of important

protective molecules such as trehalose and supportive proteins cell degradation occurs during dehydration. If the removal of water molecules occurs too quickly there is no time to accumulate protective molecules and if water loss occurs too slowly degradative enzymatic reactions can lead to the cell death before the cell enters in to a stable condition at dehydration (Cornette and Kikawada 2011).

During dehydration, anhydrobiotic organisms tend to control the rate of intracellular water loss in order to successfully enter anhydrobiosis. Some organisms such as tardigrades change their body form into a small coiled shape to minimize the exposed surface area of the body and to reduce the rate of water loss during dehydration (Wright et al., 1992). Moreover, some anhydrobiotic nematodes accumulate large amounts of macromolecules within a short period of time when the environmental water losing rate is very high. (Perry 1977; Westh and Ramlov 1991). The larva of the chironomid *P. vanderplanki* lives inside a nest made by a combination of saliva and mud particles. It facilitates a reduction in the rate of water loss during dehydration and in other words it takes more time to reduce the body water content. The extended time allows the larvae to accumulate macromolecules required to withstand the dehydration stress before entering into an anhydrobiotic stage. Upon rehydration the anhydrobiotic larvae recover completely from the desiccation event (Kikawada et al., 2005).

Furthermore, the rate of water loss can be modulated by the expression levels of water-channel proteins called aquaporines. It has been found that in *P. vanderplanki,* selective expression of aquaporin protein 1 (PvAQP1, DDBJ accession no. AB281619) is responsible for the removal of body water in controlled manner facilitating successful induction of anhydrobiosis in *P. vanderplanki* (Kikawada et al., 2008). Moreover, in plant leaves such as *Graptopetalum paraguayense*, expression

level of aquaporins remains at a very low level during desiccation. This suggests that the reduced amount of aquaporins helps to reduce water loss of the leaf by maintaining a low water permeability of the tonoplast and plasma membrane of the leaf (Ohshima et al., 2001).

C. Molecular Mechanisms in Anhydrobiosis

In order to survive in the anhydrobiotic state, two different stresses have to be successfully tolerated by the organism. One is the potential damage that occurs to the organism during the desiccation phase and the other is the stress that occurs upon rehydration. Therefore, it is important to not only protect biological structures and molecules in the dry state but also during subsequent rehydration (Franca et al., 2007; Clegg 2007). Anhydrobiosis is a survival strategy that has evolved independently among different organisms representing animals and plants. The most commonly available examples in plants are seeds or pollen grains. Bdelloid rotifers, round worms, nematodes, tardigrades, cysts of the crustacean *Artemia franciscana*, and larva of the insect *Polypedilum vanderplanki* are examples for highly desiccation tolerant animals. Most of these organisms use similar strategies to control and tolerate hyperosmotic stress during the early stages of drying as well as to stabilize their tissues during severe desiccation. These strategies include accumulation of disaccharides such as the sugar trehalose (Albertorio et al., 2007) and sucrose during drying (Koster and Leopold 1988; Hoekstra et al., 1992). Accumulation of proteins such as heat shock and late embryogenesis abundant (LEA) proteins occur concurrently to stabilize cells and tissues upon desiccation (Liang and MacRae 1999; Jönsson and Schill 2007; Gusev et al., 2011; Hand et al., 2011). During desiccation, the accumulation of macromolecules such as proteins and disaccharides such as

trehalose greatly increase the cytoplasmic viscosity of the cell and precedes the formation of bioglasses. Formations of bioglasses have been suggested to provide intracellular protection in order to prevent the denaturation of larger molecules such as the components of the plasma membrane (Burke 1986).

1.Trehalose

Accumulation of trehalose in cells and tissues is one of the major anhydrobiosis mechanisms and the level of accumulation varies among anhydrobiotic organisms. In nematode *Aphelenchus avenae* and the anhydrobiotic midge *Polypedilum vanderplanki* trehalose accumulates up to 15 %, and cysts of *Artemia fransciscana* trehalose accumulates up to 20% of their dry weight. But some species of tardigrades accumulates only around 0% - 2.3% trehalose per dry weight (Madin and Crowe 1975; Westh and Ramlov 1991; Clegg 2005; Hengherr et al., 2008; Jonsson and Persson 2010; Cornette and Kikawada 2011). Moreover, vitrification or the formation of glassy structures of trehalose has been identified as a necessary requirement for cellular preservation in some anhydrobiotic organisms. However, trehalose is not required in all anhydrobiotic organisms to be preserved in a dry state (Crowe et al., 1998; Sakurai et al., 2008). Trehalose is composed of two glucose molecules bonded by α-α 1-1 glycolytic linkage. As glucose molecules are linked by a non-reducible bond the sugar does not involve in oxidation-reduction reactions with other reactive ions. Moreover, due to this special chemical bond, the two glucose molecules form a clamshell structure and the molecule has an equal number of hydrogen binding sites on either side. This specific structure facilitates hydrogen bond formation with other macromolecules such as proteins and head groups of phospholipid bilayers (Albertorio et al., 2007). Furthermore, trehalose forms an

amorphous non-crystalline sugar glass during vitrification with highly reduced molecular mobility and chemical reaction rates. As trehalose has the highest glass transition temperature among all known disaccharides, it allows the sugar to successfully form a protective glass at warm temperatures. This glassy structure protects the cell and its organelles by minimizing the molecular mobility rate as well as the chemical reaction rates during dehydration (Crowe et al., 1998; Albertorio et al., 2007). In insects such as *P. vanderplanki*, trehalose production occurs mainly in fat body cells and the sugar is released into the hemolymph and distributed throughout the body (Wyatt 1967). Trehalose synthesis process is triggered by an increase in the ionic strength of the cell (Watanabe et al., 2003).

However, the molecular mechanism by which trehalose is responsible for the protection of cells is still not fully resolved and the exact function(s) may depend on the hydration status of macromolecules and trehalose during dehydration. Trehalose may act differently at different hydration levels. At low water levels trehalose may function by sequestering water molecules, compacting proteins, and forming hydrogen bonds with other cellular macromolecules. When the cell is further dehydrated vitrification may stabilize cellular macromolecules and prevents cellular degradation. The phospholipid bilayer of the cell membrane serves as an impermeable membrane to most water-soluble molecules, such as ions, amino acids and macromolecules including carbohydrates such as trehalose (Lodish et al., 2001). Hence, in *P. vanderplanki*, trehalose is transported across the plasma membrane using the trehalose transporter TRET1 (Kikawada et al., 2007). Some cells such as mammalian cells lack endogenous trehalose transporters (Crowe et al., 2005). Therefore transgenic expression of trehalose transporters might be helpful to increase the concentration of intracellular trehalose and thereby cellular protection during

dehydration in cells from these animals. (Kikawada et al., 2007; Chakraborty and Chang, et al., 2011; Chakraborty et al., 2012).

2. Proteins Involved in Anhydrobiosis

In recent years, considerable emphasis has been placed on the role of disaccharides such as trehalose (animals) and sucrose (plants) in the molecular mechanisms governing anhydrobiosis. However, it has become clear that the presence of sugars alone is not sufficient to confer anhydrobiosis (Hoekstra et al., 2001). Indeed, some anhydrobiotic organisms do not accumulate sugars at all during desiccation (Tunnacliffe and Lapinski, 2003). Therefore, more attention has been given to the role of other supportive proteins such as heat shock proteins and late embryogenesis abundant proteins (LEA) during desiccation (Goyal et al., 2005 a).

a. Heat Shock Proteins

Molecular chaperones are recognized as a class of proteins that bind to hydrophobic areas of non-native proteins and release them in a highly regulated manner. This action minimizes the probability of forming cytotoxic protein aggregates. One of the best known examples for chaperone proteins is the heat shock proteins (HSPs). HSPs are abundant in cells during physiologically stressful conditions (Feder and Hofmann 1999). In general, HSPs are a highly conserved group of proteins found in all kingdoms of life. They are grouped based on their molecular weight such as Hsp 10, Hsp 40, Hsp 60, Hsp 70 etc., (Li and Srivastava 2004). Laboratory experiments at high temperatures have highlighted the changes in Hsp 70 protein expression and tolerance to elevated temperature in *Drosophila melanogaster* (Bettencourt et al., 1999). Moreover, production of Hsp70 in tardigrades can be

triggered by cell dehydration (Jönsson and Schill 2007). The heat shock protein 26 was found to be upregulated in the nucleus of *Artemia franciscana* cysts during diapauses (Liang et al., 1997). In *P. vanderplanki* larva, a large increase in the levels of several heat shock proteins was observed during dehydration. These increased Hsp levels might help to increase vitrification by structuring disaccharide sugars such as trehalose during dehydration (Gusev et al., 2011).

b. Late Embryogenesis Abundant (LEA) Proteins

Late embryogenesis abundant (LEA) proteins were first discovered in mature wheat (*Triticum aestivum*) and cotton (*Gossypium hirsutum*) embryos (Cuming and Lane 1979; Dure et al., 1981). Over the past 30 years research findings have shown that LEA proteins are not restricted to plants and they are present in animal phyla as well (Tunnacliffe and Wise 2007; Shih et al., 2008; Hoekstra et al., 2001; Dure 2001). In general, LEA proteins have been classified into six different groups based on amino acid sequence and conserved motifs. Members of Group I and III have been found in arthropods while group III homologous have also been reported in nematodes such as *C. elegans*, *Steinernema feltiae* and *Aphelenchus avenae*, and the prokaryotes *Deinococcus radiodurans*, *Bacillus subtilis* and *Haemophilus influenzae* (Solomon et al., 2000; Browne et al., 2002; Goyal 2003).

According to the structural features of LEA proteins, Group I and Group II LEA proteins have considerably low sequence complexity. In contrast, they appeared to be structurally disordered in a solution (Eom et al., 1996; Soulages et al., 2002, 2003). For example, the results of differential scanning calorimetry experiments have reported that a Group I LEA protein found in soybean does not show detectable unfolding transition upon the exposure to high temperature (Russouw et al., 1997). In

addition, NMR (Nuclear magnetic resonance) spectroscopy experiments showed that a recombinant dehydrin-related LEA (DSP16) protein from the resurrection plant (*C. plantagineum*) seems to have no defined three dimensional structures in its native state (Lisse et al., 1996). However, At1g01470.1 from *A. thaliana* which is classified as a "LEA14 protein" a class of LEA proteins found in many plant species, exhibit a stable three dimensional structure in solution (Singh et al., 2005). Moreover, Fourier transform IR (FT-IR) spectroscopy results have highlighted that a LEA group III family proteins found in *Typha latifolia* pollen grain is a highly disordered protein in solution showing only random coil conformation but the conformation changes into a α-helical structure after fast drying. However, when the protein is dried at a slower rate it showed the reversible formation of both α-helical and intermolecular extended β-sheet structures (Wolkers et al., 2001).

Among the proposed functions of LEA proteins, some were shown to stabilize sugar glasses during dehydration (Wolkers et al., 2001), and prevent protein aggregation (Goyal et al., 2005 b). Furthermore, LEA proteins can act as cryoprotectants (Bravo et al., 2003; Kazuoka and Oeda, 1994; Houde et al., 1995). In addition, some LEA proteins are able to bind to lipid vesicles (Koag et al., 2003) or actin filaments (Abu-Abied et al., 2006). However, the precise molecular mechanisms responsible for the specific functions of different LEA proteins are unknown. Indeed, there are number of proposed mechanisms such as stabilization of membrane and macromolecules, water replacement, and ion sequestration (Cuming, 1999; Close, 1996).

i. Group I LEA Proteins

Group I LEA proteins have been isolated from various plants including wheat. All proteins are rich in charged amino acids (40%). The average molecular weight of group I LEA proteins is around 11.5 kDa (maximum 20.3, minimum 6.8). In this group, most proteins are acidic in nature and unstructured in aqueous solution. Previous research findings have divided group I LEA proteins into two subgroups according to the number of hydrophilic 20-mer repeats with conserved sequence [(R/G) S(R/K) GGQTRKEQLGXEGTXEM] near the carboxyl terminus (Espelund et al., 1995; Gaubier et al., 1993). For example, *Arabidopsis* expresses two genes: AtEm1, which contained four repeats of a 20-mer motif, and AtEm6, with only one 20-mer motif (Gaubier et al., 1993). Recently a group I LEA protein was found in dehydrated cysts of *A. franciscana* (Wang et al., 2007).

ii. Group III LEA Proteins

Group III LEA proteins have been found in plants, fungi, bacteria as well as in animals (Cuming 1999; Tunnacliffe and Wise 2007; Dure 2001). The average molecular weight of LEA III proteins is 25.5 kDa (maximum 67.2, minimum 7.2). The specific feature found in most LEA III proteins is a tandem-repeating 11-mer sequence. These 11-mer motifs had been proposed to be linked by ionic bridges (Dure et al., 1989). Some LEA III proteins contain the subsequent duplication and divergence of the 22-mer sequence, which gives rise to additional copies of the 11-mer sequence. A large variation in molecular weight among LEA III proteins is caused by the number of tandem repeats, which can range from 5 to more than 30. LEA III proteins were first observed in plant seeds and then found in organisms such as *Caenorhabditis elegans* and the eubacteria *Haemophilus influenzae* and

Dienococcus radiodurans. Although the molecular function of these motif has yet been uncovered the proteins carrying 11-mer motif regions seems to play a role in plant abiotic stress tolerance (Dure 2001).

iii. Structural Changes in LEA Proteins during Dehydration

In general, proteins have a well defined native structure in the hydrated state and tend to denature during desiccation. The secondary structures of several LEA proteins have been determined by circular dichroism (CD), nuclear magnetic resonance (NMR), or Fourier transform IR (FTIR) spectroscopy. Structurally, several LEA proteins were found to be largely unfolded in solution (Eom et al., 1996; Goyal et al., 2003; Soulages et al., 2002; Mouillon et al., 2006; Lisse et al., 1996) and some were shown to undergo transition to a more folded state upon dehydration (Goyal 2003; Wolkers et al., 2001; Shih et al., 2004). The structural behaviors of LEA proteins in solution and upon dehydration highlights that LEA proteins are different from a typical protein with a defined native structure. Moreover, some studies have predicted that LEA proteins adopt large amounts of defined secondary structures such as α-helix or β-sheet upon dehydration (Singh et al., 2005; Goyal et al., 2003). For example, group III LEA protein found in *Typha latifolia* pollen grain is highly disordered in solution showing a random coil conformation and changes the conformation into α-helical structure after fast drying (Wolkers et al., 2001). Similarly, in *Aphelenchus avenae* Aav-LEA1, a protein similar to group III LEA proteins seem to be natively unfolded in aqueous solution. However, FT-IR spectroscopic analysis results have showed that the protein became more folded, forming α -helical component upon dehydration (Goyal et al., 2003). To support these predictions, a study carried out using biochemical and spectroscopic analyses have

demonstrated that a LEA protein found in pea seed mitochondria (LEAM) is intrinsically disordered in solution thus is a genuine natively unfolded protein (Tolleter et al., 2007). Moreover, LEAM has been demonstrated to adopt an essentially α-helical structure upon air-drying (Li and He 2009). Most of the described functions for other natively unfolded proteins concern regulatory mechanisms that take advantage of induced conformational changes (Wright and Dyson 1999; Dunker et al., 2001).

iv. Mitochondrial Membrane Protection Role of LEA Proteins

Mitochondria are the main energy generating organelle in a healthy cell and it can be expected that the organelle is highly protected and structurally conserved during stressful conditions such as desiccation due to the fact that its functional failure would ultimately lead to cell death (Tolleter et al., 2007). Many stressful stimuli are capable of initiating apoptosis via the mitochondrial pathway by releasing pro-apoptotic proteins into the cytoplasm (Gulbins et al., 2003). The most prominent pro-apoptotic protein stored in mitochondria is cytochrome c (Liu et al., 1996). In general, the inner mitochondrial membrane is more or less impermeable to all ions including protons and other molecules such as proteins in a healthy cell (Kroemer et al., 2007). Many apoptotic signals, including reactive oxygen species, can induce the release of pro-apoptotic factors into the cytoplasm and initiate apoptosis (Gulbins et al., 2003; Kroemer et al., 2007). Therefore, protection of the inner and outer mitochondrial membrane by mitochondrial targeted LEA proteins (LEAM) could be crucial to confer tolerance to hyperosmotic stress. A study has been reported that LEAM protein is localized in the matrix space of mitochondria in pea seeds and the protein starts to accumulate just before the seed undergoes dehydration (Grelet et al., 2005).

Furthermore, studies of LEAM used for liposome assays (artificial constructed lipid bilayer similar to cell membrane) have reported that LEAM interacts with the liposome lipid bilayer and protects liposomes by maintaining its membrane integrity when subjected to drying (Tolleter et al., 2007). Moreover, both CD and FTIR spectroscopy analysis results have demonstrated that LEAM is capable of folding into α -helical structure upon desiccation. Altogether, considering the structural and biochemical properties of LEAM these data indicate strong evidence to highlight that LEAM protects the inner mitochondrial membrane during desiccation.

Considering all these facts, a deeper knowledge about the mechanisms underlying desiccation tolerance might offer valuable avenues in many areas of science. For example, bioengineered insect cells are widely used for the production of recombinant proteins such as insulin, human growth factor, erythropoietin, and interferon with an market value of over 53 billion dollars in 2010 (Pavlou 2004). Although cryopreservation is used as the long term storage method of these cells, any temperature fluctuation can decrease the cell viability causing storage and transportation difficulties. Engineering insect cells to be desiccation (severe dehydration) tolerant would negate many of these problems. Indeed, better understanding of the principles underlying desiccation tolerance in nature will be the next step towards an economical option for commercial and educational storage of insect cell lines. In order to full fill this requirement, following objectives will be tested.

D. Objectives of the Thesis

The primary objective of this thesis was evaluate any increase in cellular desiccation tolerance by expressing different LEA proteins in a non-desiccation tolerant insect cell line *Drosophila melanogaster* (fruit fly) (Kc167 cells) in the presence of trehalose.

In order to gain a deeper knowledge about the role of late embryogenesis abundant proteins during desiccation following objectives will be utilized.

1. To investigate the properties of LEA protein on the protection of macromolecular proteins such as enzymes at sub-freezing temperatures
2. To elucidate the behavior of a solution during freezing in the presence of LEA protein
3. To utilize bioinformatics technique to exploit the native structure of different LEA proteins

II. Materials and Methods

1. Kc167 Cell Maintenance

Kc167 (*Drosophila melanogaster*) cells were obtained from the Drosophila Genomics Resource Center (DGRC, Bloomington, IN). Cell culturing was carried out in 75 cm^2 cell culture flasks (Corning Corporation, Lowell, MA). Cells were cultured in BPYE composed of Shields and Sang M3 insect medium (M3+BPYE) (Sigma-Aldrich, St. Louise, MO) supplemented with 5 % fetal bovine serum (Atlanta Biologics, Lawrenceville, GA), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 25 µg/ml amphotericin B (MP Biomedicals, Solon, OH), 0.5 g/l potassium bicarbonate, 1 g/l yeast extract (Sigma-Aldrich, St. Louise, MO) and 2.5 g/l trypticase peptone (BD Biosciences, San Jose, CA) adjusted to pH 6.6 with 1M KOH. Cells were grown at 26 ^{o}C to a maximum density of 20 million cells/ml and sub-culturing was carried out starting with 1.5 million cells/ml every 3-4 days. Cell counting was done using a hemocytometer (Hauser and Son, Philadelphia, PN).

1.1 Subcloning of LEA Genes

The cDNA encoding for a group I LEA protein from *A. franciscana* was amplified by Polymerase Chain Reaction (PCR) using primers based on a previously published nucleotide sequence (Sharon et al., 2009) (ABR67402.1). The primer sequences used to perform PCR were 5'-CACCATGGAACTGTCGTCGAGTAAG-3' and 5'-TTTCTGTCTTGCGAGACCTCC-3'. Purified PCR product was cloned into the pENTR/D-TOPO cloning vector (Invitrogen Corporation, Carlsbad, CA) according to the instructions provided by the manufacturer. Several clones were isolated and sequenced after cloning. Sequencing was carried out using BigDye terminator chemistry and an ABI PRISM 3100 Genetic Analyzer (Applied

Biosystems, Foster City, CA). Primer walking was used to insure full length sequence which was obtained. Sequences were assembled using Sequencher software (Gene Codes Co., Ann Arbor, MI) and two unique clones were obtained encoding for proteins of 180 (LEA 1.1) and 197 amino acids (LEA 1.3) respectively. Both sequences were subcloned into pMT-DEST-48 destination vector separately by following the protocol given for clonase technology by the supplier (Invitrogen Corporation, Carlsbad, CA). Constructed pMT-DEST-48-LEA 1.1 and pMT-DEST-48-LEA 1.3 plasmids were used for the generation of LEA protein expressing Kc167 cells.

1.2 Generation of LEA Expressing Kc167 Cells

Kc167 cells were transfected using the subcloned plasmid vector pMT-DEST-48 containing LEA 1.3 and LEA 1.1 gene sequences. Transfection was carried out using 1 µg of plasmid DNA in 240 µl of Grace's Medium and 18 µl of Cellfectin (Invitrogen Corporation, Carlsbad, CA). After pre-incubation of the transfection mixture for 15 min at room temperature, cells were pelleted and resuspended in antibiotic and serum-free M3+BPYE medium and plated in 6-well plates (Corning Incorporated, Corning, NY).

After 24 h, the medium was exchanged with M3+BPYE medium and selective antibiotics were added after 48 h. Blasticidine 50 µg/ml (Invitrogen Corporation, Carlsbad, CA) was added to select stably transfected cells containing the pMT-DEST-48 vector. Antibiotic resistant cell lines were obtained after 2–4 weeks of selection (Kc167-LEA 1.3 and Kc167-LEA 1.1). The same experimental procedure was followed to clone a group V LEA protein gene (Menze unpublished data) into the vector pMT-DEST-48 (Invitrogen Corporation, Carlsbad, CA) and the obtained

construct was transfected into Kc167 cells. Antibiotic resistant cell lines (Kc167-LEA 5) were obtained after three weeks of selection in Blasticidine 50 μg/ml.

1.3 Western Blotting

A Western blot was performed to confirm the successful transfection of the pMT-DEST-48-LEA construct into Kc167 cells. About 10 million Kc167 (control) and Kc167-LEA 1.3 expressing cells were pelleted at 1000 g for 10 min and resuspended in 200 μl of sample buffer (2% SDS, 25% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue, and 62.5 mM Tris. HCl, pH 6.8). Samples were denatured at 95°C for 5 min and 15–20 μl of the mixture was loaded per lane on a 10% precast polyacrylamide Mini-PROTEAN TGX gel (Bio-Rad laboratories, Hercules, CA). Samples containing cellular proteins were run for 30 min at 200 V in a Bio-Rad Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane (0.2 μm) using a Bio-Rad Mini Trans-Blot apparatus in a transfer buffer containing 192 mM glycine, 20% methanol, 0.025% SDS, and 25 mM Tris. The transfer was performed for 30 min at 100 V. Membranes were stained with Ponceau S staining solution (Sigma-Aldrich, St. Louise, MO) to confirm transfer of the proteins into the membrane.

The nitrocellulose membrane containing the transferred proteins was incubated in the blocking buffer (5% fat free dry milk in TBS-T (0.1% Tween 20, 20 mM Tris.HCl, 500 mM NaCl, pH 7.4) for 1h at room temperature and washed with 1X TBS and 0.1% Tween-20 (TBS-T). Mouse anti-V5 (ab27671; Abcam, Cambridge, MA) was used as the primary antibody at 1:5000 dilution and membrane was incubated on a rocking platform overnight with the primary antibody in 5% BSA

(bovine serum albumin) containing TBS-T at 4°C. Following the incubation, the membrane was washed with TBS-T. HRP-labeled goat anti-mouse IgG2a (ab97245, Abcam, Cambridge, MA) was used as secondary antibody at a dilution of 1:20,000 and incubated on a rocking platform at room temperature for 1 h. After washing the membrane with TBS-T, proteins were visualized with LumiGlo by following the protocol of the manufacturer (Cell Signaling Technology, Boston, MA) and recorded on Hyper-film ECL (GE Healthcare, Piscataway, NJ) in a dark room.

2. Convective Droplet Drying

In order to investigate desiccation tolerance of Kc167 and Kc167-LEA protein expressing cells (Kc167- LEA 1.1, Kc167- LEA 1.3 and Kc167- LEA 5), droplet-drying experiments were conducted in the presence of 200 mM trehalose. Cells were pelleted by centrifugation at 1000g for 10 min and re-suspended in M3+BPYE medium containing 200 mM trehalose. Cells were washed once with trehalose containing medium, pelleted again and resuspended at a concentration of 20–25 million cells/ml. Ten droplets of 15 µl of the cell suspension were pipetted onto 35 mm tissue culture dishes and placed in a desiccator cabinet at ambient temperature (22–24°C) containing Drierite (W.A. Hammond Drierite Co., Xenia, OH). Samples were dried to a range of final moisture contents determined gravimetrically. Final water contents were expressed as grams water per grams of dry mass (g H_20/ g DW). After desiccation to the desired moister contents, samples were immediately rehydrated with excess M3+BYPE and cultured for 24 h at 26°C. Membrane integrity was determined by trypan blue exclusion assay (BD Biosciences, San Jose, CA). In order to quantify cell survival, cell counts after drying and recovery were normalized to cell counts obtained for samples that were treated identically but never dried. In

addition sample dry mass was determined by baking parallel samples for 24 h at 60°C. Furthermore, in some experiments convective droplet drying was carried out without adding any extracellular trehalose.

3. Induction of LEA Protein Expression in Bacteria

3.1 Transformation of LEA 1.1 Gene into Bacteria

The pENTR/D-TOPO vector constructed (refer to methods section 1.1) with the LEA 1.1 gene was transformed into One Shot Chemically Competent *E.coli* cells. Transformed bacteria were grown on 50 μg /ml kanamicin containing luria bertani (LB) plates and successfully transformed colonies were used for plasmid purification. Purified pENTR/D-TOPO plasmid was subcloned into the destination vector pET-DEST-42 using LR Clonase II enzyme mix (Invitrogen Corporation, Carlsbad, CA). Subcloned plasmid mixture was transformed into BL32 One Shot Chemically Competent *E.coli* strain following the manufacture protocol. Ampicillin containing plates (100 μg/ml) were used to select bacteria containing pET DEST-42-LEA 1.1. Successful insertion of the gene was confirmed by PCR.

3.2 PCR Confirmation of pET DEST-42-LEA 1.1

Transformed bacteria were grown on LB plates supplemented with 100 μg/ml ampicillin. Successfully transformed bacterial colonies were randomly selected after incubating the plates at 37°C for 24 h and plasmid purification was performed using S.N.A.P. MidiPrep Kit (Invitrogen Corporation, Carlsbad, CA). PCR amplification was carried out in a reaction volume of 25 μl containing 10-25 ng of plasmid DNA, 10 mM $_{d}$NTPs, 5x phusion HF buffer and HF phusion enzyme (New England Biolabs Ipswich, MA). The primer sequences used to perform PCR were 5'-

CACCATGGAACTGTCGTCGAGTAAG-3' and 5'-TTTCTGTCTTGCGAGACCTCC-3'. The PCR program consisted of an initial denaturation step at 98 °C for 30 seconds followed by 25 cycles of 10 seconds at 98°C, and annealing temperature of 72 °C for 30 seconds. A final extension step at 72 °C for 10 min was also included. The PCR products were analyzed on 1% agarose gel stained with ethidium bromide (0.5µg/ml). The gel was subjected to electrophoresis and visualized on a UV transillumintor.

3.3 LEA Protein Induction in Transformed Bacteria

pET-DEST-42 vector has been designed to contain a T7 lac promoter to drive expression of the gene of interest. Therefore, IPTG was used to induce the LEA 1.1 gene expression of the transformed bacteria containing pET-DEST-42 LEA 1.1 vector. Parameters such as incubation time period, incubation temperature and IPTG concentration were varied in order to determine the optimum conditions for high levels of LEA-1.1 protein expression. An overnight culture was grown from a single colony at 37°C at 225 rpm in LB medium supplemented with 100 µg/ml ampicillin. The bacterial starter culture was inoculated into fresh LB medium containing 100 µg/ml ampicillin to yield an absorbance value of ~0.05–0.1 at OD_{600} (~1:50 dilution of the overnight culture). Culture was grown up to an absorbance value of OD_{600} of 0.4-0.6 (2 to 3 h of incubation) and IPTG was added at a final concentration of 0.5 mM or 0.75 mM. Induced bacteria were incubated at 18°C, 30°C, or 37°C and further incubated for an additional 4 or 6 h.

In order to monitor protein expression at different parameters, 1 ml of the induced bacterial culture was pelleted at 13,000 rpm for 2 min and resuspended in 200 µl of sample buffer (2% SDS, 25% glycerol, 5% mercaptoethanol, 0.01% bromphenol

blue, and 62.5 mM Tris HCl, pH 6.8). Denatured samples were electrophoresed by following the protocol described in methods section 1.3. Proteins were stained using Coomassie Brilliant Blue R-250 staining solution (Bio-Rad Laboratories, Hercules, CA).

3.4 Purification of Bulk Culture of Induced Bacteria

To perform protein purification, 250 ml of bulk culture with induced bacteria were pelleted and resuspended in 20 ml of tractor buffer (per 1g of cell pellet) by following the protocol given in xTractor buffer kit manual (Clontech Laboratories, Mountain View, CA). The pET-DEST-42 vector has been designed to have a polyhistidine tail down-stream to LEA 1.1 gene insert. Therefore, LEA 1.1 protein was translated with the polyhistidine tail tag. A nickel column (Ni-column) was used for the protein purification as 6-histidine tags have a high affinity for nickel ions and the protein can be separated from other unwanted proteins in the bacterial lysate. Twenty ml of the protein extract was loaded onto a 50 ml Ni- column and equilibrated with equilibration buffer (0.05 M Tris.HCl, 0.3 M NaCl, 0.01 M imidazole, pH 8.5). A wash buffer was used to eluate un-bound proteins from the column (0.05 M Tris.HCl, 0.3 M NaCl, and 0.02 M Imidazole, pH 8.5). Purified LEA 1.1 protein was eluted using elution buffer (0.05 M Tris. HCl, 0.3 M NaCl, 0.25 M Imidazole, pH 8.5) and 1 ml sample fractions were collected during the elution step.

In order to identify elution fractions with a minimum amount of other unwanted proteins during purification, samples were denatured and run on a 10% precast polyacrylamide Mini-PROTEAN TGX gel (Bio-Rad laboratories, Hercules, CA) (refer methods section 1.3). The gel was stained using Coomassie Brilliant Blue R-250 staining solution. After observing the gel results protein samples with high

amounts of LEA 1.1 (containing lowest amount of other residue protein) were pooled together and dialyzed against a 40 mM HEPES solution overnight at 4 ^{0}C to remove excess imidazole from the purified protein fractions. After dialysis, purified LEA 1.1 protein concentration was determined using a spectrophotometer at 280 nm. The formula which used to calculate the protein concentration is given bellow (Layne 1957).

Concentration (mg/ml) = (1.55 x A280) - 0.76 x A260)

After determining the protein concentration, purified LEA 1.1 protein samples were stored at -80^{o}C until used in experiments.

4. Lactate Dehydrogenase Assay

L-Lactate dehydrogenase (LDH) from bovine heart (Sigma-Aldrich St. Louis, MO) was used for experiments to determine possible protective properties of LEA 1.1 protein on LDH enzymatic activity during freezing. Commercially available LDH was supplied as ammonium sulfate suspension to protect the enzyme during storage at sub-zero temperatures. Therefore, the LDH suspension was dialyzed overnight at 4^{o}C against a 40 mM HEPES buffer to remove ammonium sulfate before using the enzyme in the freezing experiment. Dialyzed LDH was next dissolved in 0.2 M Tris.HCl (pH 7.3) or 0.050 mg/ml of purified LEA 1.1 protein (dissolved in 40 mM HEPES) to yield an enzymatic reaction rate of 0.10-0.15 ΔA/min. The diluted LDH enzyme was stored at -20^{o}C and 20 µl portions were thawed after 2 h, 6 h, and 10 h time intervals. Thawed LDH samples were subjected to the enzymatic assay using a freshly prepared mixture of 0.2 M Tris·HCl, 6.6 mM NADH and 30 mM sodium pyruvate (Sigma-Aldrich St. Louis, MO). Reagent preparation and performance of

enzymatic reaction was done by following the protocol given in Worthington Enzyme Manual (Worthington Biochemical Corporation, Lakewood, NJ)

Reaction rate was determined by a decrease in absorbance resulting from the oxidation of NADH to NAD^+ due to the LDH activity. It was measured by the decrease in absorbance at 340 nm using a spectrophotometer and change of reaction rate (ΔA_{340}/min) was calculated. Graphs were plotted using Sigma-plot software.

5. Freezing Studies - Cryomicroscopy

Purified LEA 1.1 protein in 40 mM HEPES solution at a concentration of 0.050 mg/ml was used and 25 µl of the sample was loaded on a glass slide and placed on the Linkam stage for cryomicroscopic observation (Linkam Scientific Instruments Ltd, UK). The sample was then subjected to decreasing temperatures at a cooling rate of 10 °C min^{-1} while being continuously observed on an upright microscope using a 20 x objective. Images were taken from a camera attached to the upright microscope and micrographs were collected at regular intervals while subjecting the solution to different temperatures. Ice formation was confirmed using visual inspection (darkening of the image field). HEPES was dissolved up to a concentration of 40 mM in distilled water and used as the control of the experiment.

6. Bioinformatics

Protein disorder predictions were performed for the amino acid sequences of LEA 1.1 (ACX81198.1), LEA 1.3 (ABR67402.1) and LEA 5 proteins (Table 1). Amino acid sequences were compared using the ExPasy bioinformatics resource portal data base (http://www.expasy.org/proteomics). The program FoldIndex was used to analyze protein folding dynamics among LEA 1.1, LEA 1.3 and LEA 5

proteins (http://bip.weizmann.ac.il/fldbin/findex). FoldIndex is a tool to predict whether a given protein sequence is intrinsically unfolded (Prilusky et al., 2005). Hydrophobicity prediction was done by Kyte and Doolittle plots (http://gcat.davidson.edu/rakarnik/kyte-doolittle.htm) (Kyte and Doolittle 1982).

Table 1: LEA protein amino acid sequences

LEA 1.1 (ACX81198.1) Protein: MELSSSKLNRSIFKRSSSVLHWHFYRLFGKKYPSKMSEQGKLSRQEAGQRGG QARAEQLGHEGYVEMGRKGGQARAEQLGHEGYQEMGQKGGQARAEQLGT EGYQEMGQKGGQKRAELLGHEGYQEIGQKGGQTRAEQLGHEGYQEMGQKG GQTRAEQLGTEGYQEMGQKGGQTRAEQLGHEGYVKMGKLGGEARKQQMS PEDYAAMGQKGGLARQQ
LEA 1.3 (ABR67402.1) Protein: MSEQGKLSRQEAGQRGGQARAEQLGHEGYVEMGRKGGQARAEQLGHEGY QEMGQKGGQARAEQLGTEGYQEMGQKGGQKRAEQLGHEGYVEMGQKGG QTRAEQLGHEGYQEMGQKGGQTRAEQLGTEGYQEMGQKGGQTRAEQLGHE GYVKMGKLGGEARKQQMSRADYAAMGQKGGLARQQ
LEA 5 Protein: MSENIGHININANLQNVDRRDAAAIQSVERKLLGYNPPGGLASEAQSAAALN EGIGQPMNRGISTDIPAPADIDVDRGTASKDFGHVRFDVDLNQVRPEEAAALQ AAESKIEGLAPSITVGGIGSAAQSMAAFNEREQSETGPFHPGIKATEPLPGPTY YQGVELSPSALPTYAPDVSVFPPSLSTNTSNVGAVPPSITTYSPDAGANDWER VYRKTTKTTQRIAIPGGIEDIVDEGKLGEAPRTNIRSTIGNVRMD

III. Results

1.Cell Desiccation and Anhydrobiosis

Some animals tolerate near total loss of body water and enter a state known as anhydrobiosis. Many desiccation-tolerant organisms withstand water stress by intracellular accumulation of sugars such as trehalose and larger macromolecules such as Late Embryogenesis Abundant (LEA) proteins. Evidence indicates that the presence of trehalose and Late Embryogenesis Abundant (LEA) proteins may work synergistically to confer cellular protection during drying in eukaryotic cells (Sakurai et al., 2008; Goyal et al., 2003; Goyal et al., 2005 a; Close 1996). Therefore, one of the goals of my research was to evaluate any increase in cellular desiccation tolerance by expressing LEA proteins in a non-desiccation tolerant insect cell line derived from *Drosophila melanogaster* (Kc167 cells) in the presence of trehalose.

1.1 Convective Droplet Drying

Convective droplet drying experiments were performed to investigate the effect of hyperosmotic stress on membrane integrity in LEA protein expressing Kc167 (Kc167-LEA) and control Kc167 cells (*D. melanogaster*). Group I LEA protein expressing cells (Kc167- LEA 1.1 and Kc167- LEA 1.3) and group V LEA protein expressing cells (Kc167- LEA 5) were used for the experiments. Cells were suspended in M3+BPYE medium and convectively dried in droplets. Cell desiccation was carried out in a desiccator box at 0% relative humidity for various time intervals to achieve a wide range of final moisture contents recorded as g H_2O/g DW (gram H_2O/gram dry weight). Cells were rehydrated in M3+BPYE medium and cultured for 24 h and cell viability was determined by using a trypan blue exclusion assay. The number of viable cells observed at any given moisture content (g H_2O/g DW) was expressed as

percentage of viable cells found on non-desiccated control plates. The number of viable cells was reduced with decreasing moisture content. Experiments were carried out with and without 200 mM extracellular trehalose. Statistical analysis demonstrated that there was no significant difference (ANCOVA: F1, 171.13 = 37.99, p =0.5217, r^2 = 0.76) between the amount of viable cells after desiccation of Kc167 control (slope = 4.7 ± 0.3) and LEA 1.3 (slope = 4.67 ± 0.28) expressing cells in the absence of extracellular trehalose (Figure 1).

1.2 Convective Droplet Drying with Trehalose

As there was no significant effect of the expression of LEA 1.3 protein on the desiccation tolerance of Kc167 cells, 200 mM extracellular trehalose was added into the culture medium and same experimental procedure was repeated. Kc167-LEA 1.1, Kc167-LEA 1.3 and Kc167-LEA 5 cells were subjected to droplet drying after suspending each cell line in M3+BPYE medium containing 200 mM trehalose. Similarly, a control experiment was carried out using non-transfected Kc167 cells with 200 mM trehalose and the results were compared with the results of the LEA protein expressing Kc167 cell experiments.

Interestingly, after adding extracellular trehalose to the culture medium, Kc167-LEA 1.3 cells showed significant increase in viability after desiccation compared to the control cells (ANCOVA: F1, 182.06 = 39.20, $p < 0.0001$, r^2 = 0.79), consistent with the results of the individual nonlinear regression (Figure 2). Linear regression resolved two significantly different slopes of 4.8 ± 0.32 (r^2 = 0.74) for Kc167 control cells and 12.80 ± 0.65 (r^2 = 0.85) for cells expressing LEA 1.3 in the presence of 200 mM trehalose. Furthermore, the lowest moisture levels at which

viable cells were found decreased from 1.02 g H_2O/g DW for control cells to 0.36 g H_2O/g DW for Kc167 cells expressing LEA 1.3.

Similarly, convective droplet drying experiments carried out for Kc167-LEA 1.1 showed a significant increase in the viable cells compared to the control Kc167 cells after desiccation. (ANCOVA: F1, 125.03= 41.04, $p < 0.0001$, $r^2 = 0.80$) Linear regression results resolved two significantly different slopes of 4.80 ± 0.32 ($r^2 = 0.74$) for Kc 167 control cells and 14.10 ± 0.68 ($r^2 = 0.95$) for cells expressing LEA 1.1 (Figure 3). Furthermore, the lowest moisture levels at which viable cells were found decreased from 1.02 g H_2O/g DW for control cells to 0.24 g H_2O/g DW for Kc167 cells expressing LEA 1.1 Moreover, the results obtained from the convective droplet drying experiments clearly showed that the transgenic expression of LEA 5 protein significantly increased cell viability of Kc167 cells during droplet drying in presence of 200 mM trehalose compared to control Kc167 cells. (Figure 4) (ANCOVA: F1, 153.10 = 39.24, $p < 0.0001$, $r^2 = 0.77$). Linear regression results resolved two significantly different slopes of 4.80 ± 0.32 ($r^2 = 0.74$) for Kc167 control cells and 13.5 ± 0.9 ($r^2 = 0.80$) for cells expressing LEA 5. Furthermore, the lowest moisture levels at which viable cells were found decreased from 1.02 g H_2O/g DW for control cells to 0.32 g H_2O/g DW for cells expressing LEA 5 (Figure 4).

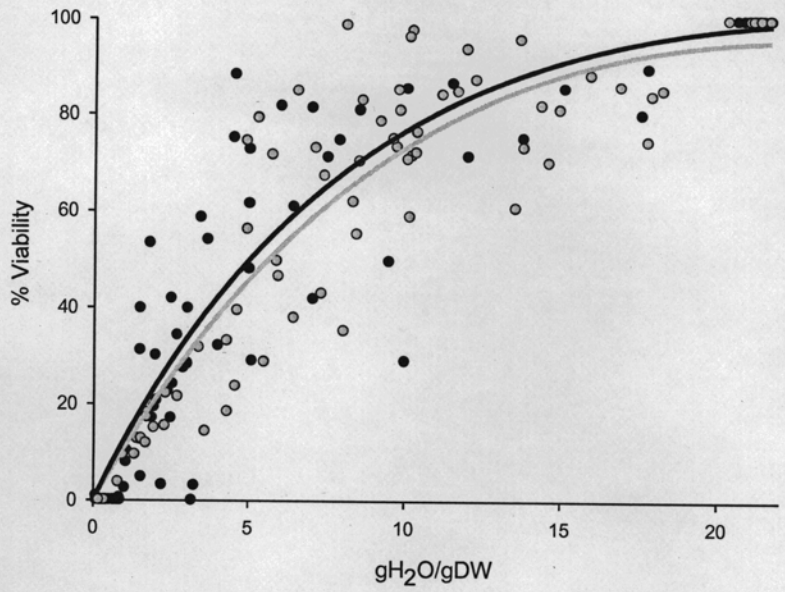

Figure 1: Response of Kc167 cells and transgenic Kc167-LEA 1.3 cells to convective droplet drying in cell culture medium without trehalose

Kc167 control (black) and LEA1.3 expressing (gray) cells were convectively dried in droplets of 15 µl cell culture medium. Cells were dried to a range of final moisture contents, rehydrated, and cultured for 24h. There was no statistically significant difference in the desiccation response between Kc167 control and LEA 1.3 expressing Kc167 cells (ANCOVA: F1, 171.13 = 37.98908, p =0.5217, r^2 = 0.76). The resolved slopes in linear regression analysis (data not shown) for Kc167 control (slope = 4.7 ± 0.3) and Kc167-LEA 1.3 cells (slope = 4.67 ± 0.28) showed no significant difference.

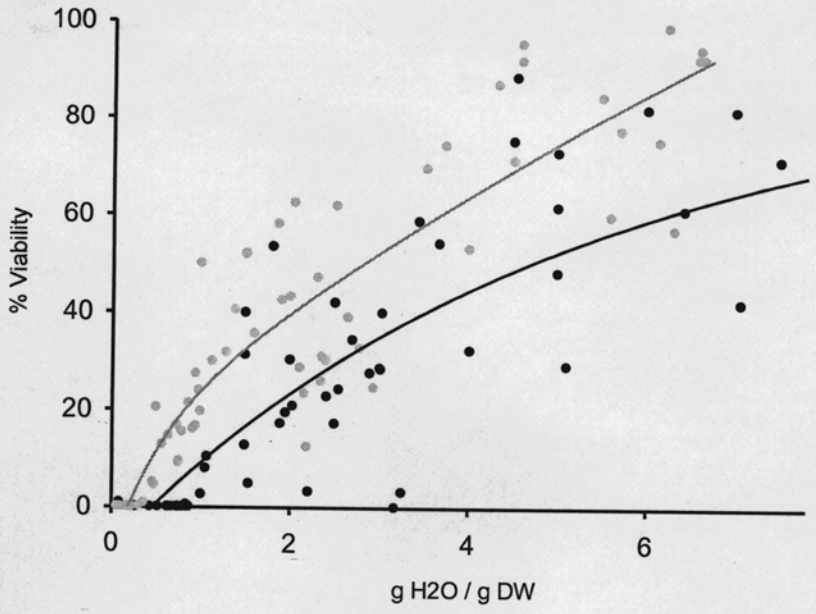

Figure 2: Response of Kc167 cells and transgenic Kc167-LEA 1.3 cells to convective droplet drying in presence of 200 mM extracellular trehalose

Kc167 control (black) and LEA 1.3 expressing (gray) cells were convectively dried in droplets of 15 µl medium with 200 mM trehalose added to the drying medium. After rehydrating, cells were incubated at 26°C for 24 h and cell counts were obtained. Statistical analysis showed a significant difference for the desiccation response among Kc167 control and LEA 1.3 expressing cells. The expression of LEA 1.3 resulted in a statistically significant increase in the viability response after desiccation (ANCOVA: F1, 182.06 = 39.20, $p < 0.0001$, $r^2 = 0.79$) with slopes of 4.80 ± 0.32 ($r^2 = 0.74$) for Kc167 control cells and 12.80± 0.65 ($r^2 = 0.85$) for Kc167-LEA 1.3 in linear regression analysis (data not shown).

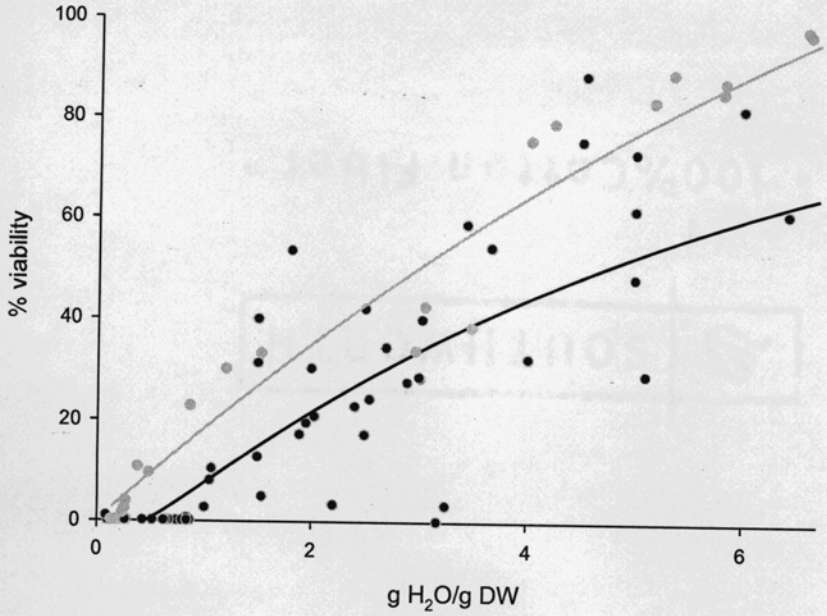

Figure 3: Response of Kc167 cells and transgenic Kc167-LEA 1.1 cells to convective droplet drying in presence of 200 mM extracellular trehalose

The viability of Kc167 control (black) and LEA 1.1 expressing Kc167cells (gray) showed a statistically significant difference after convective droplet drying. The expression of LEA 1.1 has showed led to a statistically significant increase in viability after desiccation (ANCOVA: F1, 125.03= 41.04, $p < 0.0001$, $r^2 = 0.80$) compared to the control Kc167 cells. Nonlinear regression analysis obtained with resolved two different slopes of 4.80 ± 0.32 ($r^2 = 0.74$) for Kc167 control cells and 14.10 ± 0.68 ($r^2 = 0.95$) for cells expressing LEA 1.1 (data not shown).

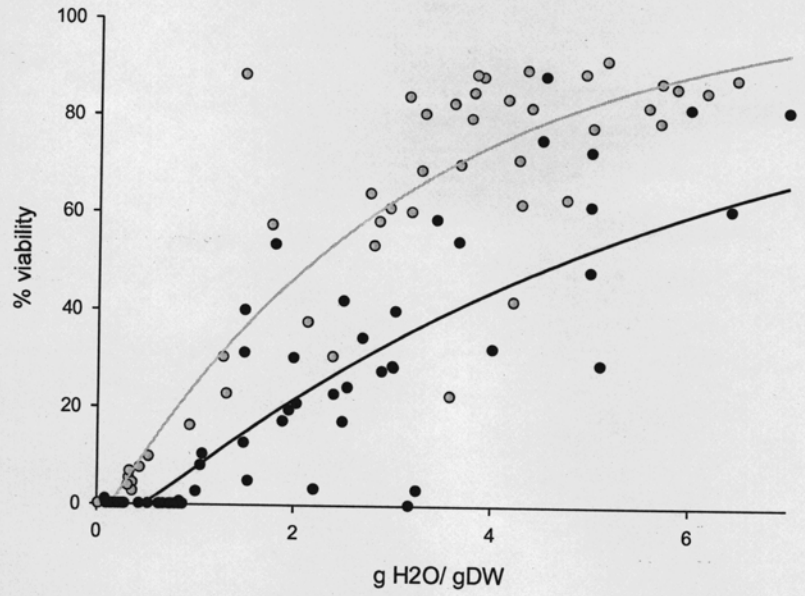

Figure 4: Response of Kc 167 cells and transgenic Kc 167-LEA 5 cells to convective droplet drying in the presence of 200 mM trehalose

The viability of Kc167 control (black) and LEA 1.1 expressing Kc167cells (gray) showed a statistically significant difference after convective droplet drying. The expression of LEA 5 led to a statistically significant increase in viability after desiccation (ANCOVA: F1, 153.10 = 39.24, $p < 0.0001$, $r^2 = 0.77$) compared to the control Kc167 cells. Nonlinear regression analysis resolved two different slopes of 4.80 ± 0.32 ($r^2 = 0.74$) for Kc167 control cells and 13.5 ± 0.9 ($r^2 = 0.8$) for cells expressing LEA 5 (data not shown).

2.Western Blotting

In order to perform convective droplet drying experiments, transgenic Kc167 cell lines harboring LEA genes were generated. This was achieved by stably inserting the pMT-DEST-48 vector which carried the LEA gene sequence into Kc167 cells. Western blot was performed with a monoclonal antibody against the V5 epitope to confirm the expression of LEA 1.3 in Kc167 cells after transfection. This was possible since the constructed vector pMT-DEST-48 LEA 1.3 carries the V5 epitope sequence. There was a prominent protein band detected with an apparent molecular weight of ~30 kDa in cellular extracts from Kc167-LEA 1.3 cells but not in control Kc167 cells (Figure 5). The molecular weight of the LEA 1.3 protein was calculated from the amino acid sequence and the calculated value for the full length protein including the V5 epitope and leader sequence was ~26 kDa. However, a study has been reported that the molecular weight of some intrinsically disordered proteins estimated by SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis) is often 1.2–1.8 times higher than the molecular mass calculated from the amino acid sequence of the protein (Tompa 2002).

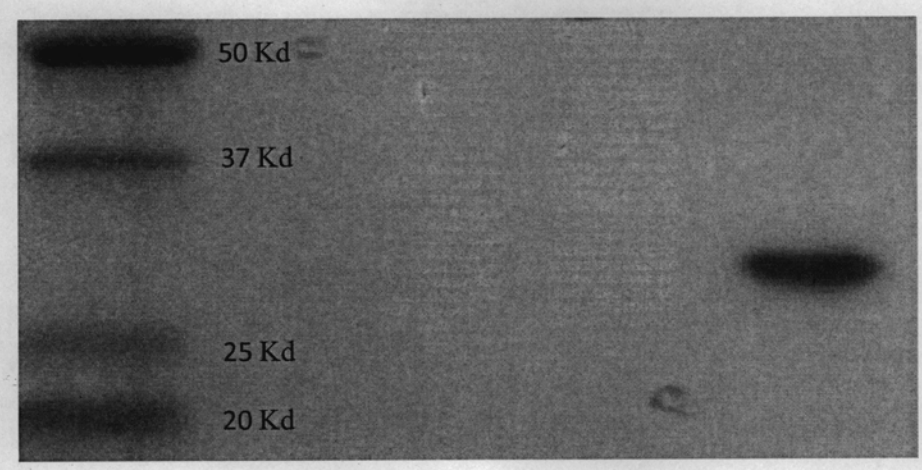

Figure 5: Western blot confirmation of LEA 1.3 protein, transgenically expressed in *D. melanogaster* cells (Kc167)

Lane a = molecular weight standards; lane b = extract of control cells probed with antibody against the V5 epitope; lane c = extract of cells stably transfected with LEA 1.3 and probed with antibody against the V5 epitope.

3. Production of Recombinant LEA Protein

3.1. Generation of Transgenic Bacteria with LEA Protein Gene

The experiments carried out for Kc167-LEA cells via convective droplet drying experiments have clearly demonstrated that there is a significant increase in cell viability when LEA proteins are expressed in non-desiccation tolerant *Drosophila* cells in the presence of extracellular trehalose. Therefore, to investigate the mechanisms by which LEA proteins protect cells, *in vitro* studies were utilized. Recombinant LEA protein production was a crucial step to perform these experiments. A transgenic *E. coli* strain harboring the LEA 1.3 gene sequence was generated and protein expression was induced by IPTG to produce recombinant LEA protein. The LEA 1.1 (ACX81198.1) gene was cloned into the vector pENTR/D-TOPO and transformed into One Shot Chemically Competent *E. coli* cells. Successfully transformed bacteria were selected with 50 μg /ml kanamycin and used for plasmid purification. Purified pENTR/D-TOPO plasmid was subcloned into the destination vector pET-DEST-42 using LR Clonase II enzyme mix and transformed into the BL32 One Shot Chemically Competent *E. coli* strain.

During recombination the ccdb suicide gene present in the pET-DEST-42 vector was replaced by the LEA 1.1 gene present in the pENTR/D-TOPO vector. The advantage of using this system is that the bacteria which carry the pENTR/D-TOPO vector with the ccdb suicide gene (non-recombinants) starts to die after transformation and the bacterial cells harboring LEA 1.1 gene in pET-DEST-42 vector survive. The purpose of transferring LEA 1.1 gene into pET-DEST-42 vector was that this particular vector carries a C-terminal polyhistidinee (6- histidine) tag. It allows the purification of the recombinant LEA 1.1 protein using a nickel column. As histidine

has a high affinity for Nickel ions, LEA 1.1 protein can be separated from other unwanted proteins during purification.

3. 2. PCR Confirmation of LEA Protein Gene Expression in Bacteria

Successfully transformed bacterial colonies were randomly selected after incubating the plates supplemented with 100 μg/ml ampicillin for 24 h after plating and plasmid purification was carried out after liquid culture overnight. PCR was performed to confirm insertion of pET-DEST-42-LEA 1.1 vector in BL32 *E. coli* strain. Figure 6 confirmed the successful insertion of the LEA 1.1 gene into bacteria as shown by the amplification of a 565 bp DNA band.

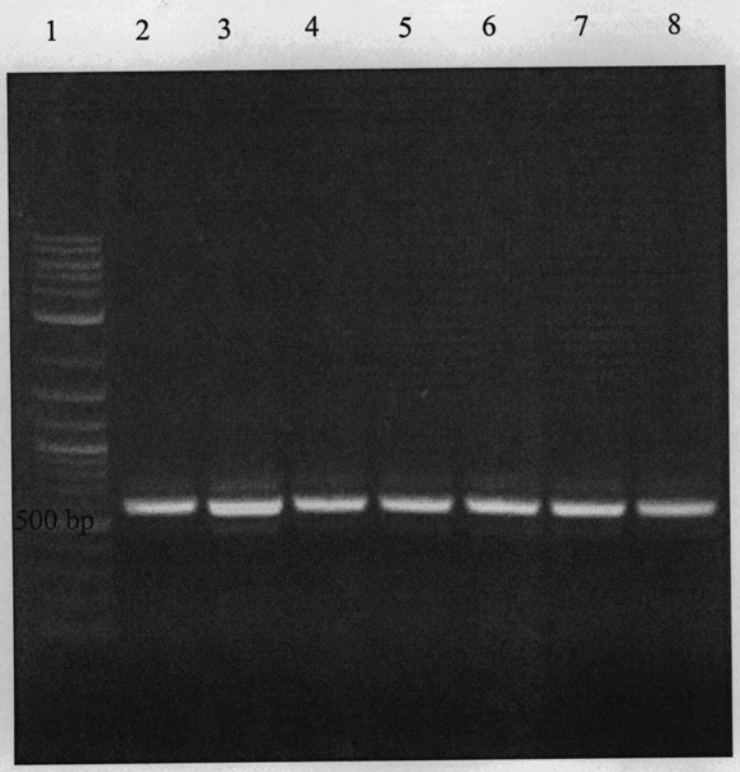

Figure 6: PCR Confirmation of LEA 1.1 gene in pET-DEST-42-LEA 1.1 vector

Lane 1= 2-Log DNA ladder, Lane 2-8 = PCR products for the extracted plasmid samples obtained from 7 randomly selected bacterial colonies after transformation of pET-DEST-42-LEA 1.1.

3.3. LEA Protein Induction in Bacteria

The gene encode for the production of LEA 1.1 protein was inserted into pET-DEST-42 destination vector and transformed into BL32 One shot *E. coli* bacterial strain. Gene expression was induced by adding IPTG. Optimum conditions for protein expression were determined by changing incubation time period (4 h and 6 h), temperature (18°C, 30°C and 37°C) and IPTG concentration (0.5 mM and 0.75 mM). Individual experiments were carried out to monitor the yield of transgenic protein after manipulation of each parameter. Protein expression was determined using denatured cellular protein samples from induced bacteria from each experiment. Samples containing LEA 1.1 protein were subjected to 10% SDS–PAGE using a Bio-Rad Mini-PROTEAN 3 Cell. Protein samples were stained using Coomassie Brilliant Blue R-250 staining solution. High yields of LEA 1.1 protein were obtained when bacteria were induced with an IPTG concentration of 0.75 mM at 30°C (Figure 7). Moreover, the highest amount of induced LEA 1.1 protein was obtained after 4 h of incubation (Figure 8).

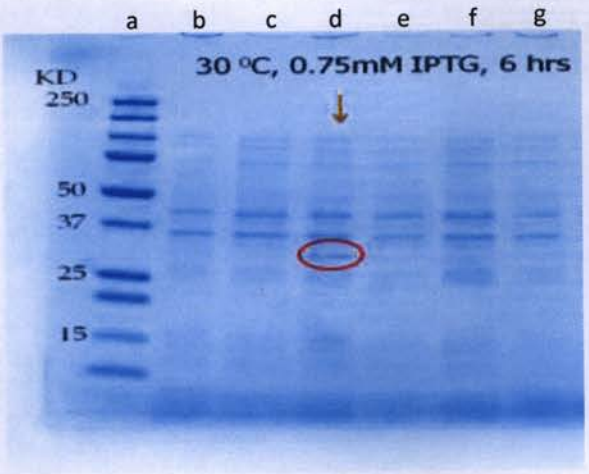

Figure 7: Optimization of LEA 1.1 protein expression in *E. coli*: response to incubation temperature

Lane a = molecular weight standards; lane b = 37°C incubation temperature, 0.75 mM IPTG harvested at 6 h post induction; lane c = 37°C at 6 h incubation un-induced sample; lane d = 30°C incubation temperature, 0.75 mM IPTG, harvested at 6 h post induction; lane e = 30°C at 6h incubation un-induced sample; lane f = 18°C, 0.75 mM IPTG harvested at 6 h post induction; lane g = 18°C at 6h incubation un-induced sample.

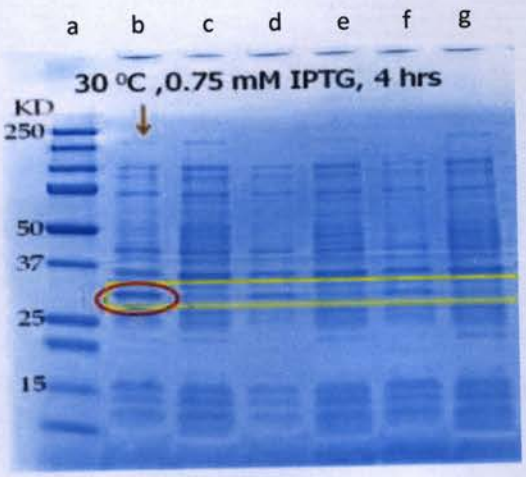

Figure 8: Optimization of LEA 1.1 protein expression in *E. coli*: protein yield at different time points after induction of protein expression

Lane a = molecular weight standards; lane b = 30°C incubation temperature 0.75 mM IPTG harvested at 4 h post induction; lane c = 30°C at 4 h incubation un-induced sample; lane d = 30°C incubation temperature 0.75 mM IPTG harvested at 6 h post induction; lane e = 30°C at 6h incubation un-induced sample; lane f = 30°C incubation temperature 0.75 mM IPTG harvested at 8 h post induction; lane g = 30°C at 8 h incubation un-induced sample.

3.4. Recombinant LEA Protein Purification

After testing a variety of conditions for transgenic protein induction in *E. coli,* the highest protein yield was obtained at 30°C, 0.75 mM IPTG, 4 h after protein induction. To purify large amounts of LEA 1.1 bulk culture of induced bacteria was used. Protein purification was performed by affinity chromatography using a nickel-column and 250 ml of induced bacteria were pelleted and resuspended in lysis buffer. The lysate was centrifuged and the supernatant was loaded into a nickel-column. Affinity chromatography was performed to purify LEA 1.1 protein by passing a buffer containing imidazole at a concentration gradient over the column. During purification, eluted protein samples were collected separately as 1 ml fractions. In order to identify elution fractions with a minimum amount of other unwanted proteins samples were subjected to 10% precast polyacrylamide Mini-PROTEAN TGX gel. The gel was stained using Coomassie Brilliant Blue R-250 staining solution. Figure 9 shows the gel of resolved proteins stained in a polyacrylamide gel. 32nd column volume contained LEA 1.1 protein with minimum amount of other residue proteins. Therefore, protein samples which obtained from column volume 32 to 36 were pooled together. The pooled fractions were dialyzed against 40 mM HEPES buffer to remove excess imidazole contained in the protein fractions. The purified LEA 1.1 protein concentration was determined using a spectrophotometer at 280 nm and calculated to be 0.050 mg/ml.

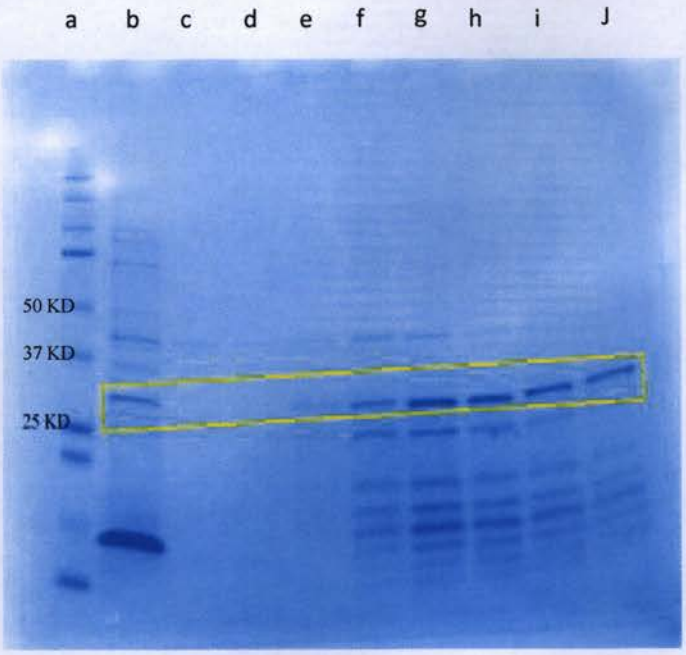

Figure 9: 10 % Polyacrylamide gel image for eluted protein sample fractions of affinity chromatography in nickel column purification

Lane a = molecular weight standards; lane b = IPTG induced bacterial cell lysate before purification; lane c-j = Eluted one ml protein sample fractions from 26^{th}-33^{rd} collected during affinity chromatography.

4. Lactate Dehydrogenase Activity

Previous studies have reported that LEA proteins can act as cryoprotectant during freezing of biomolecules (Bravo et al., 2003; Kazuoka and Oeda, 1994; Houde et al., 1995). The enzyme lactate dehydrogenase is widely used as a model enzyme to evaluate the structural properties of proteins. A study has been reported that lactate dehydrogenase can be denatured at sub-zero temperatures in the absence of cryoprotectants (Hatley and Franks 1989). Therefore, lactate dehydrogenase is dissolved with protective chemical agents such as ammonium ions to prevent the denaturation during freezing. In order to determine the properties of LEA protein on the protection of macromolecular proteins such as enzymes, lactate dehydrogenase (LDH) assays were performed. Cryoprotectant free LDH was diluted to yield a final reaction rate of 0.10-0.15 ΔA/min using purified LEA 1.1 protein and stored at -20°C. Enzyme samples were thawed after 2 h, 6 h and 10 h time intervals and enzymatic activity was measured as a decrease in absorbance at 340 nm using a spectrophotometer. Figure 9 shows a declining of LDH activity during storage time. However, LDH activity was significantly higher (n = 3, ±SE, $P < 0.05$) after co-incubation with 0.050 mg/ml LEA 1.1 protein compared to both control conditions (0.2 M Tris.HCl and 40 mM HEPES). There were two controls used for the experiment. LDH was dissolved in 0.2 M Tris.HCl which is the typical solution which enzymes are dissolved to measure enzymatic assays. Tris.HCl maintains the pH required for the enzyme activity during the reaction. Therefore LDH dissolved in Tris.HCl was used as a control. Commercially available LDH is supplied as ammonium sulfate suspension to protect the enzyme during storage at subzero temperatures. Therefore, it was dialyzed with a 40 mM HEPES buffer to remove ammonium ions before the experiment. Moreover, Purified LEA 1.1 protein was

dialyzed in 40 mM HEPES before the experiment to remove excess imidazole. Therefore, LDH dissolved in 40 mM HEPES was used as the other control. Figure 10 shows that there was no statistically significance difference in the enzyme activity among LDH stored in 0.2 M Tris.HCl (control) and the LDH stored in 40 mM HEPES (control) at any time interval. However, after 10 h, there was a significant difference of the LDH activity (n = 3, ±SE, $P < 0.05$) in the presence of 0.050 mg/ml LEA 1.1 protein compared to the LDH incubation with 0.2 M Tris.HCl (control).

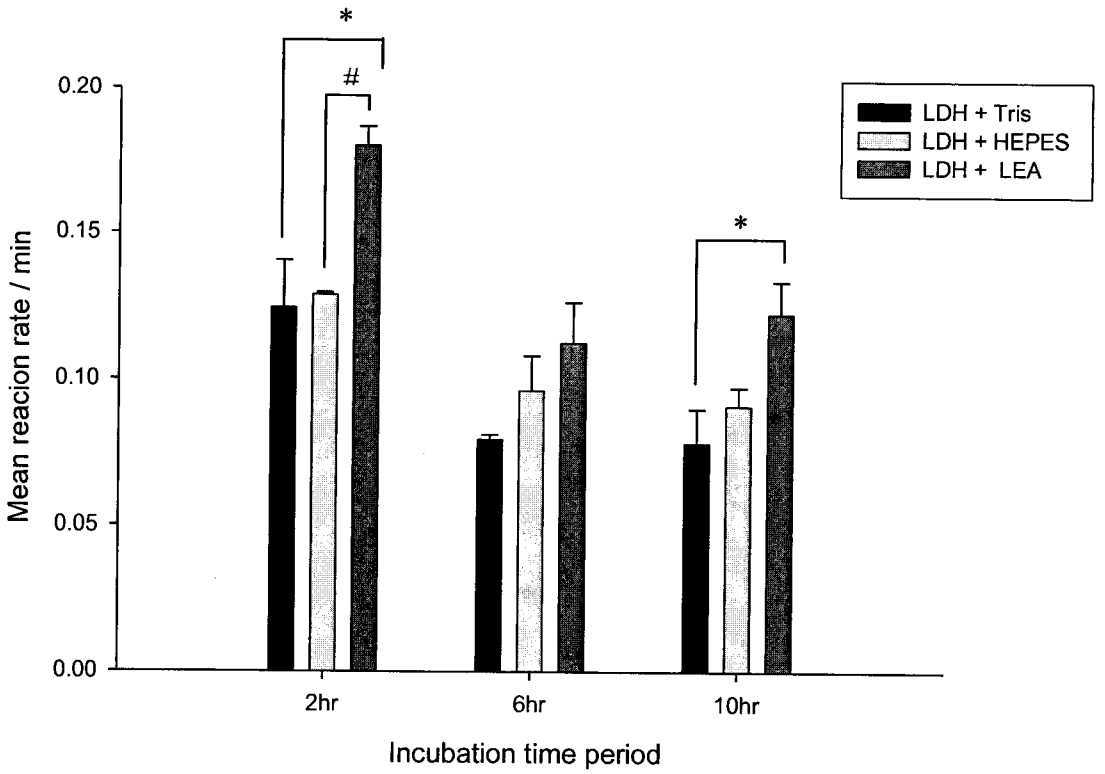

Figure 10: Lactate dehydrogenase activity after freezing at -20°C followed by thawing in presence and absence of LEA 1.1

LDH activity was plotted after incubating with Tris.HCl (control), 40 mM HEPES (control) and 0.050 mg/ml purified LEA 1.1 protein at 2 h, 6 h and 10 h of storage at -20°C.

*and # indicates significantly different means (n = 3, ±SE, $P < 0.05$).

5. Freezing Studies - Cryomicroscopy

The LDH assay results demonstrated a protective role of LEA 1.1 protein when LDH enzyme was stored at sub-freezing temperatures. It is possible that in the presence of LEA protein, solutions may freeze at a higher temperature and minimize the damage to biomolecules. Therefore, it was important to observer the behavior of a solution during freezing in the presence of LEA protein. To fulfill this goal a standard cryogenic microscope was used. For this experiment, dialyzed LEA 1.1 protein which used for the LDH assay was used at a concentration of 0.050 mg/ml as the sample and 40 mM HEPES buffer was used as the control. Samples were subjected to a decreasing temperature ramp at -10°C min^{-1} and images were taken from a camera attached to the upright microscope. While decreasing the temperature 40 mM HEPES buffer froze at -14.4°C (SE ± 0.11) showing a rectangular ice-crystal shape (Figure 11). Similarly, cryogenic microscope observation was recorded for the 0.050 mg/ml LEA 1.1 protein with HEPES which started to freeze at -17.3°C (SE ± 0.06) (Figure 12). When comparing the figure 11b and figure 12b, it is clear that the ice-crystal shape of HEPES buffer have changed from rectangular to more pentagonal shape in the presence of LEA 1.1 at 0.050 mg/ml concentration.

Figure 11: Images of 40 mM HEPES buffer before and after freezing

Image a= 40 mM HEPES buffer at -13.7°C before freezing; Image b = 40 mM HEPES buffer at -15.2°C after freezing. Each experiment was repeated three times and average freezing temperature was obtained. Magnifications: 200X.

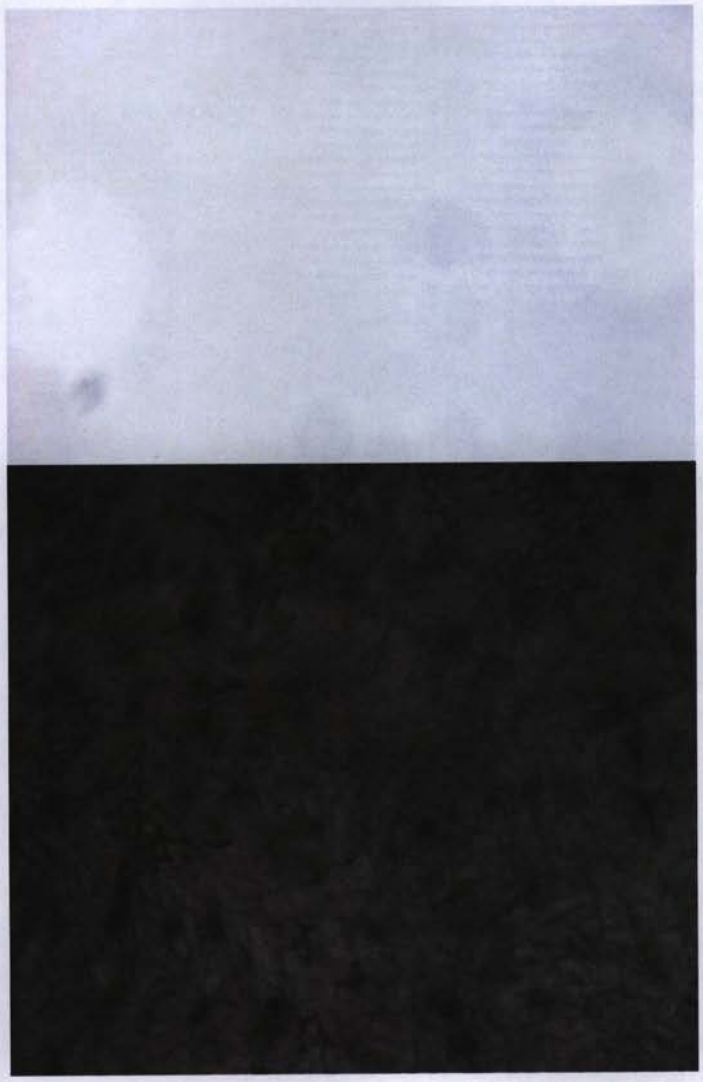

Figure 12: Images of 0.050 mg/ml LEA 1.1 protein dialyzed in 40 mM HEPES buffer before and after freezing

Image a= 0.050 mg/ml LEA 1.1 protein dialyzed in 40 mM HEPES buffer at -14.9°C before freezing. Image b= 0.050 mg/ml LEA 1.1 protein dialyzed in 40 mM HEPES buffer at -17.6°C after freezing. Each experiment was repeated three times and average freezing temperature was obtained. Magnifications: 100X.

6. LEA Protein Bioinformatics

The amino acid sequences of LEA 1.3, LEA 5 and LEA 1.1 (Table 1) were used to identify the phylogenetically closest members of these proteins using the NCBI BLAST data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi). NCBI Blast sequence analysis has shown that the closest member for LEA 1.3 was from a group I LEA protein found in *Artemia franciscana* (GenBank: ABR67402.1). Also the closest member for LEA 1.1 was from a mitochondria targeted group I late embryogenesis abundant protein found in *Artemia franciscana* (GenBank: ACX81198.1). However, BLAST results for LEA 5 showed that the closest member is a plant seed maturation protein found in *Medicago truncatula* and it involves in desiccation tolerance.

According to some research findings, LEA proteins found in group I and group III are structurally disordered in aqueous solution (Eom et al., 1996; Soulages et al., 2002, 2003). Upon desiccation, there is a structural transition from the disordered protein into a more ordered conformation (Wolkers et al., 2001; Goyal et al., 2003; Shih et al., 2004). Therefore, bioinformatics programs were utilized to judge the probability that LEA 1.3 and LEA 1.1 (group I LEA proteins) as well as LEA 5 (group V LEA protein) are disordered in aqueous solution. To investigate this, protein disorder predictions were performed to identify the extent of unfolded regions present in these three LEA proteins. According to Prilusky et al., 2005, the program FoldIndex is a simple tool to predict whether a given protein sequence is intrinsically disordered. Hence, protein folding index in Kyte-Doolittle hydrophilicity plots was constructed to compare the disordering nature of these LEA proteins using ExPASy ProtScale.

Figure 13 indicates that the LEA 1.1 protein is completely unfolded in its native state (red color). According to the protein foldIndex value, all the amino acids

in LEA 1.1 were located in the negative side of the plot. Prilusky et al., 2005 explains that all positive values represent the regions in a protein which are likely to be folded, and negative values represent those regions likely to be intrinsically unfolded. My results indicate that the LEA 1.1 protein is randomly coiled by showing negative values throughout the foldIndex plot. Moreover, similar results are shown in Figure 13 for the foldIndex values of LEA 1.3. However, LEA 5 figure demonstrated that the majority of the LEA 5 amino acids group in the positive side of the protein foldIndex plot indicating confined secondary structure motives of the protein (green color). Moreover, there were some minor unfolded areas in LEA 5 as indicated by the negative values in the foldIndex plot. Therefore, this protein appears to be a folded protein in aqueous solution (Figure 13-LEA 5).

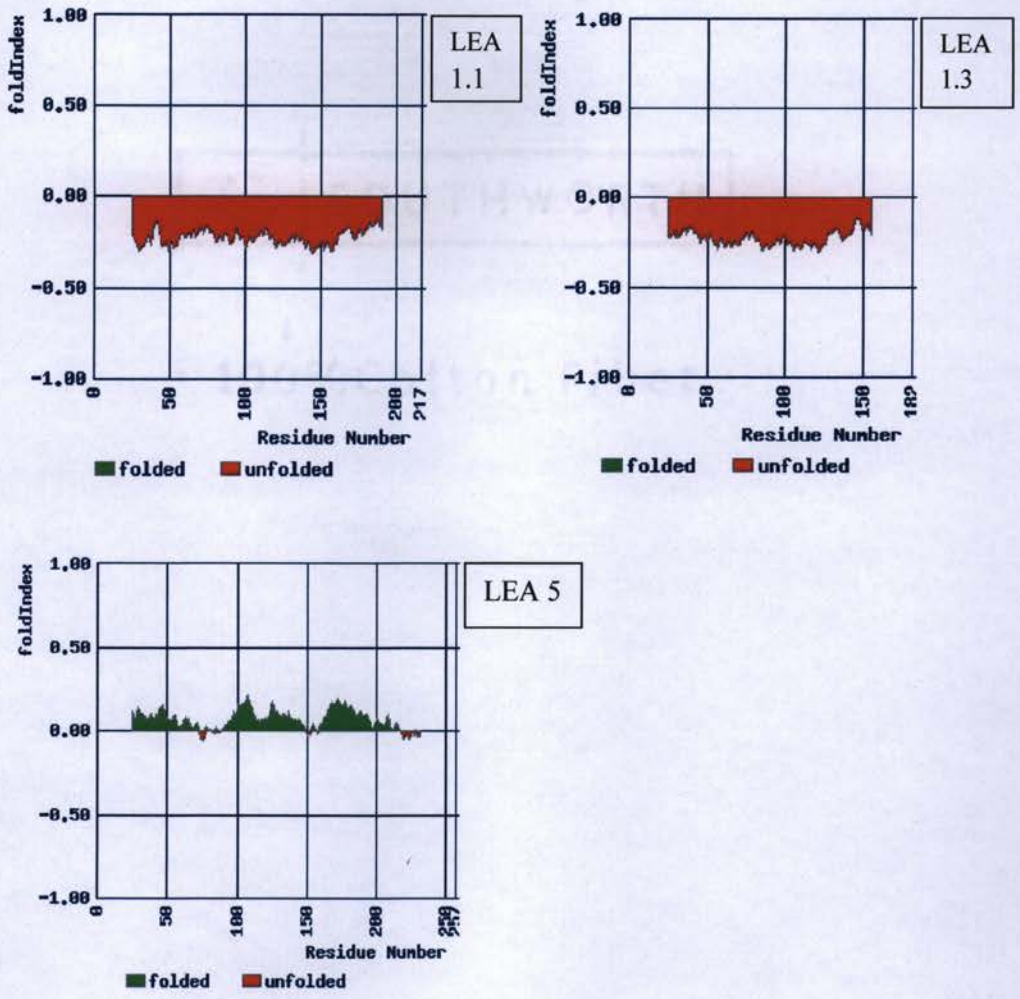

Figure 13: Protein folding index of Kyte-Doolittle plot for LEA 1.1, LEA 1.3 and LEA 5

The amino acid sequence of each LEA protein was analyzed for folded (green) and unfolded (red) protein regions using the protein folding index in Kyte-Doolittle hydrophilicity plot. The protein folding index was calculated as described in Prilusky et al., 2005.

V. Discussion

Hyperosmotic stress can create severe damages to living cells by causing mitochondrial dysfunction, disorganization of macromolecules such as proteins and degradative enzymatic reactions. All these damages and dysfunctions may lead to activation of cell death pathways in the cell (Michea et al., 2002; Copp et al., 2005). However, many organisms representing wide variety of taxonomical groups possess the ability to survive under extreme dehydration conditions using a strategy known as anhydrobiosis. (Crowe and Crowe 1992).

Anhydrobiotic organisms can survive dehydration via several different mechanisms. Accumulation of disaccharides such as the sugar trehalose in the cytoplasm and (Albertorio et al., 2007) accumulation of late embryogenesis abundant proteins (LEA) are among those survival mechanisms (Liang and MacRae 1999; Jönsson and Schill 2007; Gusev et al., 2011; Hand et al., 2011). It has been shown that trehalose and LEA proteins may work synergistically to confer cellular protection during drying in eukaryotic cells (Sakurai et al., 2008; Goyal et al., 2003; Goyal et al., 2005 a; Close 1996).

In my study, I demonstrated a significant increase in cellular desiccation tolerance when LEA proteins are expressed in a non-desiccation tolerant cell line from the fruit fly *Drosophila melanogaster* (Kc167) in the presence of extracellular trehalose (Figures 2-4). Some studies have demonstrated that LEA proteins are capable of protecting other proteins such as enzymes from inactivation and aggregation during storage at sub-freezing temperatures (Reyes et al., 2005; Reyes et al., 2008). Indeed, the results of the LDH assay conducted in the presence of purified group I LEA protein have shown an increased enzyme activity after freezing and thawing compare to the controls at 2 h and 10 h incubation time durations (Figure 9).

Therefore, a deeper knowledge of understanding the different mechanisms involved in anhydrobiosis would allow the researchers to combine the most effective strategies employed by anhydrobiotes in order to create successful desiccation tolerant cells and tissues for biomedical applications.

A. Convective Droplet Drying

Among anhydrobiotic organisms the brine shrimp *A. franciscana* is considered as an extremophile (Clegg 2011). High expression levels of multiple LEA proteins which belong to different LEA protein groups such as group I (PF00477), group III (PF02987) and group IV (PF04927) were found in *A. franciscana* embryos before the onset of desiccation (Hand et al., 2007; Sharon et al., 2009; Wu et al., 2011). Hence, all the cDNA sequences used for this study were obtained from *A. franciscana* to evaluate whether these different proteins impact cell desiccation in a similar manner. LEA 1.1 and LEA 1.3 sequences used for the current study were obtained from group I LEA gene expressed in diapause embryos of *A. franciscana* (Sharon et al., 2009). A recent study has been constructed to generate GFP-tagged LEA 1.1 and LEA 1.3 proteins expressed in Kc167 cells (Marunde et al., 2013). Using confocal images they have reported that LEA 1.1-GFP localized to the cytoplasmic compartment, while LEA 1.3-GFP accumulated within the mitochondria of Kc167 cells. Although these two proteins were localized in different compartments of the cell it seems like they both contribute to the protection of Kc167 cells in a similar manner showing a significant increase in the cell viability in convective droplet drying experiments (Figure 2 and 3).

However, convective droplet drying experiments conducted for Kc167 cells and Kc167-LEA 1.3 have showed that there was no significant effect on LEA protein

expression for the desiccation tolerance in the absence of extracellular trehalose (Figure 1). The possible reason for this observation may be due to the fact that only LEA protein expression is not sufficient enough for a significant cellular protection in the absence of trehalose. Supporting this idea, convective droplet drying studies carried out for Kc167-LEA protein expressing cells have shown a significant increase in the cell viability after adding 200 mM trehalose to the medium (Figure 2-4). Confirming these results a study has reported that group I and group III LEA proteins alone do not have any protective function against heat stress but in the presence of trehalose these LEA proteins may prevent other protein from aggregation due to water stress (Goyal et al., 2005 b). Moreover, in addition to the accumulation of LEA proteins, embryos of *A. fransiscana* have shown an accumulation of trehalose about 20% of their dry weight during desiccation (Crowe et al., 1987).

Considering all these facts my results strongly supports the initial hypothesis that the expression of LEA proteins in a non-desiccation tolerant insect cell line will increase cellular desiccation tolerance in the presence of trehalose. This may be due to the fact that trehalose and LEA proteins work synergistically to confer cellular protection during desiccation (Sakurai et al., 2008; Goyal et al., 2003; Goyal et al., 2005 a; Close 1996).

Vitrification or the formation of glassy structures has been identified as a necessary requirement for cellular preservation in some anhydrobiotic organisms such as *Polypedilum vanderplanki (*Sakurai et al., 2008). This glass structure protects the cell and its organelles by minimizing the molecular mobility rates as well as the chemical reaction rates during dehydration (Crowe et al., 1998; Albertorio et al., 2007). Trehalose is composed of two glucose molecules bonded by α-α 1-1 glycolytic linkage. Due to this special chemical bond, the two glucose molecules form a

clamshell structure and the molecule has an equal number of hydrogen binding sites on either side. This specific structure facilitates hydrogen bond formation with other macromolecules such as proteins and head groups of phospholipid bilayers (Albertorio et al., 2007). Therefore, addition of 200 mM trehalose to the culture medium possibly facilitates the hydrogen bond formation with phospholipid head groups of the cell membrane bilayer of Kc167 cells and increases membrane integrity during convective droplet drying (Figure 2-4).

Moreover, it is possible that presence of conserved trehalose transporter sequences (Kanamori et al., 2010), *D. melanogaster* cells may facilitate the transport of extracellular trehalose into Kc167-LEA cells during dehydration. This transported trehalose may act synergistically with the LEA proteins in Kc167-LEA cells during desiccation.

Structurally, several LEA proteins were found to be largely unfolded in solution (Eom et al., 1996; Goyal et al., 2003; Soulages et al., 2002; Mouillon et al., 2006; Lisse et al., 1996) and some were shown to undergo transition to a more folded state upon dehydration (Goyal 2003; Wolkers et al., 2001; Shih et al., 2004). Similar to these findings, protein foldIndex of Kyte-Doolittle plot performed for the group I LEA proteins LEA 1.1 and LEA 1.3 indicated that both these proteins are unfolded in solution (Figure 13 - LEA 1.1 and LEA 1.3).

However, it has been found that LEA proteins help to prevent aggregation of other proteins during desiccation in the presence of trehalose (Goyal et al., 2005 b) Therefore, it is possible that transgenic expression of LEA proteins in *D. melanogaster* cells (Kc167) ((Figure 5) might reduce unfolding of cellular proteins by acting as a molecular chaperon. The trehalose transported into the cell might help the

LEA proteins to prevent the formation of protein aggregates. However, the exact mechanism that governs this synergetic effect is unclear.

cDNA sequence for LEA 5 protein was also obtained from *A. fransiscana* (Menze unpublished data). Even though LEA 5 belonged to group V LEA proteins, NCBI- BLAST sequence analysis has shown that this sequence has similarities to a highly conserved region of proteins that belong to the seed maturation protein family (data not shown). Similar to late embryogenesis abundant proteins, certain plants such as soybean (*Glycine max*) have shown a rapid accumulation of seed maturation proteins during desiccation (Blackman et al., 1992). Interestingly, even though LEA 5 is different from group I LEA proteins, Kc167 cells expressing transgenic LEA 5 protein also showed a significant increase in cell viability after desiccating in the presence of trehalose (Figure 4). However, the protein foldIndex of Kyte-Doolittle plot performed for LEA 5 indicates that the LEA 5 protein is a folded protein in aqueous solution (Figure 13- LEA 5). This suggests that it is not always necessary to be unfolded for the LEA proteins to act as cellular protectors during desiccation. Moreover, these observations highlights that the LEA proteins present in *A. franciscana* responds to desiccation in a similar manner when they are transgenically expressed in *D. melanogaster* cells (Kc167) even though they have a different structure and hydrophilic/hydrophobic nature .

Most folded proteins contain a hydrophobic core with the side chain stabilizing the folded conformation and polar side chains on the surface where they interact with surrounding water molecules. LEA 5 group proteins are different among other hydrophilic LEA groups because of the hydrophobic nature. Protein disorder predictions in Figure 13- LEA 5 have indicated that it is natively a folded protein. Thus, LEA 5 proteins may have the advantage of determining their native secondary

structures. A study has reported that At1g01470.1, a LEA protein found in *Arabidopsis*, has one α-helix and seven β-sheet strands that forms two antiparallel α β sheets in its native structure (Singh et al., 2005). Therefore, future studies such as LDH assay and cryomicroscope would be helpful to identify the behavior of folded, hydrophobic proteins such as LEA 5 during freezing as LEA 5 have already showed an increased desiccation tolerance after expressing in Kc167 cells.

B. Freezing Studies

Several studies have demonstrated that LEA proteins can act as cryoprotectants at sub-freezing temperatures (Kazuoka and Oeda, 1994; Houde et al., 1995; Bravo et al., 2003). Results of the lactate dehydrogenase enzyme assay have demonstrated that addition of 0.050 mg/ml purified group I LEA protein (LEA 1.1) to LDH significantly increased enzymatic activity after freezing for 10 h at -20°C compared to controls (Figure 10). Moreover, it has been reported that group I LEA proteins can protect citrate synthase activity at sub-freezing temperatures (Goyal et al., 2005 b). However, group I LEA proteins are unstructured and have a low complexity of defined secondary structure motifs (Soulages et al., 2002). Therefore, it is possible that these features may allow LEA proteins to act as a 'molecular shield', forming a physical barrier between enzyme molecules and thereby preventing contact of exposed hydrophobic regions and aggregation (Goyal et al., 2005 b). Although the exact mechanism of action for the protection of enzymes at sub-freezing temperatures by group LEA I protein is unknown it is possible that the LEA 1.1 protein used for the present study acts in the similar manner and prevents LDH aggregation and thereby preserves the enzymatic activity at sub-freezing temperatures.

Natively unfolded proteins are likely to be randomly coiled with very little or no ordered secondary structure in solution (Uversky, 2002 a and b). Several studies have demonstrated that group I LEA proteins lack a defined structure in solution (Gilles et al., 2007; McCubbin et al., 1985; Russouw et al., 1995, 1997; Eom et al., 1996). Supporting these ideas, Figure 13 showed the disordered nature of the group I LEA proteins, LEA 1.1 and LEA 1.3 in the hydrated state. Most natively unfolded proteins form interactions between water molecules and the protein in solution. Hence, they fail to obtain defined structures (Shih et al., 2004). Considering the hydrophilic nature and random coil structure of group I LEA proteins, some researchers have proposed that these proteins may serve as water-binding proteins and thereby minimize water loss (McCubbin et al., 1985; Roberts et al., 1993). This would help to act as hydration buffers to regulate the cellular water status (Dure 1993a; Walters et al., 1997), or to interact with the surface of macromolecules as a 'water matrix' to prevent denaturation during desiccation (McCubbin et al., 1985; Cuming, 1999). Therefore it is possible that in presence of LEA proteins, solutions may freeze and form ice crystals at a lower temperature due to the fact that LEA proteins may serve as water-binding proteins and thereby minimize water loss (McCubbin et al., 1985; Roberts et al., 1993). In another way, this minimized water loss can be explained as a reduction in the water losing rate. Therefore, LEA protein might help to minimize the freezing damage to biomolecules that has to be cryopreserved. Supporting this idea the experiments conducted using a cryomicroscope have shown that 40 mM HEPES buffer froze at -14.4°C (SE ± 0.11) showing a rectangular ice crystal shape (Figure 11). When the same buffer froze in the presence of 0.050 mg/ml LEA 1.1 protein, it started to form ice crystals at -17.3°C (SE ± 0.06) (Figure 12) changing the ice crystal shape from rectangular to more pentagonal shape. These

observations point out that LEA 1.1 protein may interact with water molecules and possibly leads to reduce freezing point of the solution from -14.4°C to -17.3°C. In order to form ice crystals utilizing minimum energy requirement it is possible that the ice crystal shape changed from rectangular to pentagonal. Supporting these suggestions, a study has reported an antifreeze activity from a protein called dehydrin (PCA60) obtained from peach (*Prunus persica*) (Wisniewski et al., 1999). Dehydrins are hydrophilic, heat stable proteins which are expressed under various environmental conditions that lead to cellular dehydration (Close 1997). However, future studies will be necessary to confirm these suggestions.

V. Conclusions

The conclusions of this thesis can be summarized as follows:

1. Kc167 cells expressing the LEA 1.1, LEA 1.3 and LEA 5 proteins showed a significant increase in cell viability in the presence of 200 mM trehalose after desiccation suggesting that trehalose and LEA proteins may work synergistically to confer cellular protection during desiccation.
2. LEA 1.1 protein used for the present study protected lactate dehydrogenase enzyme activity at sub-freezing temperatures.
3. Addition of 0.050mg/ml LEA 1.1 protein reduced the freezing temperature of 40 mM HEPES buffer from -14.4°C to -17.3°C and changed the ice-crystal shape from rectangular to pentagonal shape.
4. Protein foldIndex of Kyte-Doolittle plot performed for group I LEA protein, LEA 1.1 and LEA 1.3 showed that both these proteins are unfolded at its native stage while the LEA 5 protein is likely folded protein in aqueous solution.

VI. Work Cited

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