June 2020

Insights into Desiccation Tolerance: Properties of Late Embryogenesis Abundant Proteins from Embryos of Artemia franciscana

Blase Matthew LeBlanc
Louisiana State University and Agricultural and Mechanical College

INSIGHTS INTO DESICCATION TOLERANCE: PROPERTIES OF LATE EMBRYOGENESIS ABUNDANT PROTEINS FROM EMBRYOS OF *ARTEMIA FRANCISCANA*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by
Blase Matthew LeBlanc
B.S., Louisiana State University, 2015
August 2020

To my parents, Nelson and Tammy, for providing me with these great opportunities

ACKNOWLEDGEMENTS

My greatest thanks are to Dr. Steven Hand, who guided me through my graduate career with patience, understanding, and sound advice in all areas. Without his help, none of this research would have been possible. Secondly, I would like to thank my senior graduate students from Hand Lab for welcoming me and helping to fine-tune my skills over the years. Specific thanks are extended to Dr. Michael Menze (University of Louisville) and his lab for their collaboration and insights into various aspects of my research. I would like to acknowledge Dr. Brad Marden (Research Division, Great Salt Lake Artemia, Ogden, Utah) for his help in collecting diapause embryos of *A. franciscana* from the Great Salt Lake which were used throughout my project. For their invaluable help with localization studies, I would like to thank Dr. David Burk (LSU Shared Instrumentation Facility) and the LSU School of Veterinary Medicine Histology Lab. Dr. Rafael Cueto is acknowledged for his assistance with dynamic light scattering measurements of liposomes. This research was supported by National Science Foundation grants IOS-1457061/IOS-1659970.

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ABSTRACT

LEA proteins are a family of intrinsically disordered proteins that are expressed in various life stages of anhydrobiotic organisms and have been strongly associated with survival during water stress. The brine shrimp *Artemia franciscana* is the only known anhydrobiotic animal that expresses LEA proteins from Groups 1, 3, and 6. Here, I report that AfrLEA6, a novel Group 6 LEA protein, is most highly expressed in embryos during diapause and decreases throughout pre-emergence development. Notably, there is an acute drop in expression upon termination of the diapause state and the titer of AfrLEA6 during diapause is 10-fold lower than values reported for Group 3 LEA proteins in *A. franciscana*. Investigations of the secondary structure of AfrLEA6 support classification as an intrinsically disordered protein. Drying the protein or exposure to sodium dodecyl sulfate (SDS) or trifluoroethanol (TFE) promote a large gain in secondary structure of AfrLEA6, predominated by α-helix and exhibiting minimal β-sheet structure. Physiological concentrations of trehalose, a non-reducing disaccharide known to drive protein folding, do not affect the circular dichroism spectra of AfrLEA6, AfrLEA2, or AfrLEA3m in the aqueous state. Furthermore, AfrLEA6 protects enzymes and liposomes from damage during drying. AfrLEA6 protects cytoplasmic enzymes lactate dehydrogenase and phosphofructokinase but not the mitochondrial enzyme citrate synthase; in some cases, the addition of trehalose improves protection. AfrLEA6 weakly stabilizes liposomes simulating the inner leaflet of the plasma membrane, outer mitochondrial membrane, or inner mitochondrial membrane during drying; overall protection is less than with Group 3 LEA proteins. Results of immunohistochemistry support a cytoplasmic localization of AfrLEA6. Finally, an investigation into the structure and function of AfrLEA2 at intermediate water contents reveals a protective role for LEA proteins across a range of hydration states. The ability of AfrLEA2 to stabilize PFK

during drying coincides with the gain of α-helix secondary structure as the relative dryness of the sample increases. These findings underscore the concept that multiple LEA proteins exist at different amounts and within different cellular compartments within a single organism and may act together to protect the organism during water stress.

CHAPTER 1
INTRODUCTION

1.1. Background and significance

Maintaining homeostasis during dehydration that occurs via evaporative water loss in arid climates, freezing during winter in temperate regions, and osmotic variation in aquatic ecosystems is a necessity for the survival of many organisms (Crowe et al. 1997; Crowe and Clegg 1973; Crowe and Clegg 1978; Crowe et al. 1992; Yancey 2005; Yancey et al. 1982). Without mechanisms for preserving key biological structures, desiccation leads to protein denaturation and aggregation (Goyal et al. 2005; Tapia and Koshland 2014), fusion and leakage of membranes (Crowe et al. 1992; Hoekstra et al. 2001), and damage to nucleic acids (Dinakar and Bartels 2012; Gusev et al. 2010; Leprince et al. 1995). Organisms typically avoid desiccation through osmoregulation, behavioral adaptations, storage of excess water, and the usage of specialized epithelia to prevent evaporative water loss. Certain organisms, however, have the ability to survive extended periods of almost complete desiccation in a state that is termed anhydrobiosis, or life without water (Crowe et al. 1997; Keilin 1959). The brine shrimp *Artemia franciscana* is one example of an anhydrobiont that is commonly used as a model species for studying desiccation tolerance in animals.

One mechanism for desiccation tolerance in many anhydrobionts is the accumulation of protective molecules such as late embryogenesis abundant (LEA) proteins, heat-shock proteins, and stabilizing organic solutes such as trehalose (Clegg 2005; Clegg 2011; Crowe et al. 1998; Crowe et al. 2005; Hand et al. 2011; Hand et al. 2018; Tan and MacRae 2018; Tapia and Koshland 2014; Tunnacliffe and Wise 2007). *A. franciscana* accumulates both trehalose (Carpenter et al. 1986) and LEA proteins (Hand et al. 2007; Menze et al. 2009; Warner et al. 2012; Warner et al. 2010). While the larval and adult stages are susceptible to damage from

desiccation, embryos are routinely able to survive almost complete desiccation (Clegg 2005; Crowe and Clegg 1978). When the adult females receive environmental cues such as cold temperatures, reduced photoperiod, and limited food availability they begin to release encysted embryos in a state of arrested metabolism known as diapause. The state of diapause is a programmed arrest of development that is controlled by endogenous physiological factors and may or may not involve a substantial depression of metabolism (Hand et al. 2016 and references therein). Entering diapause is a common mechanism for overwintering, because life cycle delays are beneficial for species encountering suboptimal environments. Once favorable conditions return, the embryos resume development, and adult females shift their reproductive strategy back to releasing free-swimming nauplii (Clegg and Conte 1980).

LEA proteins are a family of intrinsically disordered proteins that are expressed in developmental and/or adult life stages of anhydrobiotic organisms and have been strongly associated with survival during water stress. Before identification in anhydrobiotic animals, LEA proteins were first described in wheat (Cuming and Lane 1979) and cotton seeds (Dure et al. 1981; Galau and Dure 1981). The term “late embryogenesis abundant” was first introduced into the literature by Galau et al. (1986) due to the abundance of mRNA during late embryogenesis of cotton seeds. Initially, LEA proteins were classified into five groups based on primary structure analysis (Dure et al. 1989). With more recent bioinformatics and computational analyses, a new classification of LEA proteins into six groups was proposed which holds relevance to date (Wise 2003). Although initially studied in plants (Cuming 1999; Shih et al. 2004; Tunnacliffe and Wise 2007), LEA proteins have been found in fungi (Abba et al. 2006), animals such as nematodes (Browne et al. 2002; Gal et al. 2004; Solomon et al. 2000), brine shrimp (Hand et al. 2007), African chironomid insect larvae (Gusev et al. 2014; Kikawada et al. 2006), rotifers (Denekamp

et al. 2010; Tunnacliffe et al. 2005), springtails (Clark et al. 2007), tardigrades (Forster et al. 2009), and bacteria (Battista et al. 2001; Rodriguez-Salazar et al. 2017; Stacy and Aalen 1998). Within these organisms, LEA proteins are present across a range of subcellular compartments including the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondrion and chloroplast (Avelange-Macherel et al. 2018; Boswell and Hand 2014; Browne et al. 2002; Hand et al. 2011; Tripathi et al. 2012; Tunnacliffe et al. 2010; Tunnacliffe and Wise 2007). It is common for multiple groups of LEA proteins to exist in anhydrobiotic animals (Candat et al. 2014; Warner et al. 2012; Warner et al. 2010), In fact, *A. franciscana* is a unique anhydrobiotic organism that is the only currently known animal that expresses LEA proteins from groups 1, 3, and 6 (Hand and Menze 2015; Tunnacliffe and Wise 2007; Warner et al. 2012; Warner et al. 2010).

The first Group 6 LEA protein identified was PvLEA18 from the bean plant *Phaseolus vulgaris*, where it accumulates in the embryo radicle during early germination (Colmenero-Flores et al. 1997). Nomenclature varies (e.g. D34) when describing Group 6 LEA proteins, although all Group 6 LEA proteins are classified based on the presence of the seed maturation protein (SMP) PFAM domain PF04927. Furthermore, there has been debate over whether to include Group 6 in the LEA protein family due to their lower overall hydrophilicity and sequence differences which suggests they may have different structures and functions compared to compared to Groups 1-3 (Battaglia et al. 2008; Cuming 1999; Tunnacliffe and Wise 2007). Indeed, early *in vitro* studies showed that PvLEA18 did not have the same stabilizing effect on target enzymes during desiccation as seen with other LEA protein groups (Reyes et al. 2005). However, SMP's in plants have been associated with desiccation tolerance in seeds (Boucher et al. 2010; Chatelain et al. 2012). In *Medicago truncatula*, the SMP D34.3, accumulates in the mature seed and is correlated with long-term viability of the seed in the dried state (Chatelain et

al. 2012). The first Group 6 LEA protein from animals was identified in *A. franciscana*; termed AfrLEA6, it exhibits strong sequence homologies to D34.3 and has been recently cloned, sequenced, and expressed (Hand and Menze 2015; Janis et al. 2017; Wu et al. 2011). AfrLEA6 exhibits the lowest hydrophilicity of the LEA proteins thus far characterized in *A. franciscana* and more closely resembles the Group 6 LEA protein *Mt*PM25 (Boucher et al. 2010; Janis et al. 2017). Other defining features of AfrLEA6 include two SMP domains (pfam family PF04927, with consensus sequence pVtpeDAaavqaAEaraageartapgGvAaaaqaAAdaNer; Jaspard et al. 2012) and a proline rich region, which are predicted to be sites of self-interaction (Janis et al. 2017).

Although the exact roles of LEA proteins in desiccation tolerance are still being investigated, it has been clearly shown that LEA protein expression is linked to an organism's ability to survive desiccation (Browne et al. 2004; Gal et al. 2004; Hand et al. 2007; Menze et al. 2009). Many *in vitro* and *in vivo* studies have contributed further evidence for LEA proteins protecting desiccation sensitive structures (for reviews, see Hand et al. 2011; Hincha and Thalhammer 2012; Janis et al. 2018; Tunnacliffe and Wise 2007). For example, LEA proteins have been shown to protect lipid bilayers of various compositions during drying and freezing (Hundertmark et al. 2011; Moore and Hand 2016; Moore et al. 2016; Navarro-Retamal et al. 2018; Steponkus et al. 1998; Thalhammer et al. 2014; Tolleter et al. 2010; Tolleter et al. 2007), preserve the activity of target enzymes (Boswell et al. 2014a; Goyal et al. 2005; Grelet et al. 2005; Popova et al. 2015), and prevent protein aggregation ('molecular shielding'; Boucher et al. 2010; Goyal et al. 2005; Yuen et al. 2019). Several predictions have been made as to how LEA proteins aid in desiccation tolerance, which include stabilization of vitrified sugar glasses (Hoekstra 2005; Wolkers et al. 2001), sequestering divalent ions (Grelet et al. 2005), and

forming structural networks to resist physical stress (Goyal et al. 2003). Additionally, LEA proteins may act as a molecular shield to sterically reduce the formation of damaging protein aggregates during water stress (Goyal et al. 2005). This molecular shield activity may arise due to the high hydrophilicity and consequently, the disordered structure of LEA proteins in solution. The gain of secondary structure (α-helix, β-sheet, turns) that accompanies the dehydration of some LEA proteins may allow them to perform separate/additional roles in the dried state (Goyal et al. 2003). As water is removed, an increase in secondary structures such as α-helices, β-sheets, and hairpin loops are formed (Hand et al. 2011). In the case of Group 3 LEA proteins from *A. franciscana* (AfrLEA2 and AfrLEA3m) removal of water results in an increase in predominantly α-helix structure (Boswell et al. 2014a). Finally, synergistic effects of combining trehalose, an established stabilizer of cellular components during drying and freezing (Crowe et al. 1998; Crowe et al. 1987; Tapia and Koshland 2014) with LEA proteins have been documented (Boswell et al. 2014a; Goyal et al. 2005).

The disaccharide trehalose is a non-reducing, intracellular organic solute that is accumulated in many anhydrobiotic organisms and plays a protective role during desiccation (Crowe and Madin 1974; Tapia and Koshland 2014; Yancey et al. 1982). Although highly associated with desiccation tolerance, there are anhydrobiotic bdelloid rotifers (Caprioli et al. 2004; Tunnacliffe et al. 2005) and some tardigrades (Hengherr et al. 2008) which do not accumulate trehalose. Compared to other naturally occurring carbohydrates, trehalose has a greater ability to protect macromolecules during dehydration (Crowe et al. 1998; Crowe et al. 2005) due in part to its α 1,1 glycosidic bond which is unique among naturally occurring disaccharides (Albertorio et al. 2007). Trehalose is capable of forming vitrified sugar glasses during dehydration rather than crystals. In a glassy state, macromolecules are held in their native

conformation and avoid the denaturation typically associated with dehydration (Crowe et al. 1998). Of the naturally occurring carbohydrates, trehalose has the highest glass transition temperature (T_g). Additionally, trehalose can resist the drop in glass transition temperature seen in other vitrified sugars when exposed to small amounts of water, high humidity, or high temperature (Crowe et al. 1998). These properties make trehalose an ideal sugar for maintaining a glassy state. It has also been proposed that trehalose acts as a water replacer during drying, which prevents the typical denaturation stress experienced by proteins when losing their hydration shell (Crowe et al. 1987; Webb 1965). In addition to protecting proteins, trehalose has been shown to stabilize biological membranes during drying when present on both sides of the phospholipid bilayer (Chen et al. 2001). In the absence of specific transporters, trehalose is impermeable to membranes and must be loaded artificially into cells. Methods of membrane permeabilization vary depending on the cell or organelle. In isolated mitochondria, the mitochondrial permeability transition pore can be opened to allow trehalose loading (Liu et al. 2005). Exogenous trehalose uptake can also be achieved through the transfection of a trehalose transporter TRET1 (trehalose transporter 1) which was originally cloned from *P. vanderplankii* (Kikawada et al. 2007). Lastly, electroporation and $P2X_7$ receptor channels have successfully been utilized to load trehalose (Elliott et al. 2006; Zhou et al. 2010). Once present, trehalose is thought to stabilize phospholipid head groups and prevent lipid phase transitions during rehydration through the "water replacement hypothesis" mentioned previously (Crowe et al. 1987; Webb 1965). The ability of trehalose to stabilize proteins (Carpenter et al. 1986; Carpenter et al. 1987) and DNA (Zhang et al. 2017) in the dried state has also been documented.

1.2. Research aims of this dissertation

The primary objective of this research is to investigate and characterize the features of a novel Group 6 LEA protein found in embryos of *A. franciscana*. Overall, I aim to advance our knowledge on the role of both Group 3 and Group 6 LEA proteins in desiccation tolerance. Chapter 2 focuses on expressing and purifying AfrLEA6 by using intein-mediated purification with an affinity chitin binding tag (IMPACT). Purification allows for subsequent production of custom polyclonal antibodies against AfrLEA6 which can be used to detect endogenous protein across developmental time points in embryos. Western blot analysis of diapause and pre-emergence embryos of *A. franciscana* is used to quantify AfrLEA6 throughout early development of the embryo. Furthermore, I use circular dichroism spectroscopy to investigate the secondary structure of AfrLEA6 in the hydrated and dried states as well as in the presence of physiological concentrations of trehalose. Likewise, I investigate the secondary structure of AfrLEA2 and AfrLEA3m while in the hydrated state containing physiological concentrations of trehalose.

The aim of chapter 3 is to determine whether AfrLEA6 can stabilize target enzymes and membranes against damage from desiccation. Target enzymes are chosen based on their sensitivity to desiccation as well as the cellular compartments from which they originate. To assess protection of membranes, liposomes are created with lipid mass ratios that simulate a variety of biological membranes. With this experimental design, I can evaluate whether AfrLEA6 provides universal or specific protection to enzymes or membranes. Additionally, I investigate whether the presence of trehalose impacts the protection offered by AfrLEA6 under each circumstance.

Finally, the objective of chapter 4 is to determine the subcellular location of AfrLEA6 within diapause embryos. Using an antibody against AfrLEA6 and immunohistochemistry, I resolve the location of AfrLEA6 within the cell. Additionally, I aim to link the gain of secondary structure with protective properties of LEA proteins during drying to intermediate hydration states. Samples containing LEA protein and a target enzyme are incubated at various relative humidities to determine the degree to which LEA proteins protect a target enzyme as water is removed. In parallel, a LEA protein is dried at identical relative humidities and its secondary structure is assessed with circular dichroism. Until now, no studies have evaluated simultaneously the structural data and protective properties of a LEA protein at intermediate hydration states. By addressing this gap in the literature, I hope to help elucidate the mechanisms by which LEA proteins function in desiccation tolerance.

CHAPTER 2
STRUCTURAL PROPERTIES AND CELLULAR EXPRESSION OF AFRLEA6, A GROUP 6 LATE EMBRYOGENESIS ABUNDANT PROTEIN FROM EMBRYOS OF *ARTEMIA FRANCISCANA*[1]

2.1. Introduction

Anhydrobiotic animals like the brine shrimp *Artemia franciscana* tolerate major transitions in water content at specific points during their life cycles. During anhydrobiosis tissue water can decrease to less than 2% (Crowe and Madin 1974), which imposes substantial challenges for defending the functionality of biological structures (Crowe et al. 1997; Crowe and Clegg 1973; Crowe and Clegg 1978). One key strategy for desiccation tolerance in many anhydrobiotes is the accumulation of protective molecules such as late embryogenesis abundant (LEA) proteins, heat-shock proteins, and stabilizing organic solutes (Clegg 2005; Clegg 2011; Crowe et al. 1998; Crowe et al. 2005; Hand et al. 2011; Hand et al. 2018; Tan and MacRae 2018; Tapia and Koshland 2014; Tunnacliffe and Wise 2007). LEA proteins are a family of intrinsically disordered proteins (IDPs) that are expressed in developmental and/or adult life stages of anhydrobiotic organisms and have been strongly associated with survival during water stress. In this chapter, I investigate selected structural properties of AfrLEA6 and provide evidence for its expression in the diapause state and throughout post-diapause development of the embryo to the larval stage.

Originally discovered in plants, LEA proteins have now been identified in animals such as nematodes (Browne et al. 2002; Gal et al. 2004; Solomon et al. 2000), brine shrimp (Hand et al. 2007), African chironomid insect larvae (Gusev et al. 2014; Kikawada et al. 2006), rotifers (Denekamp et al. 2010; Tunnacliffe et al. 2005), as well as bacteria (Battista et al. 2001; Rodriguez-Salazar et al. 2017; Stacy and Aalen 1998). Similar tardigrade-specific intrinsically

[1]This chapter was adapted by permission from Springer Nature: "LeBlanc B.M., Le M.T., Janis B., Menze M.A., Hand S.C. (2019) Structural properties and cellular expression of AfrLEA6, a group 6 late embryogenesis abundant protein from embryos of Artemia franciscana. *Cell Stress Chaperon* 24:979-990" ©2019

disordered proteins have also been described (Boothby and Pielak 2017; Boothby et al. 2017). Within these organisms, LEA proteins are present across a range of subcellular compartments including the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondrion and chloroplast (Avelange-Macherel et al. 2018; Boswell and Hand 2014; Hand et al. 2011; Tripathi et al. 2012; Tunnacliffe et al. 2010; Tunnacliffe and Wise 2007). Classification schemes places them into six groups, of which *A. franciscana* uniquely expresses LEA proteins from three different groups (1, 3, and 6; Hand and Menze 2015; Tunnacliffe and Wise 2007; Warner et al. 2010, 2012).

While many questions still exist in terms of the varied functions of LEA proteins, *in vitro* and *in vivo* studies have contributed insights into their roles (for reviews, see Hand et al. 2011; Hincha and Thalhammer 2012; Janis et al. 2018; Tunnacliffe and Wise 2007). For example, LEA proteins have been shown to protect lipid bilayers of various compositions during drying and freezing (Goyal et al. 2005; Grelet et al. 2005; Hundertmark et al. 2011; Moore and Hand 2016; Moore et al. 2016; Navarro-Retamal et al. 2018; Popova et al. 2015; Steponkus et al. 1998; Thalhammer et al. 2014; Tolleter et al. 2010; Tolleter et al. 2007), preserve the activity of target enzymes (Boswell et al. 2014a; Goyal et al. 2005; Grelet et al. 2005; Popova et al. 2015), and prevent protein aggregation ('molecular shielding'; Boucher et al. 2010; Goyal et al. 2005; Yuen et al. 2019). The gain of secondary structure (α-helix, β-sheet, turns) that accompanies the dehydration of some LEA proteins may allow them to perform separate/additional roles in the dried state (Goyal et al. 2003). Finally, synergistic effects of combining trehalose, an established stabilizer of cellular components during drying and freezing (Crowe et al. 1998; Crowe et al. 1987; Tapia and Koshland 2014) with LEA proteins have been documented (Boswell et al. 2014a; Goyal et al. 2005).

The state of diapause is a programmed arrest of development that is controlled by endogenous physiological factors and may or may not involve a substantial depression of metabolism (Hand et al. 2016 and references therein). Entering diapause is a common mechanism for overwintering, because life cycle delays are beneficial for species encountering suboptimal environments. AfrLEA6 is the only Group 6 LEA protein reported thus far from an animal, and bioinformatics analyses predict distinctly different structural features compared to other LEA proteins in *A. franciscana* (Janis et al. 2017). Consequently, I evaluated secondary structural features of AfrLEA6 with circular dichroism as a function of hydration state (with and without trehalose), along with the tissue content of the protein during diapause, immediately after diapause termination, and during post-diapause development.

2.2. Methods

Collection and incubation of diapause embryos, post-diapause embryos, and larvae

Diapause embryos were collected in 2018 in the hydrated state from the surface of the Great Salt Lake (Utah) and were a generous gift from Dr. Brad Marden (Great Salt Lake *Artemia*, LLC, Ogden UT). These embryos were verified to be in diapause by performing hatching assays as outlined in Reynolds and Hand (2004). The hatch rate of diapause embryos used in these experiments were 2.9%, indicative of a strong diapause state. Diapause embryos were rinsed in lake water and stored protected from light at ambient temperature in 1.25 M NaCl containing 200 units/ml nystatin, 50 μg/ml kanamycin, and 50 μg/ml penicillin-streptomycin. Prior to use, diapause embryos were rinsed and incubated with shaking at 110 rpm in artificial seawater (35 practical salinity units; Instant Ocean ®, Blacksburg, VA) for 4 d at room temperature in the dark to allow any embryos that had terminated diapause to hatch. Exposure to

light has been associated with increased diapause breakage in the laboratory (Lavens and Sorgeloos 1987). The hatched larvae were discarded, and the remaining diapause embryos were used for experimentation.

Commercial post-diapause embryos were obtained in the dried state from Great Salt Lake *Artemia*, LLC (Ogden, UT; grade: laboratory reference standard) and were stored frozen at -20^{o}C. Prior to use, commercial post-diapause embryos were hydrated overnight in ice-cold 0.25 M NaCl. To promote development, hydrated post-diapause embryos were incubated in 0.25 M NaCl at 23^{o}C with shaking, and embryos were sampled at 2, 4 6, and 8 h. Incubation was extended through 24 h to generate free-swimming nauplius larvae. Hatched larvae were separated from embryos by phototaxis and processed separately.

Expression and purification of recombinant LEA proteins from Artemia franciscana and antibody production

The original nucleic acid sequence for AfrLEA6 (GenBank accession no. MH351624) from *A. franciscana* embryos was amplified from existing *A. franciscana* cDNA library. Primer sequences used were 5’ggcggcggccatatgATGTCTGAGAATATTGGTCATATTAACATAAATGC and 5’attagtaactagtAGTGCATCTCCCGTGATGCAGTCCATGCGGACATTCCCAAT. For the polymerase chain reaction, Q5 DNA polymerase (New England Biolabs, Ipswich, MA) was used for 25 cycles following the instructions of the manufacturer, and the PCR product obtained was cloned into pTXB1 using standard techniques and the two restriction enzymes Nde1 and Spe1 (New England Biolabs, Ipswich, MA). Recombinant AfrLEA6 was purified from *E. coli* cells (BL21) designed to work with the IMPACT protein expression system (Intein-Mediated Purification with an Affinity Chitin-binding Tag; New England Biolabs Inc., Ipswich, MA). The

vector (pTXB1) was used to add a chitin binding domain and self-cleavage protein splicing elements to the expressed fusion protein. These elements permitted binding to the chitin moiety of an affinity column, and then dithiothreitol (DTT) was used to release AfrLEA6 free of any amino acid tag (see below). Bacterial cells were grown in the presence of 0.1 mM ampicillin at 37^{o}C with shaking until the optical density at 595 nm reached 0.6. Then protein expression was induced with 0.4 mM IPTG for 2 h at 37^{o}C while shaking. Cells were sedimented by centrifugation at 5,000 x g (4^{o}C) for 15 min. The cell pellets were resuspended in 40 ml of chitin column buffer (20 mM Tris, 0.5 M NaCl, pH 8.5) containing either 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St Louis, MO) or one cOmplete^{TM} protease inhibitor cocktail tablet (Roche Applied Science; Penzberg, Germany). Cells were lysed by sonication, and cellular debris was removed by centrifugation at 20,000 x g (4^{o}C) for 30 min. The supernatant was then loaded onto a chitin resin column (S6651S; New England BioLabs Inc.; Ipswich, MA) by gravity flow (~1.0 ml/min). On-column cleavage was induced by incubating the column for 48 h with chitin column buffer containing 50 mM DTT at 4^{o}C. AfrLEA6 was eluted using chitin column buffer and dialyzed overnight against anion exchange column buffer (15 mM triethanolamine, 10 mM NaCl, pH 7.0). For further purification, the protein sample was applied to a 5 ml anion exchange column (HiTrap Q FF; GE Healthcare; Chicago, IL) using an AKTAprime plus FPLC system at a flow rate of 1.0 ml/min. After washing with 30 volumes of anion exchange column buffer, bound protein was eluted using a 50 ml linear gradient of 0-25% elution buffer (15 mM triethanolamine, 1 M NaCl, pH 7.0). The eluted protein peak was detected by absorbance at 280 nm and collected in 1 ml fractions. Pooled samples were exchanged into 10 mM potassium phosphate buffer (pH 7.5) and concentrated using Amicon Ultra-15 10K centrifugal filters (MilliporeSigma; Burlington, MA). The techniques for expressing and purifying recombinant

AfrLEA2 and AfrLEA3m followed the procedures in Boswell et al. (2014a). Polyclonal antibody against the purified recombinant AfrLEA6 was raised in chicken eggs by Aves Labs, Inc. (Tigard, OR, USA).

Circular dichroism spectroscopy

Spectra were obtained with a Jasco J-815 spectropolarimeter and analyzed using Spectra Manager™ Suite (Jasco Analytical Instruments; Easton, OH). Samples were prepared in 10 mM potassium phosphate buffer, pH 7.5, at a protein concentration of 0.15 mg/ml for AfrLEA2, AfrLEA3m, AfrLEA6, and bovine serum albumin (BSA; Sigma; A6003). For aqueous protein samples, measurements were recorded between 190-250 nm using a quartz cuvette with a 0.1 cm pathlength. Dried samples were prepared by air drying a 50 µl droplet of sample containing 0.15 mg/ml protein onto one side of a demountable quartz cuvette overnight in a dry box containing desiccant (Drierite; W. A. Hammond Drierite, Xenia, OH). For conversion of spectra for dried samples to mean residue ellipticity, a pathlength of 0.01 cm was used. Spectra for blank samples that lacked protein were collected for each experimental condition and subtracted from the respective protein spectra. Each spectrum was then converted to mean residue ellipticity and smoothed with a convolution width of 9 (Savitzky and Golay 1964). Spectra Manager™ Suite was used to subtract blank spectra, convert values to mean residue ellipticity, and smooth the curves. Analysis of secondary structure was achieved using DICHROWEB online analysis software (Whitmore and Wallace 2004; Whitmore and Wallace 2008) to apply the CONTINLL algorithm (Provencher and Glockner 1981; van Stokkum et al. 1990) and SELCON3 algorithm (Sreerama et al. 1999; Sreerama and Woody 1993). Reference dataset 7 was utilized for spectra analyses as it applies to denatured proteins with measurements between 190-250 nm wavelengths (Sreerama et al. 1999).

Quantification of AfrLEA6 expression by Western Blot analysis

Diapause embryos, post-diapause embryos (0, 2, 4, 6, and 8 h), and 24 h free-swimming nauplii were used to analyze AfrLEA6 across development. Extracts were prepared by homogenizing 100 mg of embryos or larvae in 1.9 ml of 1X Laemmli buffer with a ground-glass homogenizer. Samples were heat treated at 95^{o}C for 10 min after homogenization and then centrifuged at 20,000 x g for 20 min to remove insoluble cell debris. The supernatant was collected and frozen at -20^{o}C prior to analysis. Total protein was measured with a modified Lowry assay as described by (Peterson 1977). Equal amounts of protein (40 µg) were loaded for each time point onto an SDS polyacrylamide gel (4% stacking gel, 11% resolving gel). A standard curve was prepared with 0-40 ng of purified, recombinant AfrLEA6 to quantify AfrLEA6 across the developmental stages. Electrophoresis was performed at 125 V for 80 min in a Bio-Rad Mini Protean 3 cell. Following electrophoresis, samples were transferred to a nitrocellulose membrane at 80 V for 60 min in Towbin's buffer (25 mM tris, 192 mM glycine, 20% v/v methanol, 0.025% SDS). The nitrocellulose membranes were then blocked for one hour in 5% fat-free dry milk prepared in TBS-T (20 mM Tris, 500 mM NaCl, 0.1 % Tween 20, pH 7.6) at room temperature with rocking. The blots were then incubated overnight at 4^{o}C with AfrLEA6 polyclonal antibody (raised in chicken eggs, Aves Labs Inc.) diluted 1:50,000 in 5% dry milk prepared in TBS-T. Because α-tubulin served as a loading control, blots were incubated simultaneously with α-tubulin primary antibody raised in rabbits (Cell Signaling Technology, Danvers, MA) at a 1:1,000 dilution. Afterwards, membranes were given three 15-min rinses in TBS-T with shaking. The blots were incubated at room temperature for 1 h with two horseradish peroxide-labeled (HRP) secondary antibodies: goat anti-chicken (AvesLabs Inc., Tigard OR) at a 1:10,000 dilution and mouse anti-rabbit (Cell Signaling Technologies; Danvers, MA) at a 1:1000

dilution. The blots were developed using an Amersham ECL Prime Western Blotting Detection Kit (GE Healthcare; Chicago, IL) and imaged with a Bio-Rad ChemiDoc™ XRS+ Imager. Bio-Rad Image Lab™ Software was used for quantifying bands. Values for AfrLEA6 expression were converted to mg AfrLEA6 protein per ml embryo water by calculating the total mg of AfrLEA6 present per gram embryo (hydrated and blotted as above) and then dividing by the corrected water content of metabolically-active embryos (0.573, i.e., 57.3% embryo water) as published in Glasheen and Hand (1989). Because histochemical localization (see chapter 4) indicates AfrLEA6 is a cytoplasmic protein, this concentration unit is appropriate for estimating its effective titer in the cell.

Statistical analyses

Analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, New York, USA). Levene's test was performed to check for equal variance among data sets. To determine significant differences among treatments, a one-way ANOVA was applied. For post-hoc tests used in pair-wise comparisons, Tukey Honestly Significant Difference (HSD) was chosen.

2.3. Results

Purification of recombinant AfrLEA6

Fractions containing protein eluted from the chitin column after treatment with DTT were analyzed via SDS-PAGE to estimate purity of recombinant AfrLEA6, which has a calculated molecular mass of 27.0 kDa. Several contaminating proteins were present in the eluent with estimated molecular masses of ~50 kDa, ~60 kDa, and ~20 kDa. Heat treatment and anion exchange chromatography were investigated as techniques to remove the contaminating proteins and further enrich AfrLEA6. Heat treatment followed by centrifugation was ineffective at

precipitating these contaminating proteins (Fig. 2.1 A). Anion exchange chromatography with a 0-250 mM NaCl gradient eluted a protein peak between 130-180 mM NaCl. The leading edge of the peak (130-150 mM NaCl) contained AfrLEA6 without visible contaminating bands; the purity of the pooled fractions was sufficient for production of AfrLEA6 primary antibody (Fig. 1A). The trailing edge of the peak at about 160-180 mM NaCl (Fig. 2.1 A) showed contamination. The removed contaminants likely included the intact fusion protein composed of AfrLEA6 bound to the intein (22 kDa) and the chitin binding domain protein (CBD, 6 kDa), along with free intein and intein fused with CBD. A Western blot of purified AfrLEA6 versus the native protein in an embryo extract documented equivalent migration at approximately 27 kDa (Fig. 2.1 B).

Secondary structure of LEA proteins

The secondary structure of AfrLEA6 was investigated under various solution conditions and in the dried state. AfrLEA6 exists as an intrinsically disordered protein in solution with a minimum ellipticity of 200 nm that is characteristic of disordered, random-coil proteins (Fig. 2.2 A). When interactions with water are decreased by drying or by the addition of 70% trifluoroethanol (TFE; a chemical de-solvating agent), AfrLEA6 gains significant secondary structure (α-helix, β-sheet, turns). A similar outcome was promoted by adding 2% SDS. A substantial increase in the proportion of α-helix was indicated in the CD spectra by double minima at 208 and 222 nm and a maximum at 191 nm (Fig. 2.2 A). The proportion of α-helices increased from 3.9% in the aqueous state to 47.1% in the dried state (Fig. 2.2 B). Similarly, the proportion of α-helix structure increased from 3.9% to 34% and 44.3% after the additions of 2%

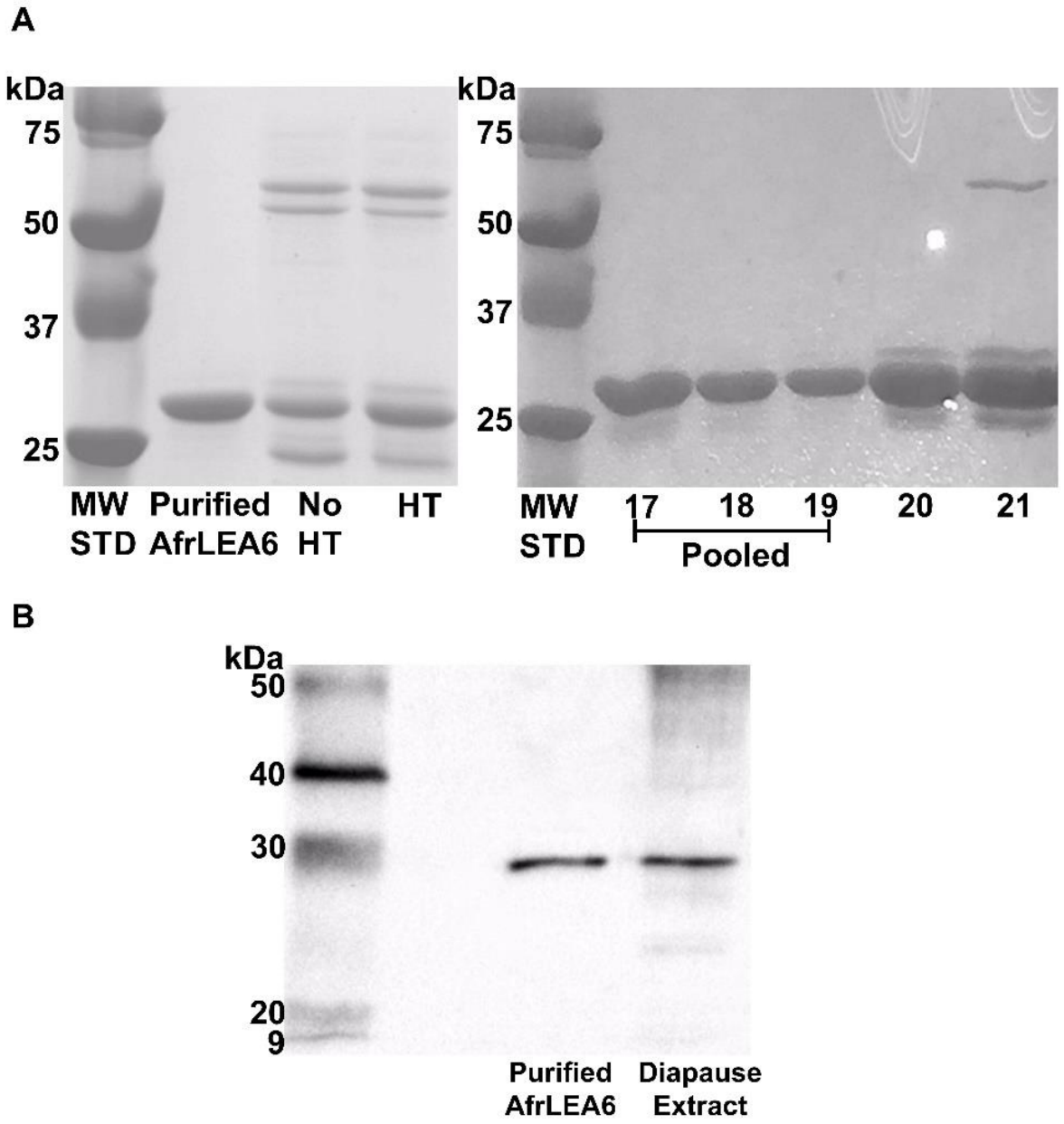

Figure 2.1. Purification of recombinant AfrLEA6. (A) *Left panel*: SDS-PAGE analysis of purified protein after IMPACT column chromatography combined with anion exchange chromatography. Evidence that heat treatment (HT) is ineffective for removing contaminants present after IMPACT chromatography alone. Total protein loaded per lane was 40 µg. MW STD = ladder of molecular weight standards. *Right panel*: SDS-PAGE analysis of 1-ml fractions eluted from the anion exchange column (HiTrap Q FF resin) that contained AfrLEA6. Contamination is present in the trailing edge of the peak, which was not pooled. Each lane was loaded with 10 µl of eluant. (B) Western blot of purified, recombinant AfrLEA6 versus the native protein in a diapause embryo extract shows equivalent migration. Approximately 40 ng of purified AfrLEA6 and 40 µg total protein from an embryo extract prepared in Laemmli buffer were loaded in respective lanes. Ladder of MW standards shown at left.

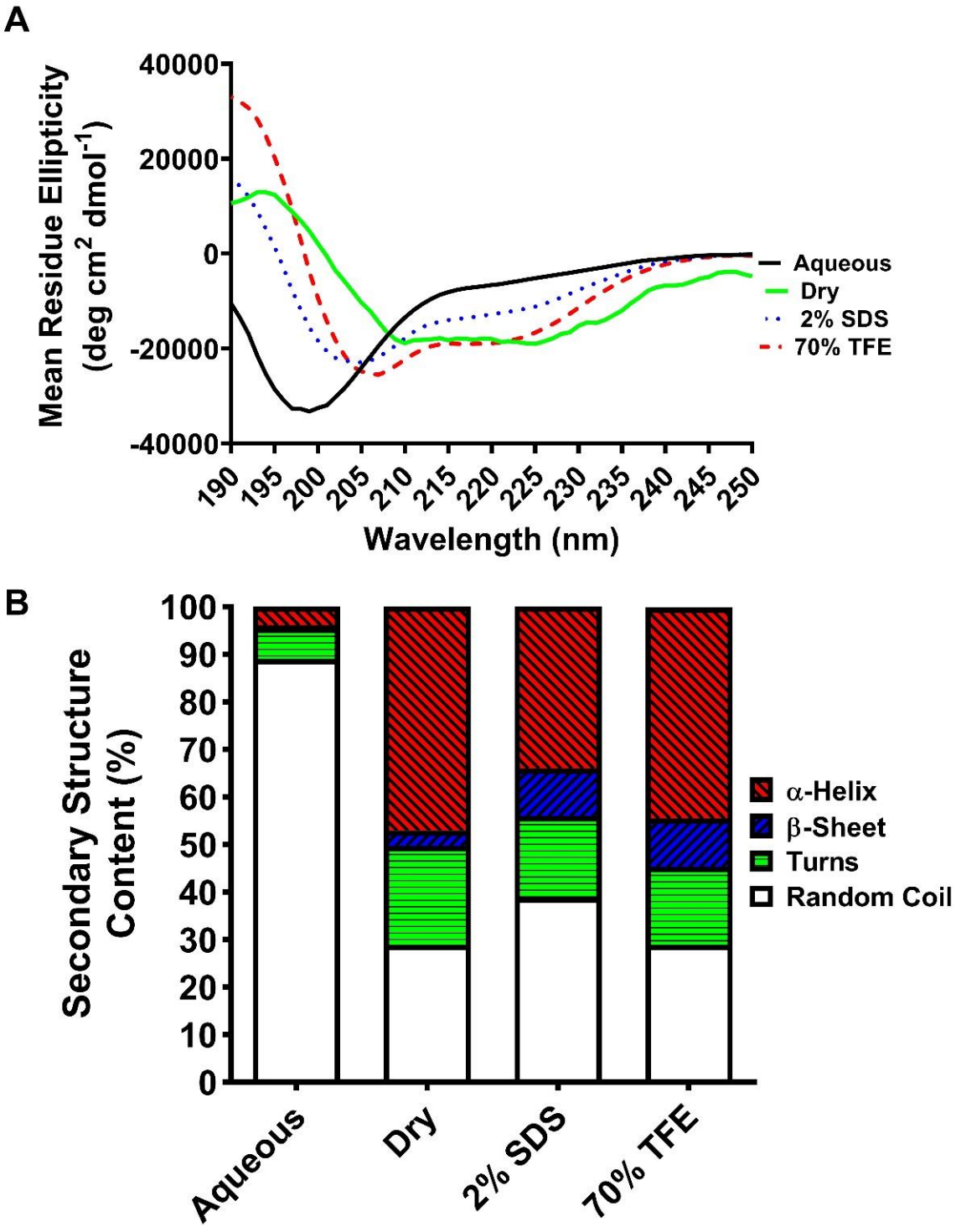

Figure 2.2. (A) Circular dichroism (CD) spectra of 0.15 mg/ml AfrLEA6 in the aqueous and dried state as well as in the presence of 2% SDS and 70% TFE. Lines smoothed via the Savitzky-Golay method with convolution width 9. (B) Dichroweb analysis of secondary structures in CD spectra of AfrLEA6 using the Contin-LL method (see text).

SDS and 70% TFE, respectively (Fig. 2.2 B). These data were substantiated by comparisons to bovine serum albumin (BSA), which is a typical globular protein known to exhibit secondary structure in solution and to AfrLEA2, which is a positive control previously shown to behave as an intrinsically disordered protein (Boswell et al. 2014a). As expected, the structure of BSA in solution was predominantly α-helix in aqueous solution and did not substantially change after treatment with 2% SDS or 70% TFE (also see Boswell et al. 2014a; Takeda et al. 1987; Fig. 2.3) . In aqueous solution, there were noteworthy differences in secondary structure between AfrLEA6 and AfrLEA2 (Fig. 2.3). Overall, AfrLEA6 exhibited a lower percentage of secondary structure (11%) when compared to AfrLEA2 (26.7%), the major difference being the reduced content of β-sheet for AfrLEA6 (0.5%) versus AfrLEA2 (15.6%). AfrLEA6 gained more total secondary structure in 70% TFE (71.1%) than in 2% SDS (61.1%), whereas the percentages for AfrLEA2 were the same across the two treatments (70 versus 71%).

Secondary structures of AfrLEA2, AfrLEA3m, and AfrLEA6 were also investigated in the aqueous state in the presence of trehalose. In embryos of *A. franciscana*, trehalose concentrations can reach at least 340 mM (Glasheen and Hand 1989) or higher, so it was of interest to evaluate whether this stabilizing organic solute might promote gain of structure in LEA proteins. In all cases, the structure of LEA proteins was unaffected by the presence of trehalose (Fig. 2.4). The CD spectra of LEA proteins remained indicative of a disordered, random-coil structure. The α-helix structure of BSA remained unchanged as well.

Protein expression of AfrLEA6 during development

The quantity of AfrLEA6 present in each developmental sample was determined by comparing band intensities to a standard curve of purified, recombinant AfrLEA6 (Fig. 2.5 A-C). These quantities were converted to units of mg LEA protein per ml embryo water. The *in vivo*

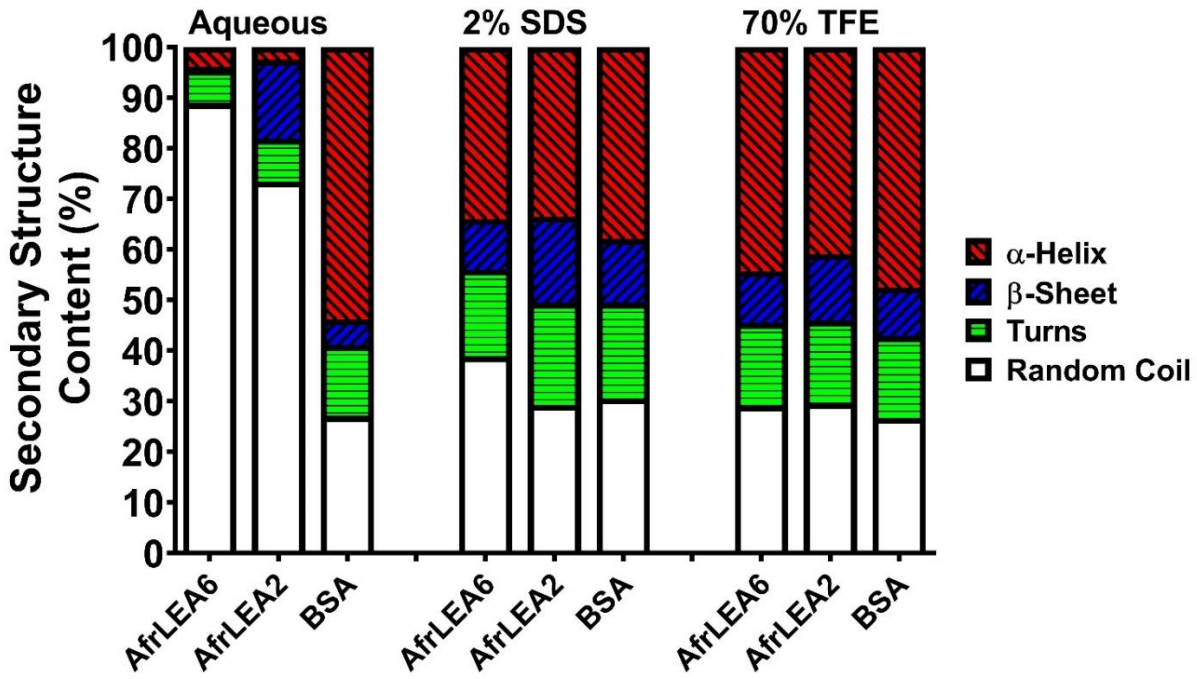

Figure 2.3. Comparison of secondary structure content for AfrLEA6, AfrLEA2, and bovine serum albumin (BSA) based on CD spectra recorded in the aqueous state, presence of 2% SDS and presence of 70% TFE. All proteins were measured at a concentration of 0.15 mg/ml. Analysis of secondary structure was performed using the Dichroweb server with the Contin-LL method (see text).

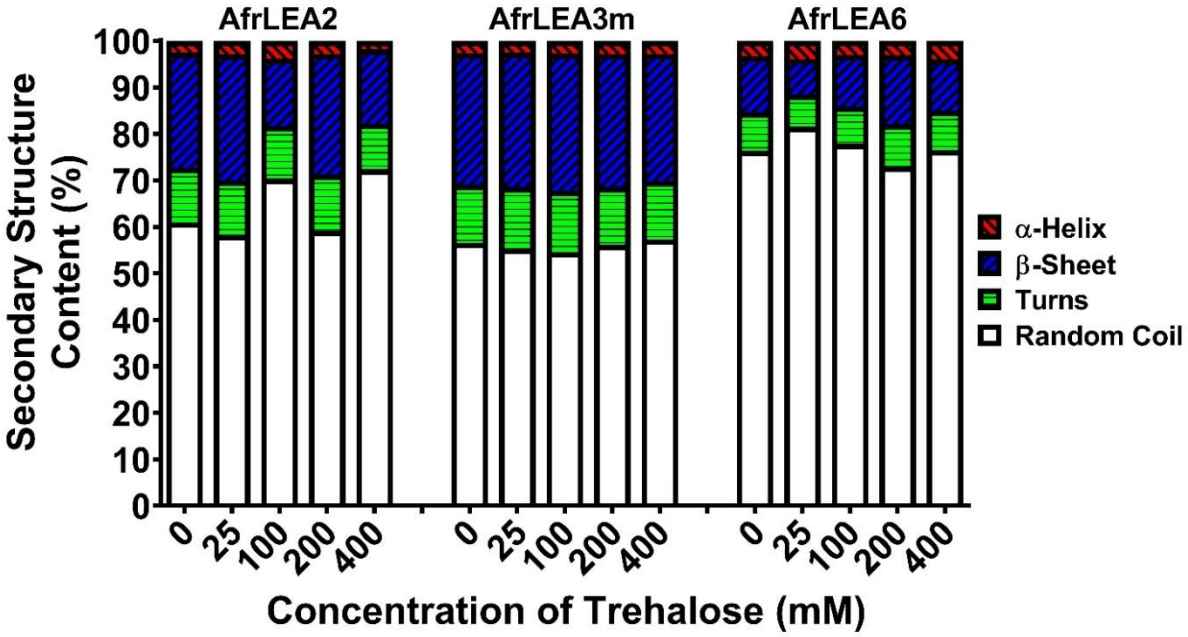

Figure 2.4. Comparison of secondary structure content for AfrLEA2, AfrLEA3m and AfrLEA6 based on CD spectra recorded in the presence of various concentrations of trehalose in the aqueous state. All proteins were measured at a concentration of 0.15 mg/ml. Analysis of secondary structure was performed using the Dichroweb server with the Contin-LL method (see text).

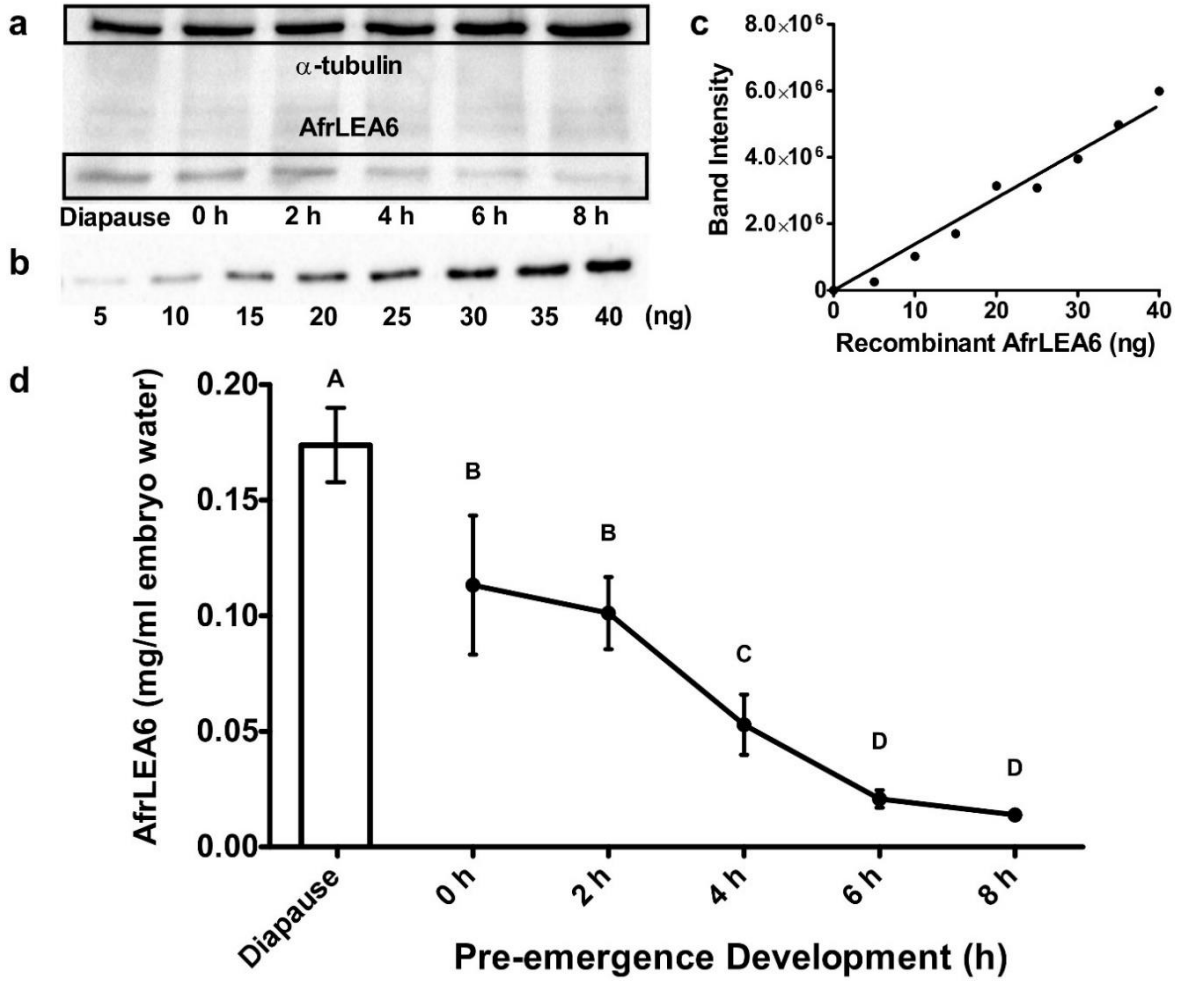

Figure 2.5. Quantification of AfrLEA6 in extracts of *A. franciscana* by Western blot analysis using an AfrLEA6 polyclonal antibody. (A) AfrLEA6 was quantified in extracts from diapause embryos and post-diapause embryos through 8 h of pre-emergence development. Total protein loaded per lane was 40 µg. α-tubulin is included as a loading control for each time point. AfrLEA6 was too low in 24 h free-swimming nauplii to reliably quantify. (B, C) Quantification of AfrLEA6 in extracts was based on a standard curve with 5-40 ng of purified recombinant AfrLEA6. Bio-Rad Image Lab™ software was used to compare band intensities. (D) AfrLEA6 concentrations from diapause throughout 8 h of pre-emergence development. All values were normalized to α-tubulin and then expressed per ml embryo water as described in Methods. Data are presented as means ± SD. Different letters above means indicate statistically significant differences (one-way ANOVA, Tukey, $P<0.01$, $n = 3$).

titer of AfrLEA6 was highest during diapause and decreased throughout pre-emergence development (Fig. 2.5 D) until it was no longer quantifiable in 24 h nauplii (data not shown). AfrLEA6 was present in diapause embryos at a concentration of 0.173 ± 0.016 (mean ± SD; *n*=3) mg per ml embryo water and decreased significantly to a concentration of 0.113 ± 0.03 (mean ± SD; *n*=3) mg per ml embryo water by 0 h of pre-emergence development – a reduction of 35% (Fig 2.5 D). By 8 h of pre-emergence development, the titer of AfrLEA6 had been reduced to 8% of the diapause values, corresponding to 0.014 ± 0.0025 (mean ± SD; *n*=3) mg per ml embryo water.

2.4. Discussion

Despite sequence and bioinformatic evidence for ArfLEA6 indicating substantially less hydrophilicity than other LEA proteins from *A. franciscana*, results presented here demonstrate that AfrLEA6 is indeed intrinsically disordered in aqueous solutions and gains structure as water is removed. Further, I show for the first time that the very high physiological concentration of trehalose known to exist in embryos of *A. franciscana* is insufficient to promote the gain of secondary structure for any of the three LEA proteins (AfrLEA2, AfrLEA3m, AfrLEA6) evaluated in aqueous solution. One might predict that the reduced hydrophilic poise of AfrLEA6 would render it more likely to initiate folding, particularly considering the strong ability of trehalose to force protein assembly/folding through preferential exclusion from the peptide backbone (Street et al. 2006; Xie and Timasheff 1997). AfrLEA6 exhibits its highest concentration *in vivo* during the diapause state, drops acutely at diapause termination, and then the content declines progressively during development to the larval stage.

CD studies indicate that AfrLEA6 is clearly intrinsically disordered in solution based on an estimated 89% content of random coil. Further the α-helix content of AfrLEA6 increased from 4% in solution to 47% when dried, 44% in TFE, and 34% in SDS. Solutions containing 2% SDS and 70% TFE have been documented to induce α-helix conformation in LEA proteins (Ismail et al. 1999; Kentsis and Sosnick 1998; Shih et al. 2012; Shih et al. 2004; Tolleter et al. 2007). In the presence of either solution, AfrLEA6 adopts a majority α-helix conformation. By comparison to Group 3 LEA proteins, Boswell et al. (2014a) estimated the total α-helix content of AfrLEA3m in the aqueous state was 2%, which increased to 41% and 36% after treatment with 2% SDS or 70% TFE, respectively. In the dried state, however, α-helix content only increased to 18%. Gains of structure were also seen for AfrLEA2 under identical conditions. However, note that recombinant AfrLEA2 and AfrLEA3m in that study contained up to 10% sequence that was atypical of the mature protein (hexa-histidine tag, amphipathic mitochondrial targeting sequence, etc.), so precise comparisons of percentages to AfrLEA6 (all native sequence due to purification by the IMPACT expression system/chitin binding column) should be limited. Recent bioinformatics predictions place AfrLEA6 at the top of the most hydrophobic LEA proteins found in *A. franciscana,* with AfrLEA2 being second. Despite its high hydrophobicity, the DisEMBL prediction of overall disorder for AfrLEA6 was 80.9% (Janis et al. 2017). In summary both experimental data and bioinformatics strongly support a classification of AfrLEA6 as an IDP.

As mentioned earlier, trehalose is a stabilizing organic solute that is present in embryonic stages of *A. franciscana.* At concentrations that are physiologically relevant to these embryos in the hydrated state (≥340 mM), trehalose has been shown to drive folding equilibrium towards the native state of globular proteins (Auton et al. 2011; Xie and Timasheff 1997). LEA protein

folding in the presence of molecular crowding agents at high concentration (≥2 M) has been successfully demonstrated (Bremer et al. 2017; Navarro-Retamal et al. 2018). However, CD spectra gathered for AfrLEA2, AfrLEA3m, and AfrLEA6 in solutions of trehalose across physiological concentrations indicate no impact of trehalose on the structure of LEA proteins or BSA. If trehalose were to play a role in promoting a gain of secondary structure in LEA proteins, it likely would be seen at higher concentrations that are approached as cellular water is removed during desiccation. Further investigations into the secondary structure of LEA proteins at intermediate hydration values, with and without trehalose, could be warranted.

Based on data presented, AfrLEA6 protein content is highest during the diapause state and undetectable in free-swimming nauplii. This pattern parallels the loss of desiccation tolerance seen at the larval stages through adults, which is consistent with a protective role of LEA proteins during times of desiccation. According to LeBlanc et al. (2019), there is an acute 38% decrease in AfrLEA6 during the termination of diapause with H_2O_2. This observation suggests that H_2O_2 triggers an immediate degradation of selected proteins, which apparently includes AfrLEA6 [H_2O_2 treatment of commercial post-diapause embryos has less impact (29% decrease; M. Le, B. LeBlanc, S. Hand, unpublished]. Disruption of key macromolecules could initiate physiological signaling events necessary for resumption of development and metabolism. For example, it has been speculated that the small heat shock protein p26 in *A. franciscana* embryos might bind proteins critical to the maintenance of diapause, or perhaps sequester a signaling protein important for diapause termination (King and MacRae 2012). Finally, considering that the cellular titer of AfrLEA6 in diapause and post-diapause embryos is approximately ten-fold lower than for AfrLEA2 and AfrLEA3m (cf. Boswell et al. 2014b),

AfrLEA6 apparently promotes its functions in *A. franciscana* at a much lower content than these Group 3 LEA proteins.

Based on sequence homologies between AfrLEA6 and SMPs that are associated with desiccation tolerance in plants (Boucher et al. 2010; Chatelain et al. 2012), potential functions of AfrLEA6 may involve the prevention and dispersion of protein aggregates during desiccation and/or their rapid dissolution during rehydration similar to *Mt*PM25. Contribution to a glassy state during desiccation as with pollen D-7 LEA protein (Wolkers et al. 2001) is also possible. The amino acid sequence of AfrLEA6 contains features associated with proteins known to undergo liquid-liquid phase separations in the hydrated state under certain conditions (Janis et al. 2018; Janis et al. 2017). An attractive hypothesis may be that the protein is involved in the partitioning of signaling peptides or other biomolecules that regulate diapause in the cyst. Due to the increasing concentration of solutes in the drying cell, AfrLEA6 may precipitate from solution into another liquid phase that partitions such biomolecules at moderate to low water content and releases them as it dissolves upon rehydration. In any case, these results underscore the concept that multiple LEA proteins exist at different amounts and within different cellular compartments within a single organism and may act together or separately to protect the organism during events of water stress.

CHAPTER 3
PROTECTION OF TARGET ENZYMES AND LIPOSOMES BY A GROUP 6 LEA PROTEIN FROM DIAPAUSE EMBRYOS OF *ARTEMIA FRANCISCANA*

3.1. Introduction

Maintaining homeostasis during water stress events such as dehydration is a necessity for many organisms. Embryos of the anhydrobiotic brine shrimp *Artemia franciscana* can withstand almost complete water loss and remain viable for years in the dried state (Clegg et al. 1978; Crowe and Clegg 1973; Crowe and Clegg 1978). The mechanisms by which *A. franciscana* survives extreme water loss continue to be studied, but one key mechanism is the accumulation of late embryogenesis abundant (LEA) proteins (Hand et al. 2007; Hand and Menze 2015; Hand et al. 2011; Janis et al. 2018; Tunnacliffe and Wise 2007). LEA proteins are a large family of proteins that were first discovered in cottonseeds (Dure et al. 1981; Galau and Dure 1981). Aside from brine shrimp, expression of LEA proteins has been documented in anhydrobiotic bacteria (Battista et al. 2001; Rodriguez-Salazar et al. 2017; Stacy and Aalen 1998), fungi (Abba et al. 2006), and animals such as nematodes (Browne et al. 2002; Gal et al. 2004; Solomon et al. 2000), rotifers (Denekamp et al. 2010; Tunnacliffe et al. 2005), and African chironomid insect larvae (Gusev et al. 2014; Kikawada et al. 2006). Many LEA proteins are characterized as intrinsically disordered proteins (IDPs) which lack secondary structure in solution but gain structure as water is removed. Current classification schemes divide LEA proteins into 6 main groups based on shared sequence motifs, and *A. franciscana* is unique among animals in expressing LEA proteins from groups 1, 3, and 6 (Hand and Menze 2015; Tunnacliffe and Wise 2007; Warner et al. 2012; Warner et al. 2010). Although the exact roles by which LEA proteins function in desiccation tolerance are not clear, several have been proposed. For example, LEA proteins are thought to interact with trehalose to stabilize vitrified sugar glasses by increasing the

glass transition temperature (T_g) (Hoekstra 2005; Wolkers et al. 2001). Additionally, LEA proteins may sequester divalent ions (Grelet et al. 2005), stabilize membranes (Tolleter et al. 2010; Tolleter et al. 2007), form structural networks to reduce physical stress (Goyal et al. 2003), and act as a molecular shield to sterically reduce harmful protein aggregation (Boucher et al. 2010; Goyal et al. 2005). These roles are supported by *in vitro* studies showing the ability of LEA proteins to preserve the activity of desiccation-sensitive enzymes (Boswell et al. 2014a; Goyal et al. 2005; Grelet et al. 2005; Popova et al. 2015), prevent protein aggregation (Boucher et al. 2010; Goyal et al. 2005; Koubaa et al. 2019; Yuen et al. 2019), and protect lipid bilayers of various composition during freezing and drying (Hundertmark et al. 2011; Moore and Hand 2016; Moore et al. 2016; Navarro-Retamal et al. 2018; Steponkus et al. 1998; Thalhammer et al. 2014; Tolleter et al. 2010; Tolleter et al. 2007). Here, I assess the ability of a Group 6 LEA protein from embryos of *A. franciscana* to protect desiccation sensitive enzymes and liposomes simulating types of mammalian membranes from damage incurred during desiccation.

In some cases, the combination of the naturally occurring sugar, trehalose, and LEA proteins results in much greater degrees of protection. Trehalose is accumulated in *A. franciscana* and is well-known for protecting some, but not all, anhydrobiotic organisms from damage during desiccation (Albertorio et al. 2007; Crowe et al. 1998; Crowe et al. 2005; Tapia and Koshland 2014). There are some anhydrobiotic bdelloid rotifers (Caprioli et al. 2004; Lapinski and Tunnacliffe 2003), tardigrades (Hengherr et al. 2008), as well as bakers' yeast (Ratnakumar and Tunnacliffe 2006) which do not accumulate trehalose. Due to its high T_g, trehalose is excellent at forming and maintaining stabilizing sugar glasses during dehydration. Trehalose prevents the typical denaturation stress experienced by proteins when losing their hydration shell by replacing water molecules in the hydrogen bonds (Crowe et al. 1987; Pereira

and Hunenberger 2008; Webb 1965). The ability of trehalose to stabilize enzymes (Carpenter et al. 1986; Carpenter et al. 1987; Piszkiewicz et al. 2019) as well as membranes (Chen et al. 2001; Crowe et al. 1985) has been studied extensively.

This chapter focuses on assessing the protective properties of AfrLEA6, a recently characterized Group 6 LEA protein from embryos of *A. franciscana*. AfrLEA6 is currently the only known Group 6 LEA protein in animals and shares sequence homology with seed maturation proteins (SMPs), such as D34.3, that are associated with desiccation tolerance in plants (Chatelain et al. 2012; Janis et al. 2017; Wu et al. 2011). AfrLEA6 was previously characterized as being a diapause-specific IDP which exhibited a gain of predominantly α-helix structure upon drying, and its titer was found to be 10-fold lower than Group 3 LEA proteins in *A. franciscana* (LeBlanc et al. 2019). Potential functions of AfrLEA6 may involve the prevention and dispersion of protein aggregates during desiccation and/or their rapid dissolution during rehydration like the SMP protein *Mt*PM25 (Chatelain et al. 2012). Therefore, I evaluated the ability of AfrLEA6 (with or without trehalose) to protect three desiccation-sensitive enzymes as well as liposomes simulating the inner leaflet of the plasma membrane, the inner mitochondrial, and the outer mitochondrial membranes.

3.2. Methods

Purification of rabbit muscle phosphofructokinase

Rabbit muscle phosphofructokinase (rmPFK) was purified by combining techniques previously described by Ling et al. (1966) and Hand and Somero (1982). It is important to note that the ATP-affinity resin (N6-[(6-aminohexyl)-carbamoyl-methyl]ATP-Sepharose

4B) often used as a key step for PFK purification (cf., Ramadoss et al., 1976; Hand and Somero, 1983; Carpenter et al. 1986) is no longer commercially available; neither is the purified enzyme. Frozen rabbit was obtained from a local butcher, and 115 g of hind leg muscle was processed in an ice-cold meat grinder. Then, 100 g of ground muscle was combined with 300 ml of ice-cold homogenization buffer (30 mM KF, 1 mM EDTA) and homogenized in a stainless-steel Waring blender at high speed for two 30 s intervals. Following homogenization, the sample was centrifuged at 25,000 x g (4^{o}C) for 30 min. The majority of PFK activity sediments to the pellet with previously frozen rabbit muscle. Consequently, PFK was solubilized from pellets and activated by resuspension in 200 ml of activation buffer (30 mM KF, 1 mM EDTA, and 0.1 M ammonium sulfate, pH 8.0), re-homogenization at medium-high speed for 15 s, and incubation at 37^{o}C in a circulating water bath for 30 min with constant stirring. Following solubilization and activation, the preparation was centrifuged at 25,000 x g (4^{o}C) for 30 min. The supernatant containing activated PFK was decanted, titrated from pH 6.6 to pH 8.0 with NaOH, and centrifuged at 175,000 x g (4^{o}C) for 4.5 h in a Beckman Optima L-90K ultracentrifuge. The pellets containing PFK were resuspended in a small volume of Buffer I [50 mM tris-phosphate, 0.2 mM EDTA, 5 mM dithiothreitol (DTT), pH 8.0] and re-dissolved by gently stirring at room temperature for 1 h. Centrifugation at 25,000 x g (4^{o}C) for 5 min was used to remove any undissolved material.

Prior to isopropanol precipitation, the supernatant containing PFK was diluted in Buffer I to match the units/ml present in the activated supernatant prior to the ultracentrifugation step. The diluted PFK was transferred to a 45^{o}C circulating water bath and stirred constantly until the temperature of the solution reached 40^{o}C. Over the next 10 min, 1/10 volume of room temperature isopropanol was added with constant stirring. Following the addition of isopropanol,

the solution was stirred for another 10 min at 45^{o}C. The solution was then cooled to 20^{o}C in an ice water bath and centrifuged at 10,000 x g (25^{o}C) for 20 mins. The supernatant containing PFK was transferred to a -4^{o}C alcohol ice bath and stirred constantly until the temperature of the solution reached 0^{o}C. Over the next 10 min, 1/10 volume of -4^{o}C isopropanol was added with constant stirring, then the solution was stirred for an additional 20 min at -4^{o}C. The suspension was then centrifuged at 10,000 x g (-4^{o}C) for 20 min in a pre-cooled rotor to pellet PFK. The pellet was re-dissolved in 1/30 volume of Buffer II (100 mM tris-phosphate, 0.2 mM EDTA, 5 mM DTT pH 8.0) based on the diluted volume immediately prior to isopropanol precipitation. The re-dissolved pellet was dialyzed for 1 h at 4^{o}C against 1 L of Buffer II, then the dialysis solution was changed and PFK was dialyzed overnight (4^{o}C; <16 h) with gentle stirring. The dialyzed preparation was centrifuged at 10,000 x g (4^{o}C) for 20 min to clarify the sample.

The clarified PFK sample was further purified by anion exchange chromatography using a 5ml HiTrap® DEAE-Sepharose FF column (GE Healthcare; 17505501) attached to an ÄKTA Prime Plus FPLC system (GE Healthcare). The column was rinsed and equilibrated with 5 bed volumes of Buffer II. Sample was loaded at a flow rate of 0.5 ml/min, and the column was rinsed with Buffer II until the absorbance returned to baseline. PFK was then eluted in a single peak with a step gradient of Buffer III (300 mM tris-phosphate, 0.2 mM EDTA, 5 mM DTT pH 8.0). All fractions under the elution peak were pooled, and an aliquot was taken for protein determination by a modified Lowry assay as described by Peterson (1977). The remainder of the purified PFK was precipitated from solution at 0^{o}C by adding crystalline ammonium sulfate to 70% saturation with stirring over a period of 30 min; the preparation was given an additional 30 min without stirring on ice to obtain maximum precipitation. The 70% saturated suspension containing PFK was centrifuged at 10,000 x g (4^{o}C) for 20 min and the supernatant discarded.

The pellet was re-dissolved in PFK dialysis buffer (100 mM sodium phosphate buffer, containing 1 mM EDTA, 5 mM DTT, pH 8.0) to give a final concentration of 3.5 mg protein/ml. The concentrated PFK sample was then heat treated in a circulating water bath at 65^{o}C for 5 min. After heat treatment, the sample was centrifuged at 10,000 x g (4^{o}C) for 20 min to remove precipitated protein. Purified PFK was precipitated with ammonium sulfate as above and stored at 4^{o}C for future use. Assessment of recovery and specific activity of PFK is shown in Table 3.1. Values for intermediate steps in the purification reflect the known, reversible inactivation of PFK due to changes in buffer constituents, temperature, and pH at various steps (Aaronson and Frieden 1972; Bock and Frieden 1974; Ling et al. 1965). SDS-PAGE (4% stacking gel, 11% resolving gel) at 125 V for 80 min in a Bio-Rad Mini Protean 3 cell demonstrated the enzyme to be 99 % pure after the final heat treatment step, based on Coomassie Blue staining and a comparison of band intensities using Quantity One Basic software v 4.6.9 (Bio-Rad Laboratories; Fig. 3.1 A, B).

Protection of target enzymes by AfrLEA6

AfrLEA6 was expressed and purified as previously described (LeBlanc et al. 2019). Lactate dehydrogenase (LDH) from rabbit muscle was obtained commercially (Sigma-Aldrich; L2500). Prior to use, LDH was exchanged into LEA storage buffer (LSB; 20 mM HEPES, 50 mM NaCl, pH 7.5) using an Amicon® Ultra-0.5 ml centrifugal filter (MilliporeSigma; UFC501024). LDH activity was assayed at 25^{o}C in a 2 ml reaction volume that contained 50 mM Tris-HCl (pH 7.3), 3 mM sodium pyruvate, and 150 µM NADH. The reaction was initiated by the addition of LDH, and the change in absorbance at 340 nm (ΔA_{340}) was recorded for 2 min. One unit is defined as the conversion of 1 µmol of pyruvate to product per min. The control activity prior to drying was assessed by adding 5 µl of 0.025 mg/ml LDH with or without

Table 3.1. Specific activity, yield, and enrichment of rabbit muscle phosphofructokinase after various purification steps. Catalytic activity was measured at 25°C. One unit is defined as the production of 1 µmol of fructose 1,6-bisphosphate/min. Yield is expressed as a percentage of the total enzyme units in the activated supernatant. Fold purification was calculated by dividing the specific activity at each step by the specific activity of the activated supernatant.

Purification Step	Total Volume (ml)	Total Activity (units)	Protein Conc. (mg/ml)	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)	Fold Purification
Activated Supernatant	153	4820	3.41	522	9.24	100	1.00
Ultracentrifugation	12.5	2830	32.9	411	6.89	58.8	0.74
Isopropanol Precipitation	4.20	569	5.26	22.1	25.8	11.8	2.79
DEAE Chromatography	9.50	1380	1.35	12.8	107	28.5	11.6
Heat Treatment	3.55	1340	3.20	11.4	118	27.8	12.8

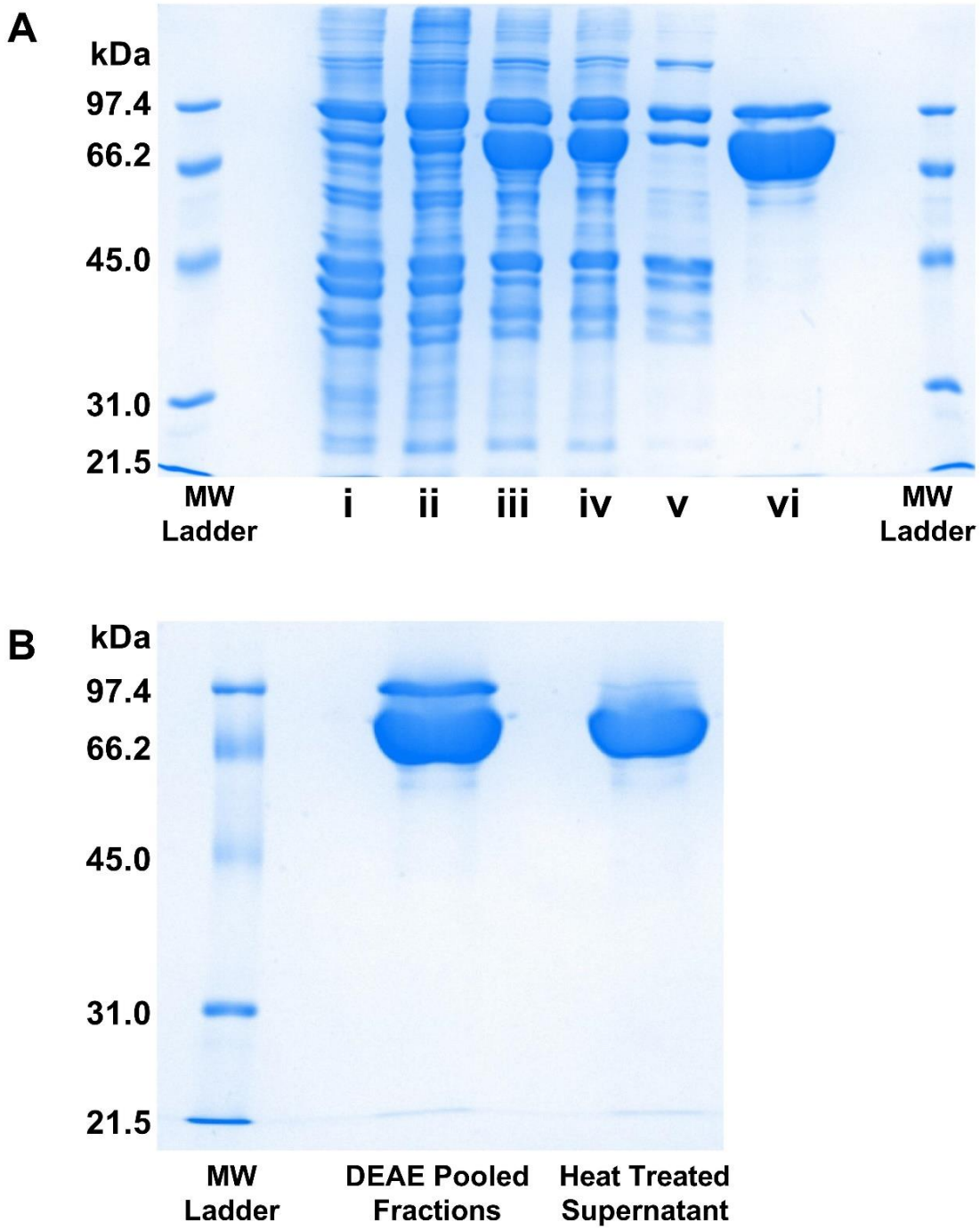

Figure 3.1. Purity of rabbit muscle phosphofructokinase (mass of PFK monomer, 80 kDa) as estimated by SDS-PAGE at various steps of the purification process (A) Ladder of MW standards shown at left and right. Middle lanes represent samples from the following steps of purification: (i) activated supernatant, (ii) post-ultracentrifugation pellet, (iii) post-isopropanol precipitation, (iv) pre-DEAE chromatography, (v) DEAE column rinse, (vi) final pooled fractions from DEAE column. (B) SDS-PAGE analysis of samples before and after heat treatment. Ladder of MW standard shown at left. All gels were stained with Coomassie Blue, and 20 µg total protein was loaded per lane.

protectants into a 2 ml final volume of reaction mixture. Then, 10 µl droplets of 0.05 mg/ml LDH with or without protectants were dried in 0.6 ml microcentrifuge tubes for 1 wk at room temperature in a dry box containing desiccant (Drierite; W.A. Hammond Drierite). Samples were rehydrated for 1 h on ice by adding 20 µl of ice-cold 0.5 x LSB. For measurements of activity after drying, 5 µl of the rehydrated LDH was added to a final reaction volume of 2 ml. Residual LDH activity after drying and rehydration was reported as a percentage of the rate of non-dried controls.

Citrate synthase (CS) from porcine heart was obtained commercially (Sigma-Aldrich; C3260), and activity was assayed at 25ºC according to Srere (1966). Prior to use, CS was buffer exchanged into 1 M Tris-HCl (pH 8.1) using an Amicon® Ultra-0.5 ml centrifugal filter (Millipore Sigma; UFC501024). The 1 ml reaction volume contained 0.1 M Tris-HCl (pH 8.1), 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 0.15 mM Acetyl Coenzyme A. The reaction was initiated by the addition of 0.5 mM oxaloacetate, and the change in absorbance at 412 nm (ΔA_{412}) was recorded for 2 min. One unit is defined as the conversion of 1 µmol of substrate to product per min. The control activity prior to drying was assessed by adding 5 µl of 0.025 mg/ml CS with or without protectants into a 1 ml final volume of reaction mixture. Then, 10 µl droplets of 0.05 mg/ml CS with or without protectants were dried in 0.6 ml microcentrifuge tubes for 24 h at room temperature in a dry box containing desiccant (Drierite; W.A. Hammond Drierite). After 24 h, samples were rehydrated with 10 µl of H_2O and drying was repeated for an additional 24 h. Double-dried samples were rehydrated for 1 h on ice by adding 20 µl of ice-cold 0.5 M Tris-HCl (pH 8.1). Double-drying has been used previously to generate adequate damage to CS in similar studies (Boswell et al. 2014a; Pouchkina-Stantcheva et al. 2007). For measurements of activity after drying, 5 µl of the rehydrated CS was added to a final reaction volume of 1 ml. Residual CS

activity after drying and rehydration was reported as a percentage of the rate of non-dried controls.

Purified phosphofructokinase (PFK) from rabbit muscle was dialyzed against PFK dialysis buffer and assayed for activity at 25^{o}C using the procedure described by Bock and Frieden (1974). The 1 ml reaction volume contained a 33 mM tris-acetate buffer (pH 8.0), with final concentrations of 40 mM KCl, 4 mM NH_4Cl, 2 mM ATP, 2 mM fructose-6-phosphate, 2 mM magnesium acetate, 0.16 mM NADH, 2.5 units/ml of glycerol-3-phosphate dehydrogenase (Sigma-Aldrich; G6751), 2 units/ml of aldolase (Sigma-Aldrich; A8811), and 25 units/ml of triosephosphate isomerase (Sigma-Aldrich; T2391). Prior to use, all accessory enzymes were dialyzed against 100 mM tris-acetate buffer (pH 8.0) containing 0.1 mM EDTA. The reaction was initiated by the addition of PFK, and the change in absorbance at 340 nm (ΔA_{340}) was recorded for 3 min. One unit is defined as the production of 1 µmol of fructose 1,6-bisphosphate/min (one-half the measured rate of the enzymatically coupled NAD^+ production). The control activity prior to drying was assessed by adding 2.5 µl of 0.15 mg/ml PFK with or without protectants into a 1 ml final volume of reaction mixture. Then, 10 µl droplets of 0.15 mg/ml PFK with or without protectants were placed in 2 ml microcentrifuge tubes and dried for 24 h at room temperature in a dry box containing desiccant (Drierite; W.A. Hammond Drierite). Samples were rehydrated for 1 h on ice by adding 20 µl of ice-cold 0.5 x PFK dialysis buffer. For measurements of activity after drying, 5 µl were added to a final reaction volume of 1 ml to account for a two-fold dilution of PFK during rehydration. Residual PFK activity after drying and rehydration was reported as a percentage of the rate of non-dried controls. Bovine serum albumin (BSA; Sigma-Aldrich, product code A6003) was used as a control protein for comparison with all target enzymes as described above during drying studies.

Preparation of liposomes

The following naturally-derived species of lipid were obtained from Avanti Polar Lipids (Alabaster, AL) and were used to prepare liposomes: L-α-phosphatidylcholine (PC; 840055; bovine liver), L-α-phosphatidylethanolamine (PE; 840026; bovine liver), L-α-phosphatidylinositol (PI; 840042; bovine liver), L-α-phosphatidylserine (PS; 840032; porcine brain), cardiolipin (CA; 840012; bovine heart), and cholesterol (Cho; 700000; ovine wool). The lipid ratios used in these experiments were based on membrane compositions reported for mammalian cells (Horvath and Daum 2013; van Meer et al. 2008). Lipids in chloroform were mixed in mass ratios according to Table 3.2 to simulate phospholipid compositions of the inner leaflet of the plasma membrane, outer mitochondrial membrane, and inner mitochondrial membrane (Moore et al. 2016). To serve as a non-biological control, liposomes consisting of 100% PC were prepared. For each preparation, 20 mg of total lipids were combined, and the chloroform was evaporated under a stream of dry nitrogen. Combined lipids were stored overnight at room temperature under vacuum to evaporate residual solvent. Next, lipids were rehydrated for 1 h (70^{o}C) with intermittent vortexing in 1 ml of 10 mM TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)-2-propanyl]amino]ethanesulfonic acid) buffer (pH 7.4) containing 0.1 mM EDTA and 100 mM 5(6)-carboxyfluorescein (CF; Sigma-Aldrich; 21877). Rehydrated lipids were sonicated with 5 cycles of 5 s pulses at 35% amplitude to improve the size distribution during the extrusion step. Small unilamellar liposomes of 100 nm diameter were then prepared using an Avanti mini-extruder kit (Avanti Polar Lipids; 610000). First the sonicated lipids were passed through two stacked polycarbonate membranes of 1 μm pore size a total of 10 times at 70^{o}C, which served to entrap the 100 mM CF dye. Maintaining the extruder and lipids at 70^{o}C was sufficiently high to ensure all phospholipids were in the liquid crystalline phase. Liposomes

Table 3.2. The lipid composition of liposomes designed to simulate mammalian membranes. Mixtures are expressed as percentages (w/w). PC-phosphatidylcholine; PE-Phosphatidylethanolamine; PI-phosphatidylinositol; PS- Phosphatidylserine; CL- Cardiolipin; Cho- Cholesterol

Membrane type	Lipid species	Mixture
Outer mitochondrial membrane	PC PE PI	55% 30% 15%
Inner mitochondrial membrane	PC PE CL	50% 30% 20%
Inner leaflet of the plasma membrane	PC PE PS Cho	50% 30% 10% 10%

were then extruded 21 times at 70ºC through two stacked polycarbonate membranes of 0.1 µm pore size. To remove excess CF that was not entrapped during extrusion, 0.5 ml of the liposome mixture was loaded onto an Illustra NAP-5 Sephadex G-25 column (GE Healthcare; 17085301), which was equilibrated in TEN buffer (10 mM TES, 50 mM NaCl, 0.1 mM EDTA, pH 7.4). Liposomes were eluted from the column by gravity flow using 0.5 ml of room temperature TEN buffer. The final concentration of the prepared liposomes was approximately 10 mg lipid/ml. A uniform size distribution of 100 nm was verified with dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern UK; Fig. 3.2A).

Assay of liposome leakage

Liposomes were combined with AfrLEA6 at various protein:lipid mass ratios with or without 250 mM trehalose (19:1 sugar:lipid mass ratio; Pfanstiehl, T-104-4). Lysozyme (Sigma-Aldrich; L6876) been shown to retain its native structure when dried and to not interact with membranes (Nagendra et al. 1998; Pap et al. 1996); consequently, it was used as a negative control at the same protein:lipid mass ratios as AfrLEA6. The final concentration of liposomes in the sample mixtures was approximately 5 mg lipid/ml. For leakage assays, 2 µl droplets of the samples were placed in wells of opaque 96 well plates (PerkinElmer; OptiPlate 96-F LBS) and dried overnight at room temperature in a dry box containing desiccant (Drierite: W.A. Hammond). Liposomes were resuspended in 120 µl of TEN buffer by repeated aspiration. One 50 µl aliquot of each liposome sample was added to another 96 well plate that contained 250 µl TEN buffer. A second 50 µl aliquot was added to a different 96-well plate that contained 250 µl TEN buffer with 1.2% Triton X-100 to fully lyse the liposomes and generate maximum fluorescence (F_T). CF fluorescence was measured with a Victor^3 1420 multilabel plate reader (PerkinElmer) set to an excitation wavelength of 485 nm and emission wavelength of 535 nm.

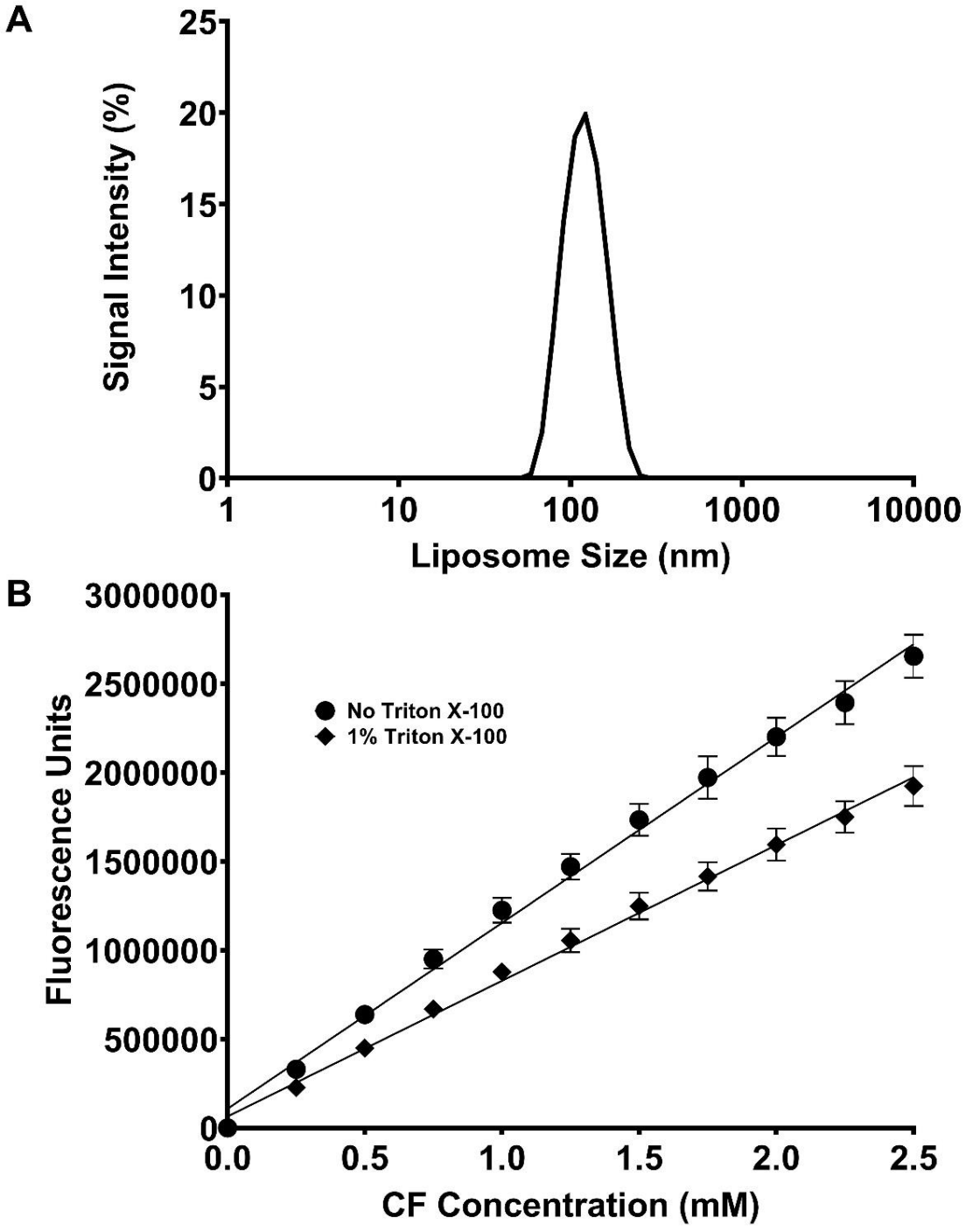

Figure 3.2. Size assessment of large unilamellar vesicles after extrusion and the carboxyfluorescein (CF) standard curve used to correct values for quenching by Triton X-100. (A) Size distribution of liposomes was measured by dynamic light scattering (DLS) immediately after preparation. (B) A standard curve comparing the fluorescence of known concentrations of CF with or without a final concentration of 1% Triton X-100.

CF fluorescence is quenched at high concentrations within the liposomes, but leakage and dilution in the surrounding buffer results in a large increase in fluorescence (Weinstein et al. 1986). The final concentration of 1% Triton X-100 used for lysis resulted in fluorescence quenching (Moore and Hand 2016; Moore et al. 2016), so a standard curve of known CF concentrations with and without 1% Triton X-100 was used to correct F_T values (Fig. 3.2 B). Specifically, CF concentrations of lysed samples calculated from the best fit line with detergent were then inserted into the equation for the line without detergent to solve for the corrected F_T values. CF leakage in the rehydrated samples (F_o) was expressed as a percentage of the corrected fluorescence in samples containing 1% Triton X-100 (F_o/F_T x 100). In certain cases, correction of values with the standard curve led to control leakage values above 100%.

Statistical analyses

Analyses were performed using GraphPad Prism version 8.4.0 for Windows (GraphPad Software, San Diego, California USA). Welch's ANOVA was performed followed by Dunnett's T3 post-hoc multiple comparisons test to determine significant differences among means. Pair-wise comparisons were made among mean values and to their respective controls. Significance level was set at $p \leq 0.05$.

3.3. Results

Protection of enzyme activity by AfrLEA6 during desiccation

AfrLEA6 was tested at various concentrations for its capacity to protect desiccation sensitive enzymes during drying and rehydration. I assessed whether AfrLEA6 could preferentially protect one enzyme over another by choosing enzymes that originate from different subcellular compartments. After drying LDH for 1 wk in the absence of protectants

followed by rehydration, the residual activity was 32.8 ± 3.4 % (mean ± SD), which served as the control. When dried in the presence of 400 µg/ml AfrLEA6, residual activity of LDH increased to 59.4 ± 1.6 % (Welch's ANOVA plus Dunnett's T3 test, $P<0.05$; Fig. 3.3). Protection by AfrLEA6 at both 40 and 400 µg/ml was statistically greater than protection by BSA ($P<0.05$). The presence of 100 mM trehalose during drying afforded more protection to LDH than either 400 µg/ml AfrLEA6 or BSA alone, as indicated by an increased residual activity of 74.8 ± 1.1 % ($P<0.05$). The combination of trehalose with AfrLEA6 resulted in a residual activity of 81.3 ± 1.0 %, which was statistically greater than either trehalose alone or BSA plus trehalose ($P<0.05$; Fig. 3.3).

Phosphofructokinase was dried for 24 h in the absence of protectants. After rehydration, the residual activity of PFK was 1.5 ± 0.4 % (mean ± SD), which served as the control. At the lowest protein concentration tested, no combination of protein or trehalose resulted in protection. When PFK was combined with 40 µg/ml AfrLEA6, residual activity increased significantly from control to 20.1 ± 2.1 % (Welch's ANOVA plus Dunnett's T3 test, $P<0.05$; Fig. 3.4). At 400 µg/ml AfrLEA6, residual activity increased further to 39.2 ± 2.8 %, which was significantly higher than 27.0 ± 2.6 % when 400 µg/ml BSA was used alone ($P<0.05$). A small increase from control activity to 7.1 ± 2.9 % residual activity was noted after PFK was dried with trehalose alone ($P<0.05$). When trehalose was combined with AfrLEA6, however, there was a statistically significant decrease in residual activity to 31.3 ± 2.5 % when compared to AfrLEA6 alone ($P<0.05$; Fig. 3.4). Compared to the residual activity of PFK when BSA was used alone, the combination of BSA plus trehalose resulted in a significantly higher residual activity of 41.7 ± 1.7 % ($P<0.05$; Fig. 3.4). Overall, AfrLEA6 protected PFK better than BSA at both 40 and 400

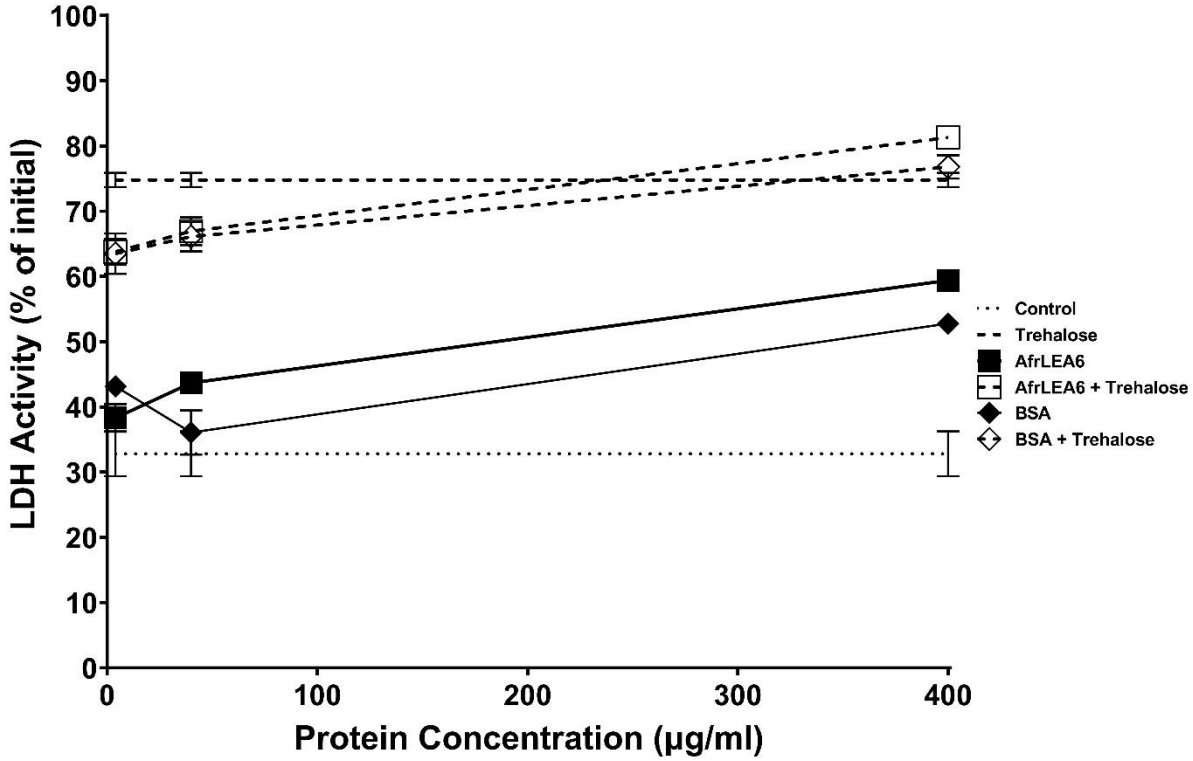

Figure 3.3. Residual lactate dehydrogenase (LDH) activity after drying for 1 wk at room temperature in the presence or absence of various protectants. Data is expressed as a percentage of the initial LDH activity before drying (mean ± SD; n=9). Concentrations of protectant proteins with or without 100 mM trehalose were 4, 40, or 400 µg/ml. Error bars were omitted where they were smaller than the marker. Symbols denoting statistical significance were omitted from the figure for clarity. At protein protectant concentration of 400 µg/ml, residual activity in the presence of AfrLEA6 alone was statistically higher than BSA alone, and residual activity in the presence of AfrLEA6 plus trehalose was statistically higher than protection by BSA plus trehalose (Welch's ANOVA plus Dunnett's T3 test, $P<0.05$).

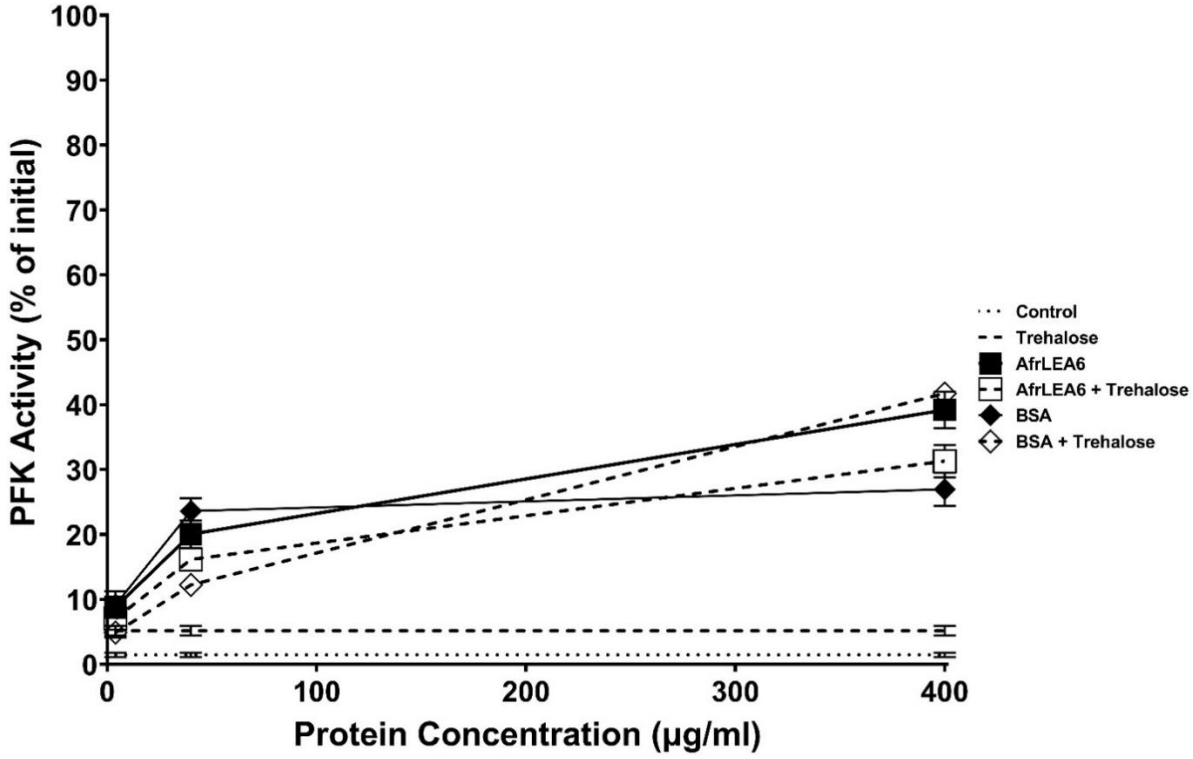

Figure 3.4. Residual phosphofructokinase (PFK) activity after drying for 24 h at room temperature in the presence or absence of various protectants. Data are expressed as a percentage of the initial PFK activity before drying (mean ± SD; n=9). Concentrations of protectant proteins with or without 100 mM trehalose were 4, 40, or 400 µg/ml. Error bars were omitted where they were smaller than the marker. Symbols denoting statistical significance were omitted from the figure for clarity. At a protein protectant concentration of 400 µg/ml, residual activity in the presence of AfrLEA6 alone was statistically higher than BSA alone (Welch's ANOVA plus Dunnett's T3 test, $P<0.05$). Residual activity in the presence of BSA plus trehalose was statistically higher than AfrLEA6 plus trehalose ($P<0.05$).

µg/ml concentrations (P<0.05), but BSA plus trehalose provided statistically identical protection to AfrLEA6 alone (P>0.05) and better protection compared to AfrLEA6 plus trehalose (P<0.05).

Citrate synthase was double-dried over a 48-h period to achieve adequate damage, and the residual activity in the absence of protectants served as the control. After rehydration, the control activity of CS was 6.7 ± 1.7% (mean ± SD). Residual activity of CS after incubation with either AfrLEA6 or BSA was statistically identical to control conditions (Welch's ANOVA plus Dunnett's T3 test, P<0.05; Fig. 3.5). When compared to control activity, the presence of 100 mM trehalose resulted in a large increase of residual activity to 55.7 ± 2.2 % (P<0.05; Fig. 3.5). The presence of 400 µg/ml AfrLEA6 increased residual activity of CS to 71.1 ± 1.8 % (P<0.05) but was not statistically different from BSA (P>0.05; Fig. 3.5). Overall, AfrLEA6 provides significant protection to CS when combined with 100 mM trehalose but does not statistically differ from the protection offered by BSA in any case (P>0.05).

Protection of liposomes by AfrLEA6 during desiccation

Liposomes simulating the outer mitochondrial membrane, inner mitochondrial membrane, inner leaflet of plasma membrane, and a non-biological mixture were dried overnight at varying mass ratios of AfrLEA6:lipid to test its ability to stabilize lipid bilayers during desiccation. Liposomes simulating the outer mitochondrial membrane were dried overnight without protectants, and CF leakage after rehydration was 90.0 ± 6.9 % (mean ± SD). Compared to control, drying liposomes in the presence of AfrLEA6 at a 0.4 protein:lipid mass ratio resulted in a decrease in CF leakage to 82.4 ± 1.8 % (Welch's ANOVA plus Dunnett's T3 test, P<0.05; Fig. 3.6). At the highest protein:lipid mass ratio, leakage when AfrLEA6 was present was statistically lower than when lysozyme was present (P<0.05). When liposomes were dried with 250 mM trehalose, CF leakage decreased from control values to 68.5 ± 3.1 % (P<0.05). The

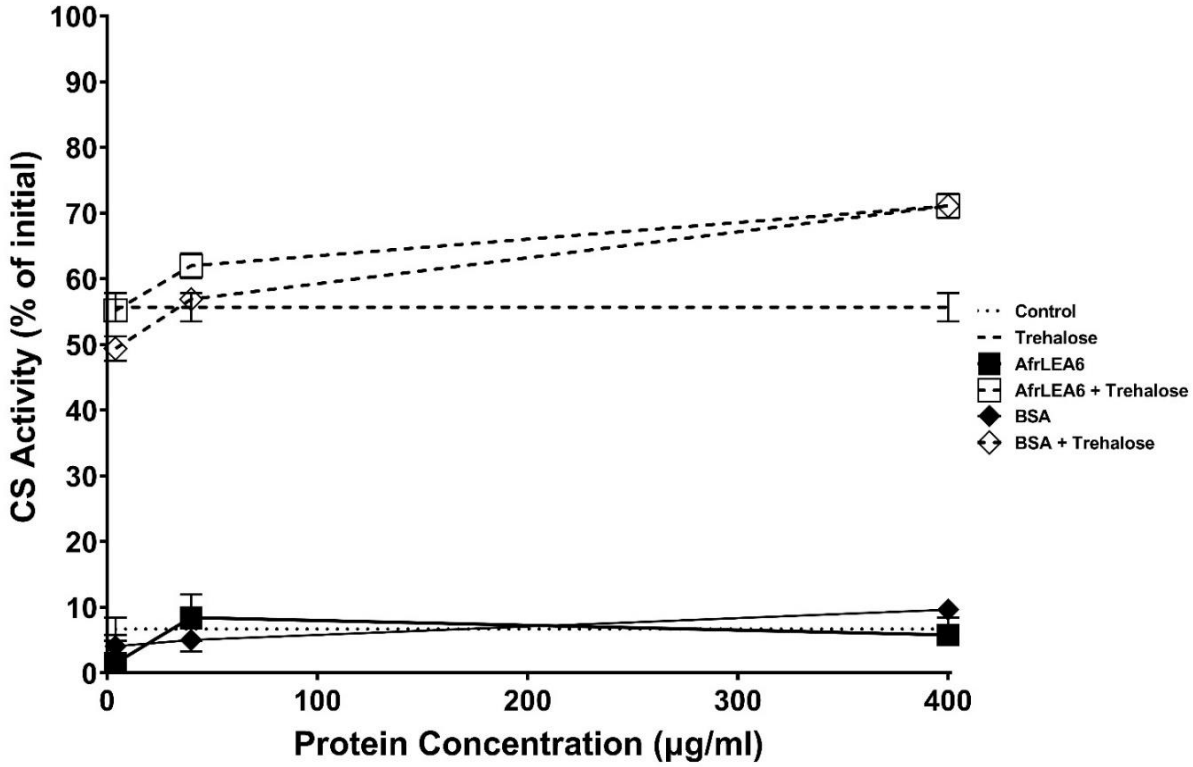

Figure 3.5. Residual citrate synthase (CS) activity after double-drying over 48 h at room temperature in the presence or absence of various protectants. Data are expressed as a percentage of the initial CS activity before drying (mean ± SD; n=9). Concentrations of protectant proteins with or without 100 mM trehalose were 4, 40, or 400 µg/ml. Error bars were omitted where they were smaller than the marker. Symbols denoting statistical significance were omitted from the figure for clarity. At a protein protectant concentration of 400 µg/ml, residual activity in the presence of AfrLEA6 alone was statistically identical to residual activity in the presence of BSA alone, and AfrLEA6 plus trehalose was statistically identical to BSA plus trehalose (Welch's ANOVA plus Dunnett's T3 test, $P>0.05$).

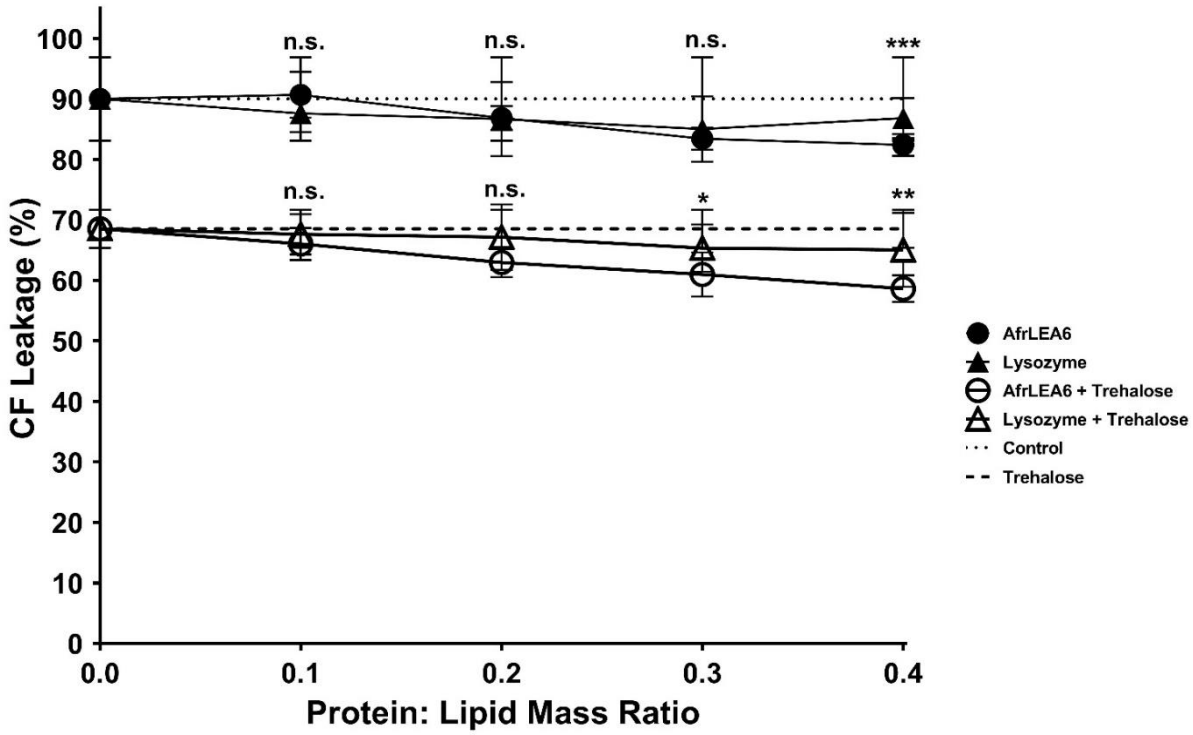

Figure 3.6. Leakage of carboxyfluorescein (CF) from liposomes simulating the outer mitochondrial membrane (OMM) after overnight drying and rehydration. Approximately 5 mg/ml liposomes were combined with AfrLEA6, lysozyme, and/or 250 mM trehalose at various protein:lipid mass ratios prior to drying. The stability of liposomes was assessed by measuring the fluorescence of released dye and expressing it as a percentage of dye released after total solubilization of the membrane with detergent (mean ± SD; n=24). Error bars were omitted where they were smaller than the marker. Asterisks denote statistically significant differences in the means of AfrLEA6 and lysozyme (Welch's ANOVA plus Dunnett's T3 test, * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; n.s. = not significant).

combination of trehalose and AfrLEA6 further decreased CF leakage to 58.6 ± 2.2 % (P<0.05; Fig. 3.6). Lastly, drying with lysozyme plus trehalose resulted in a statistically higher leakage of 65.0 ± 6.1 % compared to AfrLEA6 plus trehalose (P<0.05).

Liposomes that simulated the inner mitochondrial membrane were dried overnight without protectants as well, and CF leakage after rehydration was 102.3 ± 2.4 % (mean ± SD). The presence of AfrLEA6 during drying resulted in a decrease of CF leakage from control to 89.0 ± 4.4 % (Welch's ANOVA plus Dunnett's T3 test, P<0.05), but protection by AfrLEA6 was statistically identical to lysozyme across all tested protein:lipid mass ratios (P>0.05; Fig. 3.7). When IMM liposomes were dried in the presence of 250 mM trehalose, CF leakage decreased to 77.4 ± 2.1 % from control leakage (P<0.05). The combination of AfrLEA6 plus trehalose resulted in increased protection compared to trehalose as indicated by a significant decrease in CF leakage to 61.9 ± 1.5% (P<0.05; Fig. 3.7). Leakage of liposomes dried with AfrLEA6 plus trehalose was also statistically lower than the leakage of 70.0 ± 3.5 % seen when liposomes were dried with lysozyme and trehalose (P<0.05).

Liposomes that simulated the inner leaflet of the plasma membrane were dried overnight without protectants, and control CF leakage after rehydration was 100.0 ± 1.2 % (mean ± SD). At the highest protein:lipid mass ratio, the presence of AfrLEA6 alone during drying resulted in a significant decrease in CF leakage to 86.0 ± 1.5 % compared to control (Welch's ANOVA plus Dunnett's T3 test, P<0.05; Fig. 3.8). However, the protective effects seen when AfrLEA6 was present did not differ statistically from when lysozyme was present (P>0.05). Compared to control, a significant decrease to 62.7 ± 1.1% leakage was observed when liposomes were mixed with 250 mM trehalose (P<0.05). Similarly, trehalose alone resulted in a statistically lower leakage than lysozyme plus trehalose (P<0.05). When AfrLEA6 was combined with trehalose

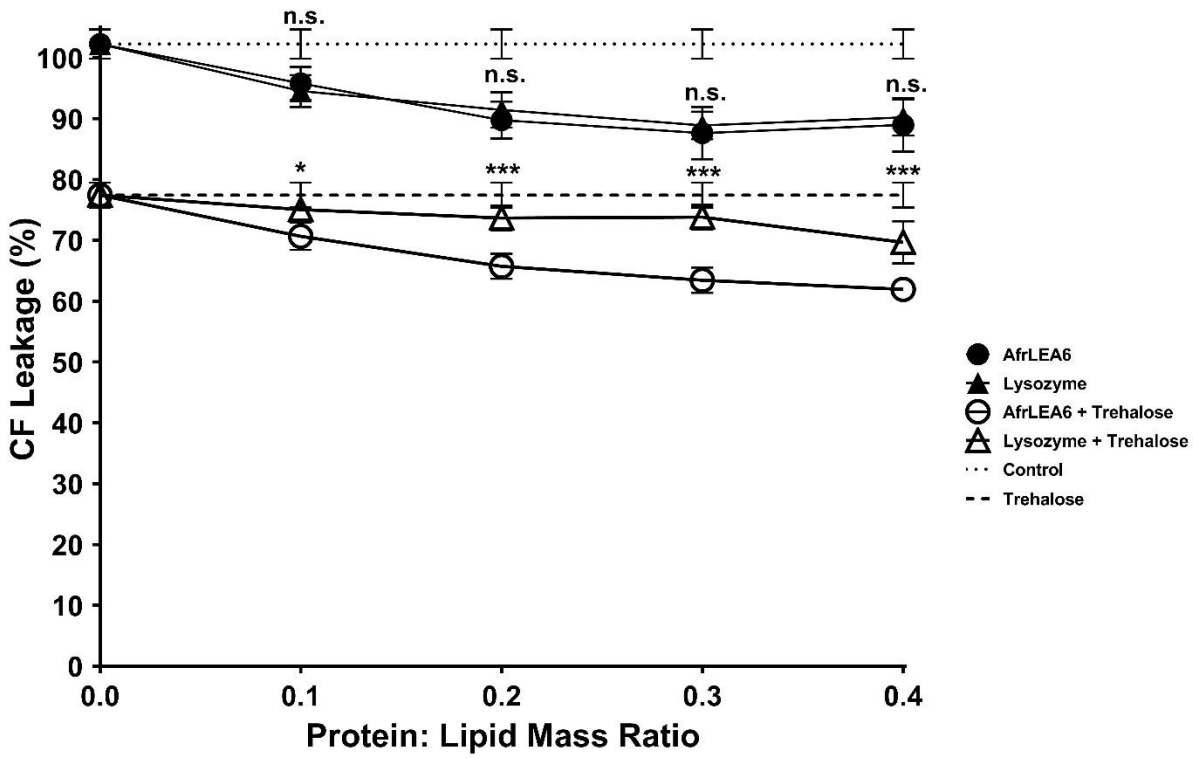

Figure 3.7. Leakage of carboxyfluorescein (CF) from liposomes simulating the inner mitochondrial membrane (IMM) after overnight drying and rehydration. Approximately 5 mg/ml liposomes were combined with AfrLEA6, lysozyme, and/or 250 mM trehalose at various protein:lipid mass ratios prior to drying. The stability of liposomes was assessed by measuring the fluorescence of released dye and expressing it as a percentage of dye released after total solubilization of the membrane with detergent (mean ± SD; n=8-16). Error bars were omitted where they were smaller than the marker. Asterisks denote statistically significant differences in the means of AfrLEA6 and lysozyme (Welch's ANOVA plus Dunnett's T3 test, * = $P<0.05$; *** = $P<0.001$; n.s. = not significant).

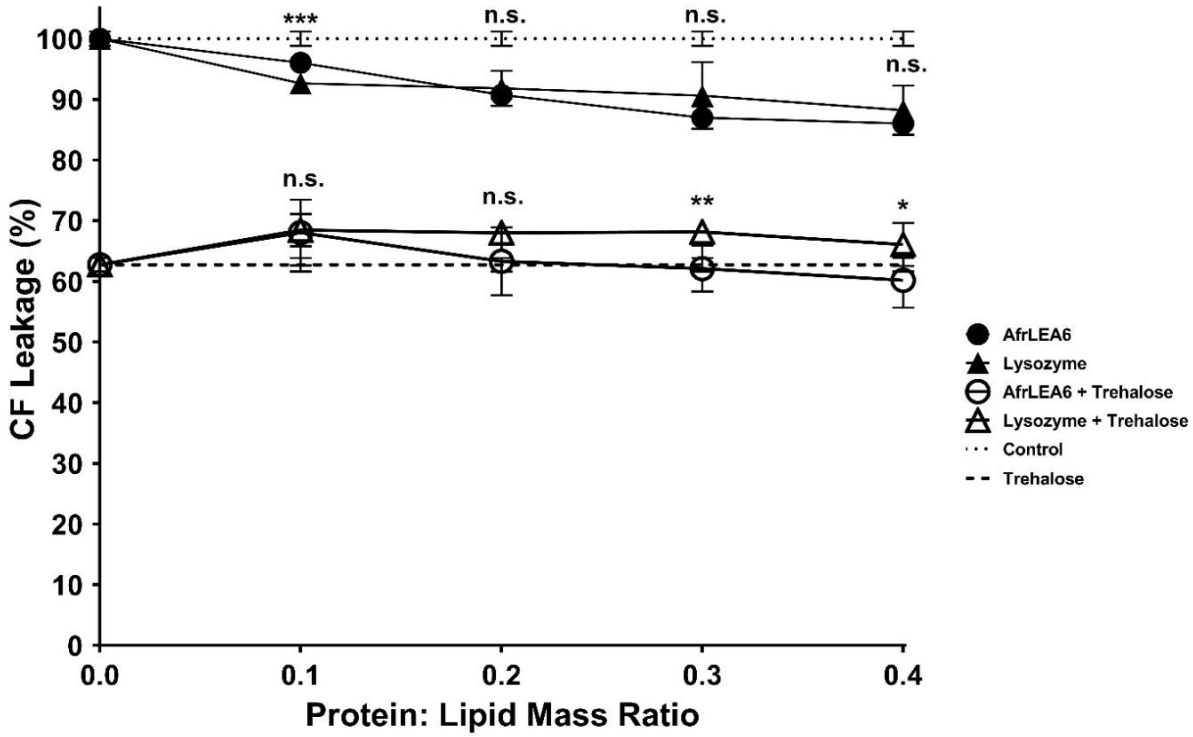

Figure 3.8. Leakage of carboxyfluorescein (CF) from liposomes simulating the inner leaflet of the plasma membrane (ILPM) after overnight drying and rehydration. Approximately 5 mg/ml liposomes were combined with AfrLEA6, lysozyme, and/or 250 mM trehalose at various protein:lipid mass ratios prior to drying. The stability of liposomes was assessed by measuring the fluorescence of released dye and expressing it as a percentage of dye released after total solubilization of the membrane with detergent (mean ± SD; n=8-16). Error bars were omitted where they were smaller than the marker. Asterisks denote statistically significant differences in the means of AfrLEA6 and lysozyme (Welch's ANOVA plus Dunnett's T3 test, * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; n.s. = not significant).

the CF leakage was 60.2 ± 4.5 %, which was statistically identical to trehalose alone (P>0.05) and statistically lower than lysozyme plus trehalose (P<0.05; Fig. 3.8).

After drying and rehydration of 100% PC liposomes with no protectants, control leakage was 89.5 ± 3.3 % (mean ± SD). When dried with AfrLEA6 at the highest protein:lipid mass ratio of 0.4, CF leakage was statistically lower at 82.4 ± 2.1 % (Welch's ANOVA plus Dunnett's T3 test, P<0.05; Fig. 3.9). However, decreases in leakage across the tested protein:lipid mass ratios were statistically identical when lysozyme was present (P>0.05). The presence of 250 mM trehalose resulted in a remarkable decrease in CF leakage from control to 39.9 ± 4.5 % (P<0.05). The combination of trehalose and AfrLEA6 reduced leakage to 44.3 ± 3.6 % from control (P<0.05), but this reduction was identical to values of trehalose alone as well as lysozyme plus trehalose (P>0.05; Fig. 3.9).

3.4. Discussion

My results demonstrate the ability of AfrLEA6 to protect two out of three of the desiccation sensitive enzymes tested during drying. AfrLEA6 alone protected both LDH and PFK during drying but did not protect CS. The combination of trehalose and AfrLEA6 enhanced the protection for both LDH and CS but decreased the stabilization of PFK. The overall magnitude of protection from AfrLEA6 was less than previously observed for Group 3 LEA proteins (Boswell et al. 2014a; Grelet et al. 2005). For liposomes, AfrLEA6 stabilized those simulating the OMM, both with and without trehalose, to a degree surpassing lysozyme. AfrLEA6 alone was no better than lysozyme alone in protecting IMM liposomes, but offered greater protection when combined with trehalose, compared to lysozyme plus trehalose. Finally, with ILPM liposomes, AfrLEA6 alone did not enhance protection over lysozyme alone but was

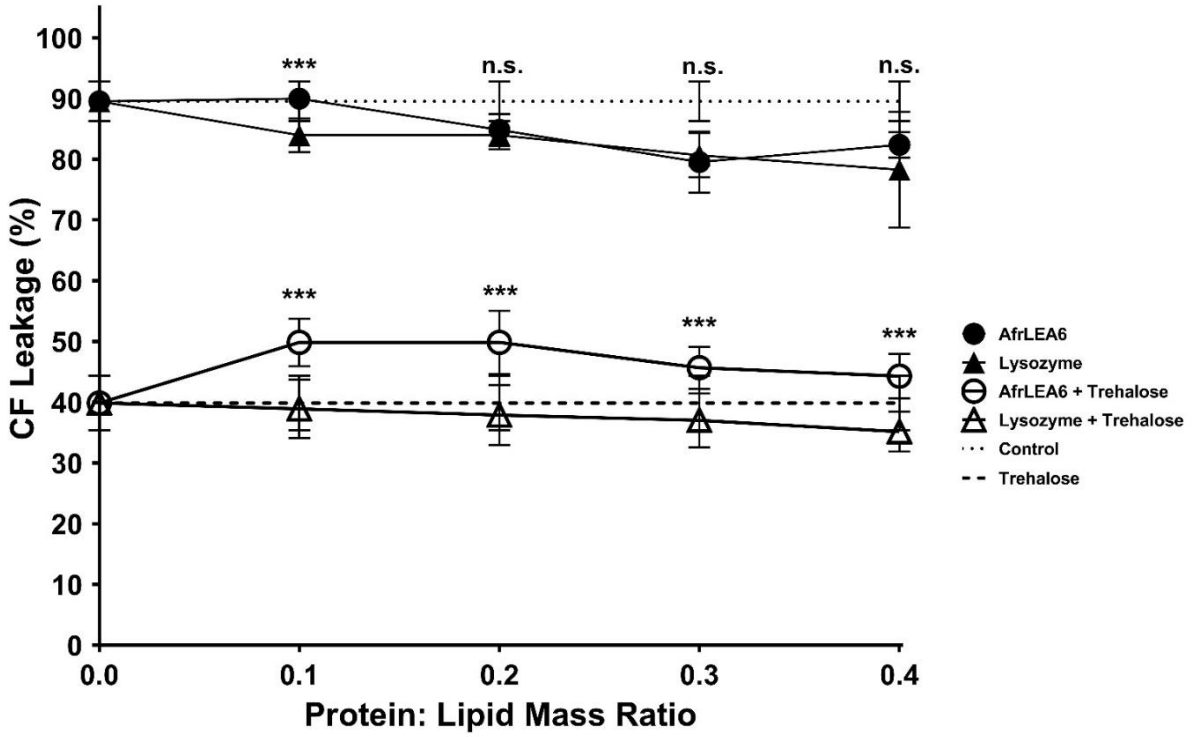

Figure 3.9. Leakage of carboxyfluorescein (CF) from liposomes consisting of 100% phosphatidylcholine after overnight drying and rehydration. Approximately 5 mg/ml liposomes were combined with AfrLEA6, lysozyme, and/or 250 mM trehalose at various protein:lipid mass ratios prior to drying. The stability of liposomes was assessed by measuring the fluorescence of released dye and expressing it as a percentage of dye released after total solubilization of the membrane with detergent (mean ± SD; n=16-24). Error bars were omitted where they were smaller than the marker. Asterisks denote statistically significant differences in the means of AfrLEA6 and lysozyme (Welch's ANOVA plus Dunnett's T3 test, *** = $P<0.001$; n.s. = not significant).

slightly better in combination with trehalose compared to lysozyme plus trehalose. Overall, AfrLEA6 was far less effective at stabilizing liposomes against damage incurred during desiccation compared to previous work with Group 3 LEA proteins (Moore et al. 2016; Tolleter et al. 2010; Tolleter et al. 2007).

Protection of target enzymes

As a cytoplasmic LEA protein (see Chapter 4), I tested for the capacity of AfrLEA6 to preferentially protect the cytoplasmic enzymes (LDH and PFK) over a mitochondrial enzyme (CS). Indeed, AfrLEA6 was found to offer protection to LDH that was significant and higher than the protection from BSA with or without trehalose (Fig. 3.3). BSA is classically used to stabilize enzymes under a variety of conditions, e.g. Chang and Mahoney (1995), and the potential of BSA to protect target enzymes during desiccation has been documented (Boswell et al. 2014a; Goyal et al. 2005; Reyes et al. 2005). In comparison to previous studies with Group 3 LEA proteins from *A. franciscana*, AfrLEA6 protects LDH just as well as AfrLEA2 or AfrLEA3m with or without trehalose (Boswell et al. 2014a).

Regarding PFK, protection by AfrLEA6 alone was better than BSA at 40 and 400 µg/ml concentrations (Fig. 3.4). The residual activity of PFK after drying with Group 3 LEA proteins from *A. franciscana* was of a similar magnitude, although it is noteworthy that PFK in this study was much more sensitive to desiccation than seen in Boswell et al. (2014a). When considering the difference between control (no protectant) and AfrLEA6, it can be argued that AfrLEA6 alone may have more protective capacity than either AfrLEA2 or AfrLEA3m. However, when these latter Group 3 LEA proteins were combined with trehalose, both preserved nearly 100% of PFK activity when dried with trehalose – a dramatic, synergistic increase over either protein alone (Boswell et al. 2014a). Unexpectedly, the combination of trehalose and protectant proteins

did not yield the strong synergistic increase anticipated. The PFK results suggest that differences exist between AfrLEA6 and Group 3 proteins in the protection afforded in combination with trehalose.

Residual activity of CS after double-drying was remarkably low, and in contrast to LDH and PFK, was not recoverable with any concentration of either AfrLEA6 or BSA alone (Fig. 3.5). These findings contrast with previous work showing the ability of BSA to protect CS (Boswell et al. 2014a; Goyal et al. 2005); perhaps a 3-fold lower residual activity obtained during control drying in my experiment contributed to this difference. With the addition of trehalose, there was a substantial increase in residual activity after drying which was improved further by the combination of AfrLEA6 or BSA with trehalose (Fig. 3.5). Group 3 LEA proteins were much more effective at preventing damage incurred during desiccation with or without trehalose compared to AfrLEA6 (Boswell et al. 2014a). One interesting observation from the protein stabilization data is that the cytoplasmically localized AfrLEA6 protected both cytoplasmic enzymes (LDH and PFK) better than it did the mitochondrial enzyme CS. It would be informative to expand the numbers of target enzymes from both compartments to see if this initial pattern holds.

Liposome stabilization

I investigated the ability of AfrLEA6 to stabilize lipid bilayers of different compositions during drying, and I was especially interested to determine if AfrLEA6 could display preferential protection to liposome mimicking membranes contacted by AfrLEA6 *in vivo* (ILPM and OMM). In the case of Group 3 LEA proteins from *A. franciscana*, the protection was primarily dependent on whether liposomes were composed of physiological lipid ratios compared to a non-biological composition of 100% PC (Moore et al. 2016). Liposomes of 100% PC were hardly

protected at all by Group 3 proteins (including one from pea seeds; Tolleter et al., 2010), perhaps due to the tight packing of PC in the hydrated state at high, non-physiological concentrations (Hincha et al. 1999). For liposomes composed of 100% PC, both AfrLEA6 and lysozyme provided an identical reduction in CF leakage (Fig. 3.9). These results were similar to what was reported for Group 3 LEA proteins (Moore et al. 2016); in my study trehalose alone provided more protection to 100% PC liposomes than reported. Similar to Group 3 LEA proteins, AfrLEA6 did not display preferential stabilization across the three physiological compositions of liposomes (ILPM, OMM, IMM). All three were weakly protected against drying damage (Figs. 3.6, 3.7, 3.8). Although Group 3 and Group 6 LEA proteins from *A. franciscana* do not display preferential stabilization of liposomes, lipid preferences have been shown regarding other LEA proteins. For example, Tolleter et al. (2010) suggested that cardiolipin found in the IMM may facilitate the preferential stabilization of IMM liposomes seen with PsLEAm, a Group 3 protein targeted to the mitochondrial matrix in pea seeds. Likewise, COR15 is an intrinsically disordered stress protein that is targeted to the chloroplast and shows specificity for protecting liposomes modeling chloroplast membranes during freezing (Thalhammer et al. 2014).

The basis for the pronounced decrease in ability of AfrLEA6 to protect liposomes compared to Group 3 proteins likely arises from key differences in charge distribution in AfrLEA6. AfrLEA6 is far less hydrophilic than Group 1 or 3 LEA proteins (Janis et al. 2017). In addition to the two Pfam-SMP domains found toward the N-terminus that contain protein-binding MoRF sites, there is a large middle region representing 11% of the total sequence that is highly enriched in proline residues; prolines are known to be α-helix and β-sheet disruptors (Janis et al. 2017). These features may reduce the propensity of AfrLEA6 to form amphipathic α-helices that are observed for Group 3 proteins and predicted to interact with phospholipid head

groups. Modeling predicted that AfrLEA2 and AfrLEA3m likely form amphipathic α-helices during drying that display parallel bands of positively and negatively charged amino acids (Moore et al. 2016), closely resembling the amphipathic α-helices for PsLEAm (Tolleter et al. 2010; Tolleter et al. 2007). Another Group 3 protein, COR15, is thought to confer freeze tolerance through formation of amphipathic α-helices during crowding conditions and subsequent interactions of these α-helices with monogalactosyldiacylglycerol in chloroplast membranes (Thalhammer et al. 2014; Thalhammer et al. 2010).

Alternative roles for AfrLEA6

Based on low titer *in vivo*, it was expected that AfrLEA6 might perform its function at a lower concentration than other LEA proteins. However, any significant protective effects were typically seen at only the highest concentrations tested. Overall, our findings suggest that AfrLEA6 may contribute to desiccation tolerance in *A. franciscana*, but the magnitude of stabilization of both target proteins and liposomes is less than seen with Group 3 LEA proteins from *A. franciscana*. Recent experiments have shown the ability of AfrLEA6 to undergo liquid-liquid phase separation (LLPS), wherein a protein droplet is formed that can selectively exclude certain targets (Janis et al. 2019). Furthermore, Janis et al. (2019) provide evidence for AfrLEA6 forming a hydrogel that transitions into a reversible glassy state upon full desiccation. Janis et al. (2019) offer an attractive hypothesis that AfrLEA6 may undergo LLPS to include and protect desiccation sensitive targets within embryos of *A. franciscana*, which reinforces the perspective that LEA proteins may fulfill different roles in combination to confer desiccation tolerance to an organism.

CHAPTER 4
SUBCELLULAR LOCALIZATION OF AFRLEA6 AND PROPERTIES OF AFRLEA2 AT INTERMEDIATE HYDRATION STATES

4.1. Introduction

Artemia franciscana is an anhydrobiont that can survive long periods of severe water stress in its embryonic stage (Crowe et al. 1997; Crowe and Clegg 1973; Crowe and Clegg 1978; Crowe and Madin 1974). The ability of embryos to survive desiccation can be partly attributed to the accumulation of protective macromolecules such as late embryogenesis abundant (LEA) proteins (Hand et al. 2011; Hand et al. 2018; Tunnacliffe and Wise 2007). A strong body of evidence concludes that anhydrobiotic organisms express multiple LEA proteins, and that LEA proteins are localized to various subcellular compartments such as the cytoplasm, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondrion, and chloroplast (Avelange-Macherel et al. 2018; Boswell and Hand 2014; Hand et al. 2011; Tripathi et al. 2012; Tunnacliffe et al. 2010; Tunnacliffe and Wise 2007). The subcellular localization of LEA proteins is thought to play an important role in protecting biological structures from damage incurred during desiccation and rehydration (Hand and Hagedorn 2008; Menze and Hand 2009). Recently, we have identified, expressed, and purified AfrLEA6, a novel Group 6 LEA protein from *A. franciscana* (Hand and Menze 2015; Janis et al. 2017; LeBlanc et al. 2019; Wu et al. 2011). AfrLEA6 shares strong sequence homology with the seed maturation protein (SMP) D34.3 from *Medicago truncatula* which has been associated with long-term desiccation tolerance in the mature seed (Chatelain et al. 2012). AfrLEA6 is currently the only known example of a Group 6 LEA protein in animals, and it was characterized as an intrinsically disordered protein (IDP) that is strongly associated with the diapause state (Janis et al. 2017; LeBlanc et al. 2019). An unresolved issue regarding protection by LEA proteins is whether gain of secondary structure during drying is a prerequisite for functions like stabilization of macromolecular targets (cf. Chakrabortee et al. 2007;

Chakrabortee et al. 2012; Hand and Menze 2015; Hand et al. 2011; Li and He 2009; Liu et al. 2011; Marunde et al. 2013; Tunnacliffe and Wise 2007) . In this chapter, I evaluate the intracellular localization of AfrLEA6 and assess the structure and function of a LEA protein during intermediate levels of drying.

LEA proteins commonly feature spans of amino acids that contribute to their strong hydrophilicity (Janis et al. 2017). Consequently, LEA proteins are typically classified as intrinsically disordered proteins which fold when interactions with water are removed (Tompa and Kovacs 2010; Uversky and Dunker 2010). The folding of animal LEA proteins during dehydration was first observed in anhydrobiotic nematodes, where Goyal et al. (2003) used fourier-transform infrared (FTIR) spectroscopy to show that aavLEA1 gained secondary structure upon dehydration that was fully reversible upon rehydration. Further studies discovered that LEA protein folding was dependent on the drying kinetics. For example, Wolkers et al. (2001) provide evidence that faster rates of drying led to a higher proportion of α-helix structure, whereas slower rates increased β-sheet as well as α-helix. Based on observations of LEA protein secondary structure in the fully hydrated and dry states, simulations were used to model the structure of LEA proteins at intermediate water contents (Li and He 2009). Li and He's model showed a 66 amino acid region of AavLEA1 folding as a function of % weight of water to weight of protein at various intermediate steps. In this study, I provide evidence that AfrLEA6 is located in the cytoplasm of diapause embryos and that gain of secondary structure by a LEA protein as a function of hydration state is linked to its ability to protect the desiccation sensitive enzyme phosphofructokinase (PFK). Specifically, the proportion of α-helix gained by AfrLEA2 during incremental drying is correlated with enhanced residual activity for PFK dried and quantified upon rehydration. Finally, I describe the resolution of a technical problem associated

with chitin-based affinity chromatography (i.e., the IMPACT system) used for AfrLEA6 purification that should be generally applicable.

4.2. Methods

Preparation of diapause embryos

Diapause embryos were collected in the hydrated state from the surface of the Great Salt Lake (Utah). Diapause embryos were rinsed in lake water, transferred to 1.25 M NaCl containing 200 units/ml nystatin, 50 μg/ml kanamycin, 50 μg/ml penicillin-streptomycin, and stored protected from light at room temperature. These embryos were verified to be in diapause by performing hatching assays as outlined in Reynolds and Hand (2004). The hatch percentage of diapause embryos used in these experiments was 2.9%, indicative of a strong diapause state. Prior to use, diapause embryos were rinsed and incubated with shaking at 110 rpm in artificial seawater (35 practical salinity units; Instant Ocean ®, Blacksburg, VA) for 4 d at room temperature in the dark to allow any embryos that had terminated diapause to hatch. Exposure to light has been associated with increased diapause breakage in the laboratory (Lavens and Sorgeloos 1987). The hatched larvae were discarded, and the remaining diapause embryos were used for experimentation.

The diapause embryos were dechorionated as described by Kwast and Hand (1996) and Reynolds and Hand (2004) with an antiformin solution (1% hypochlorite, 0.4 M NaOH, and 60 mM Na_2CO_3) for 15 min at room temperature with occasional stirring. The antiformin solution was removed and embryos were rinsed three times with ice-cold 0.25 M NaCl. Next, embryos were incubated in an ice-cold solution of 1% w/v sodium thiosulfate for 5 min to inactivate any residual hypochlorite. The 1% sodium thiosulfate was removed, and embryos were rinsed twice

with ice-cold 0.25 M NaCl. Embryos were incubated for 5 min in an ice-cold solution of 0.25 M NaCl containing 40 mM HCl, filtered, and rinsed three times with ice-cold 0.25 M NaCl to remove residual HCl.

Fixation, immunostaining, and microscopy

Prior to fixation, the embryos were incubated as described by Hofmann and Hand (1990) and Reynolds and Hand (2004) for 20 min in a 2 M sucrose solution to reduce turgor pressure, then nicked with a 000-gauge insect pin while under a dissecting microscope to allow entry of the fixative. Embryos must be nicked because the embryo wall is only permeable to water and low molecular weight gases (Clegg and Conte 1980). Embryos were then transferred to a fixing solution (0.2 M KPO_4, 4% paraformaldehyde, and 1 M sucrose, pH 7.8) and allowed to incubate at room temperature for at least 1.5 h. Fixed embryos were embedded in paraffin and 2 µm sections were mounted on microscope slides.

Embryo sections were deparaffinized by two 10 min washes with xylene and rehydrated with 5 min incubations in successively decreasing concentrations of ethanol (100%, 95%, 70%, 50%), ending with one 5 min incubation in phosphate buffered saline (PBS). Sections were first blocked for 1 h at room temperature with normal rabbit serum (NRS; Jackson ImmunoResearch, West Grove PA; 011-000-120) diluted to 5% v/v with PBS. To visualize AfrLEA6, sections were treated with chicken polyclonal anti-AfrLEA6 IgY (AvesLabs Inc., Tigard OR). Simultaneously, sections were treated with mouse monoclonal anti-VDAC1 IgG (AbCam, Cambridge MA; ab14734) to visualize the voltage-dependent anion channel (VDAC) which is an integral membrane protein present in the outer mitochondrial membrane. Both primary antibodies were used at a 1:200 dilution in 5% NRS, and sections were incubated with both antibodies overnight at 4^{o}C. The next morning, sections were washed six times in 10 min increments by gently

rocking in PBS at 4ºC. Next, sections were incubated with fluorescent secondary antibodies for 1 h at 4ºC. Alexa Fluor-488 conjugated rabbit, anti-chicken IgG (Jackson ImmunoResearch; 303-545-003) was used at a 1:500 dilution to visualize AfrLEA6, whereas Rhodamine (TRITC) conjugated rabbit, anti-mouse IgG (Jackson ImmunoResearch; 315-025-003) was used at a 1:500 dilution to visualize the mitochondria. Sections were washed again as previously described to remove unbound secondary antibodies. Samples were sealed using 1 drop of ProLong Gold Antifade with DAPI (Cell Signaling Technology; Danvers, MA; 8961S) to visualize the nuclei. Prior to microscopy, the slides were cured in the dark for 24 h.

Microscopy was performed using a Leica TCS SP8 confocal microscope with white light lasers capable of both differential interference contrast (DIC) and fluorescence microscopy. To visualize nuclei, AfrLEA6, and mitochondria, the three lasers were set at excitation wavelengths of 358 nm at 0.25% power, 488 nm at 15% power, and 547 nm at 3% power, respectively. Three hybrid detectors were utilized with gating set to 405-458 nm, 504-543 nm, and 561-706 nm to detect emission signals. Yolk platelets were visualized using DIC microscopy. Image analysis was performed using Leica Application Suite X (LAS X).

Detection of trace amounts of chitin binding domain in AfrLEA6 preparations

Recombinant AfrLEA6 was purified with an IMPACT protein expression system (Intein-Mediated Purification with an Affinity Chitin-binding Tag; New England BioLabs Inc., Ipswich, MA) as previously described (LeBlanc et al. 2019). With this procedure, a chitin binding domain (CBD) and self-cleavage protein splicing element (intein) were added to AfrLEA6 to create an expressed fusion protein. These elements permit binding of AfrLEA6 to the chitin moiety of the affinity column (New England BioLabs Inc.; S6651S) but are normally retained on the column when ArfLEA6 is cleaved and eluted. Consequently, it was possible that trace amounts of the

CDB fused to intein (approx. 28 kDa total) were released from the column in addition to the purified AfrLEA6 (27 kDA), which could co-migrate on SDS-PAGE. To test for this possibility, an antibody against CBD was used in Western blot analysis. Samples of AfrLEA6 after the last purification step (anion exchange chromatography, cf., LeBlanc et al. 2019) were loaded onto SDS polyacrylamide gels (4% stacking gel, 11% resolving gel). Electrophoresis was performed at 125 V for 80 min in a Bio-Rad Mini Protean 3 cell. Following electrophoresis, samples were transferred to a nitrocellulose membrane at 80 V for 60 min in Towbin's buffer (25 mM tris, 192 mM glycine, 20% v/v methanol, 0.025% SDS). The nitrocellulose membranes were then blocked for one hour in 5% fat-free dry milk prepared in TBS-T (20 mM Tris, 500 mM NaCl, 0.1 % Tween 20, pH 7.6) at room temperature with rocking. Then, blots were incubated overnight at 4^{o}C with either chicken anti-AfrLEA6 polyclonal antibody (Aves Labs Inc.) diluted 1:50,000 or mouse anti-CBD monoclonal antibody (New England BioLabs Inc.; E8034S) diluted 1:1,000 in 5% dry milk prepared in TBS-T. Afterwards, membranes were given three 15-min rinses in TBS-T with shaking. The blots were incubated at room temperature for 1 h with one of two horseradish peroxide-labeled (HRP) secondary antibodies: goat anti-chicken (AvesLabs Inc.; H-1004) at a 1:10,000 dilution or goat anti-mouse (Abcam; ab205719) at a 1:5,000 dilution. The blots were developed using an Amersham ECL Prime Western Blotting Detection Kit (GE Healthcare; Chicago, IL) and imaged with a Bio-Rad ChemiDocTM XRS+ Imager

Removal of antibodies against chitin binding proteins by antiserum adsorption

A. franciscana embryos are known to contain chitin binding proteins in the outer cuticular membrane (Ma et al. 2013). Consequently, two samples of dechorionated diapause embryos (approximately 0.5 g each) were inspected under a dissecting microscope to verify that their embryonic cuticles were intact. After confirming integrity, the embryos were settled by

gravity and the aqueous medium was removed. Polyclonal chicken anti-AfrLEA6 IgY was diluted 1:200 with 5% v/v NRS, and then 1.0 ml was added to the first sample of dechorionated diapause embryos. The embryo-antibody mixture was gently rocked for 1 h at room temperature in a 2 ml microcentrifuge tube. After 1 h, the adsorbed antiserum was separated from the embryos and transferred to a second tube containing the second sample of dechorionated diapause embryos. The process was repeated and the 2X adsorbed antiserum was separated from the dechorionated embryos and used for subcellular localization studies of AfrLEA6.

Protection of phosphofructokinase by AfrLEA2 during intermediate drying

The target enzyme PFK was used to assess protection at intermediate hydration states due to its fragile polymerization state (Aaronson and Frieden 1972; Bock and Frieden 1974; Bock and Frieden 1976a; Bock and Frieden 1976b; Carpenter and Hand 1986a; Carpenter and Hand 1986b; Frieden et al. 1976; Hand and Somero 1982; Hand and Somero 1983; Parr and Hammes 1975; Parr and Hammes 1976) and extreme sensitivity to water stress (Boswell et al. 2014a; Carpenter and Crowe 1988; Carpenter and Crowe 1989; Carpenter et al. 1986; Carpenter et al. 1987). AfrLEA2 used in this experiment was expressed and purified according to Boswell et al. (2014a); (Boswell et al. 2014b) and was chosen over AfrLEA6 due to the significant protection it offered to phosphofructokinase (PFK) when combined with trehalose. Intermediate hydration states were achieved by incubation of protein solutions in sealed chambers each controlled at a specific relative humidity (RH). One chamber contained distilled water to achieve 100% RH, and one chamber contained Drierite desiccant (calcium sulfate) to achieve 0% RH. The saturated salt solutions used to achieve intermediate RH values were 85% (potassium chloride), 75% (sodium chloride), 33% (magnesium chloride), and 11% (lithium chloride; Winston and Bates, 1960; Young 1967). Chambers were equilibrated for 24 h at room temperature with the desired

saturated solution (containing excess salt crystals) prior to experiments. Then, one 50 µl droplet containing 100 mM trehalose, 0.15 mg/ml PFK, and 0.4 mg/ml AfrLEA2 prepared in PFK dialysis buffer (100 mM sodium phosphate buffer, containing 1 mM EDTA, 5 mM DTT, pH 8.0) was placed on a 12 mm diameter glass disk and transferred to a raised platform inside of each airtight chamber. Samples without protein stabilizers (AfrLEA2 or BSA) that contained 100 mM trehalose and 0.15 mg/ml PFK were placed on separate glass disks and transferred into the corresponding chambers alongside samples containing AfrLEA2.

For activity measurements of control (never dried) PFK, additional aliquots of sample mixtures were kept sealed in microfuge tubes at room temperature and assayed for activity at the termination of RH incubation (48 h). For measurements of PFK activity after drying and rehydration, samples were incubated for 48 h at room temperature, removed from the chambers, placed on ice, and rehydrated for 1 h by adding PFK dialysis buffer to a final volume of 100 µl (2X diluted over original volume). Samples were assayed for PFK activity at 25^{o}C using the procedure described by Bock and Frieden (1974). Briefly, the 1 ml reaction volume contained final concentrations of 33 mM tris-acetate buffer (pH 8.0), 40 mM KCl, 4 mM NH_4Cl, 2 mM ATP, 2 mM fructose-6-phosphate, 2 mM magnesium acetate, 0.16 mM NADH, 2.5 units/ml of glycerol-3-phosphate dehydrogenase (Sigma-Aldrich; G6751), 2 units/ml of aldolase (Sigma-Aldrich; A8811), and 25 units/ml of triosephosphate isomerase (Sigma-Aldrich; T2391). Prior to use, all accessory enzymes were dialyzed against 100 mM tris-acetate buffer (pH 8.0) containing 0.1 mM EDTA. The reaction was initiated by the addition of 5 µl of dried/rehydrated samples, or 2.5 µl of control (never dried) samples, and the change in absorbance at 340 nm (ΔA_{340}) was recorded for 3 min. One unit is defined as the production of 1 µmol of fructose 1,6 bisphosphate/min (one-half the measured rate of the enzymatically coupled NAD^+ production).

Residual PFK activity after intermediate drying was reported as a percentage of the rate of the control samples.

Secondary structure analysis of AfrLEA2 after intermediate drying

Experiments to analyze the secondary structure of AfrLEA2 after drying to intermediate hydration states were performed in parallel with experiments on protection of PFK. One 50 µl droplet containing 0.55 mg/ml AfrLEA2 and 100 mM trehalose was prepared in 10 mM potassium phosphate buffer, pH 7.5, and placed on a demountable quartz window for cuvettes with 0.01 cm path length (Z805693; Sigma Aldrich). The concentration of AfrLEA2 was raised to 0.55 mg/ml to match the total protein contained in samples for the PFK stabilization series above. To subtract the trehalose spectra from the spectra of AfrLEA2, samples containing 100 mM trehalose alone were prepared and incubated simultaneously in the humidity chambers. All samples were incubated simultaneously in the RH chambers for 48 h at room temperature. After incubation, the demountable quartz windows were quickly removed from the chamber and sealed in the cuvettes with the supplied cover slips.

Spectra were obtained with a Jasco J-815 circular dichroism spectropolarimeter and analyzed using Spectra Manager™ Suite (Jasco Analytical Instruments; Easton, OH). Spectra Manager™ Suite was used to subtract blank spectra, convert values to mean residue ellipticity, and smooth the curves. Measurements were recorded between 190-250 nm and converted to mean residue ellipticity using a pathlength of 0.01 cm. Spectra were smoothed with a convolution width of 9 (Savitzky and Golay 1964). Analysis of secondary structure was achieved using DICHROWEB online analysis software (Whitmore and Wallace 2004; Whitmore and Wallace 2008) to apply the CONTINLL algorithm (Provencher and Glockner 1981; van Stokkum et al. 1990) and SELCON3 algorithm (Sreerama et al. 1999; Sreerama and Woody

1993). Reference dataset 7 was utilized for spectra analyses as it applies to denatured proteins with measurements between 190-250 nm wavelengths (Sreerama et al. 1999).

4.3. Results

Subcellular localization of AfrLEA6

The cytoplasm of diapause embryos is dominated by yolk platelets of ~2.5 µm diameter, and the fluorescence visualized upon treatment with anti-AfrLEA6 IgY and fluorescent secondary antibody was dispersed in the cytoplasmic space surrounding these platelets (Fig. 4.1 A). There was no significant co-localization of AfrLEA6 with the DAPI-stained nuclei, but in some cases, there seemed to be limited areas of overlapping fluorescence with the mitochondria (Fig. 4.1 B-D). Surprisingly, fluorescence was also prominent in the embryonic cuticular layer (ECL; Fig. 4.1 A). The ECL is a non-cellular, multi-layered structure composed predominantly of chitin. At high magnification, the intense fluorescence imaged in the ECL with anti-AfrLEA6 antibody was exceptionally evident in the outer and inner cuticular membranes, with virtually no staining of the fibrous layer that resides in between (Fig. 4.1 E). Consequently, fractions taken from the last step of the AfrLEA6 protein purification were analyzed via Western blot using anti-AfrLEA6 IgY or a mouse anti-CBD monoclonal antibody to check for trace contamination of AfrLEA6 with CBD-intein. Western blot analysis confirmed co-migration of CBD-intein in the anion exchange fraction containing AfrLEA6 (Fig. 4.2 A). Accordingly, immunohistochemistry was repeated with chicken polyclonal anti-AfrLEA6 IgY that had been adsorbed to remove contaminating anti-CBD IgY (see Methods). Usage of the pre-adsorbed antiserum against

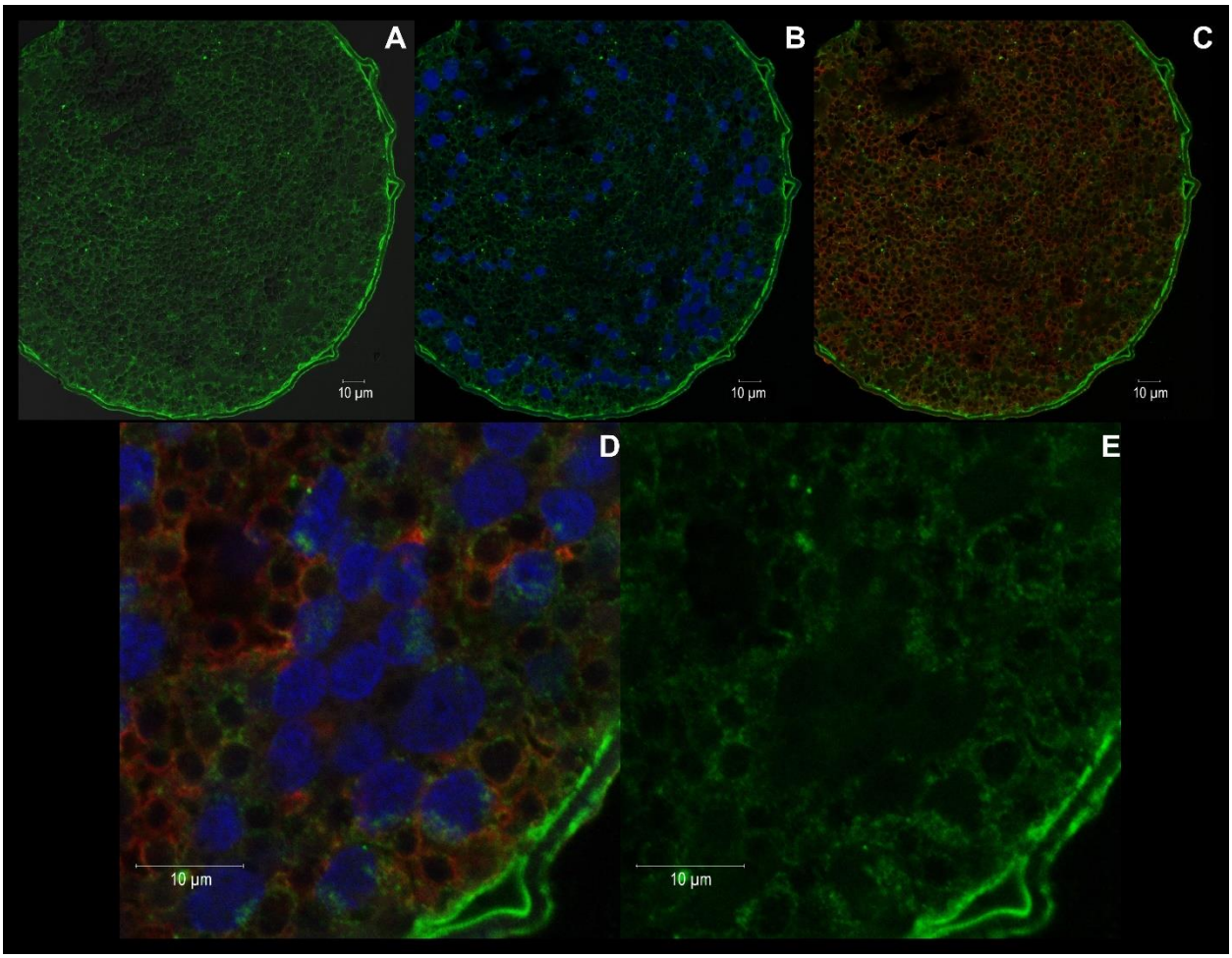

Figure 4.1. Images of a diapause embryo from *A. franciscana* obtained with confocal fluorescence and/or differential interference contrast microscopy. Merged images show the location of (A) AfrLEA6 (anti-AfrLEA6 antibody, green) and spherical yolk platelets (gray) of ~2.5 µm diameter, (B) AfrLEA6 and nuclei (DAPI-stained, blue), or (C) AfrLEA6 and mitochondria (anti-VDAC antibody, red). (D) Combined images of AfrLEA6, nuclei, and mitochondria at high magnification shows AfrLEA6 located in the cytoplasm, independent of nuclei or mitochondria. (E) A high magnification image of immunofluorescence obtained with anti-AfrLEA6 antibody alone showing staining throughout the cytoplasm and in the ECL, especially in the outer and inner cuticular membrane.

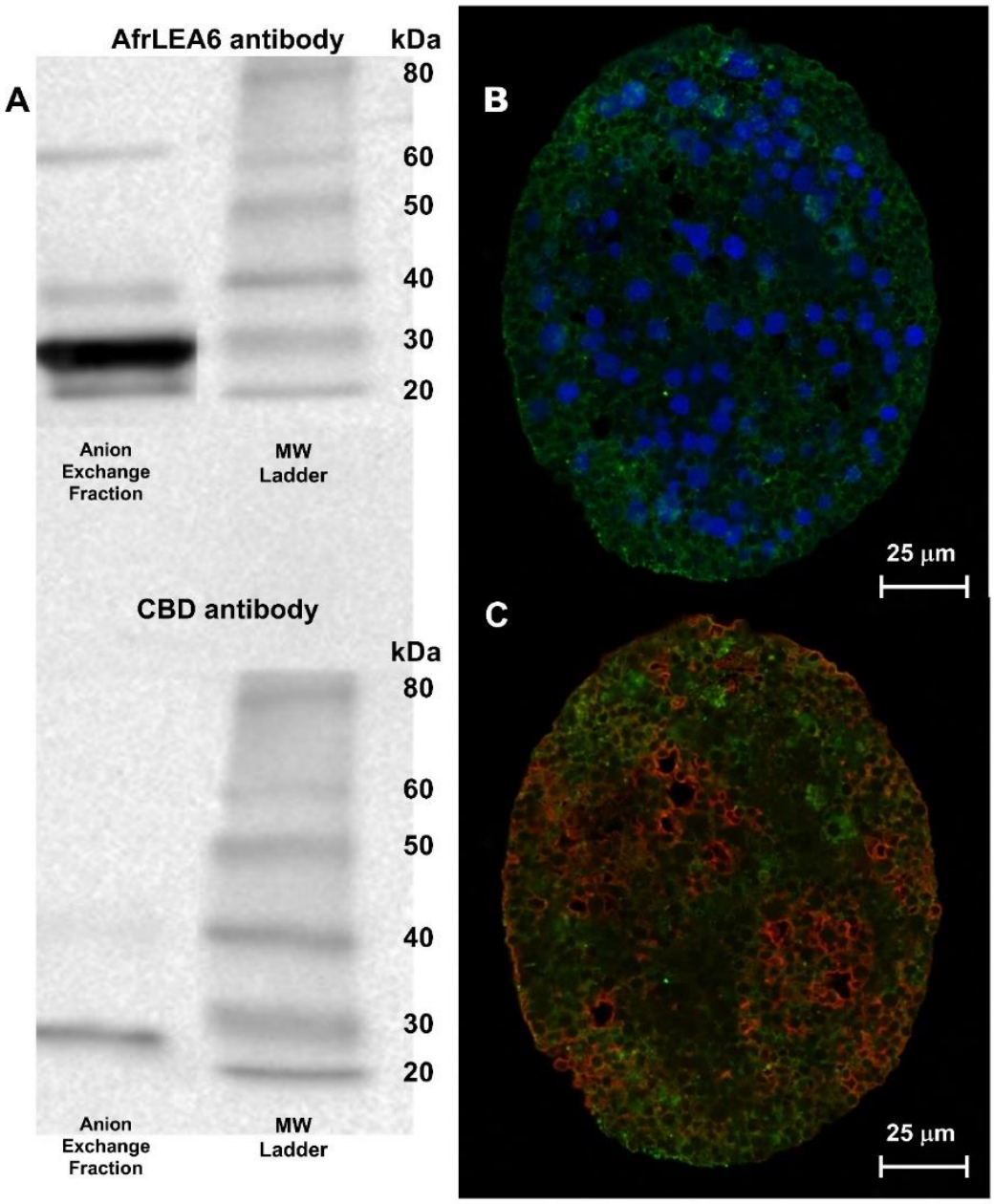

Figure 4.2. The co-migration of AfrLEA6 and CBD-intein during SDS-PAGE and removal of fluorescence in the ECL by using pre-adsorbed antiserum. (A) Western blot analysis of pooled fractions and purified, recombinant AfrLEA6 from anion exchange chromatography. Blots were treated with either anti-AfrLEA6 or anti-CBD primary antibodies. The lanes labeled "anion exchange fraction" were overloaded with 20 µl of sample to detect the presence of trace amounts of CBD-intein (28 kDa). Images of a diapause embryo from *A. franciscana* obtained with confocal fluorescence microscopy after treatment with pre-adsorbed antiserum show AfrLEA6 alongside (B) nuclei (DAPI-stained, blue) or (C) mitochondria (anti-VDAC antibody, red). Fluorescence in the ECL is no longer observed after using the pre-adsorbed antiserum, but ArfLEA6-specific fluorescence in the cytoplasm is not diminished.

AfrLEA6 resulted in the complete elimination of fluorescence in the outer cuticular membrane, while retaining full intensity of AfrLEA6 staining in the cytoplasm (Fig. 4.2 B, C). Additionally, the overlap of AfrLEA6 with mitochondria fluorescence (i.e., VDAC staining) was likely coincidental because the volume of free cytoplasm is so constrained among yolk platelets and mitochondria; AfrLEA6 fluorescence can clearly be seen in areas where no mitochondrial fluorescence exists (Fig. 4.2 C). This experimental determination of a cytoplasmic localization is supported by the targeting prediction we acquired with the bioinformatics software TargetP, which indicates a cytoplasmic location of AfrLEA6 with 80.6% confidence.

Secondary structure and protective effects of AfrLEA2 during intermediate drying

Analyses of the structure of AfrLEA2 during drying reveal that as RH decreases, there are major shifts in the makeup of total secondary structure (Fig. 4.3). Generally, the percentages of turns and random coil remained similar despite differences in RH. Changes were observed primarily in the percentage of α-helix and β-sheet. As RH decreased from 100% to 33%, the proportion of α-helix progressively increased from 5.7% to 36.1%. In contrast, the proportion of β-sheet decreased from 33.7% to 5.8% across the same range (Fig. 4.3). Unexpectedly however, the proportion of α-helix decreased at 11% and 0% RH compared to the values recorded at 33%. Simultaneously, the percentage of β-sheet increased at 11% and 0% RH (Fig. 4.3).

The residual activity of PFK was assessed after drying to intermediate hydration states in the presence or absence of protein protectants plus trehalose. Equilibration for 48 h without any protectants other than trehalose showed that residual activity of PFK decreased as a function of dryness (Fig. 4.4). Residual activity with trehalose was highest at 100% RH with a value of 75.8 ± 2.7% (mean ± SD; n=3), which decreased to 8.4 ± 0.6% (mean ± SD; n=3) when equilibrated at 0% RH (Fig. 4.4). PFK activity was protected much better in samples containing AfrLEA2,

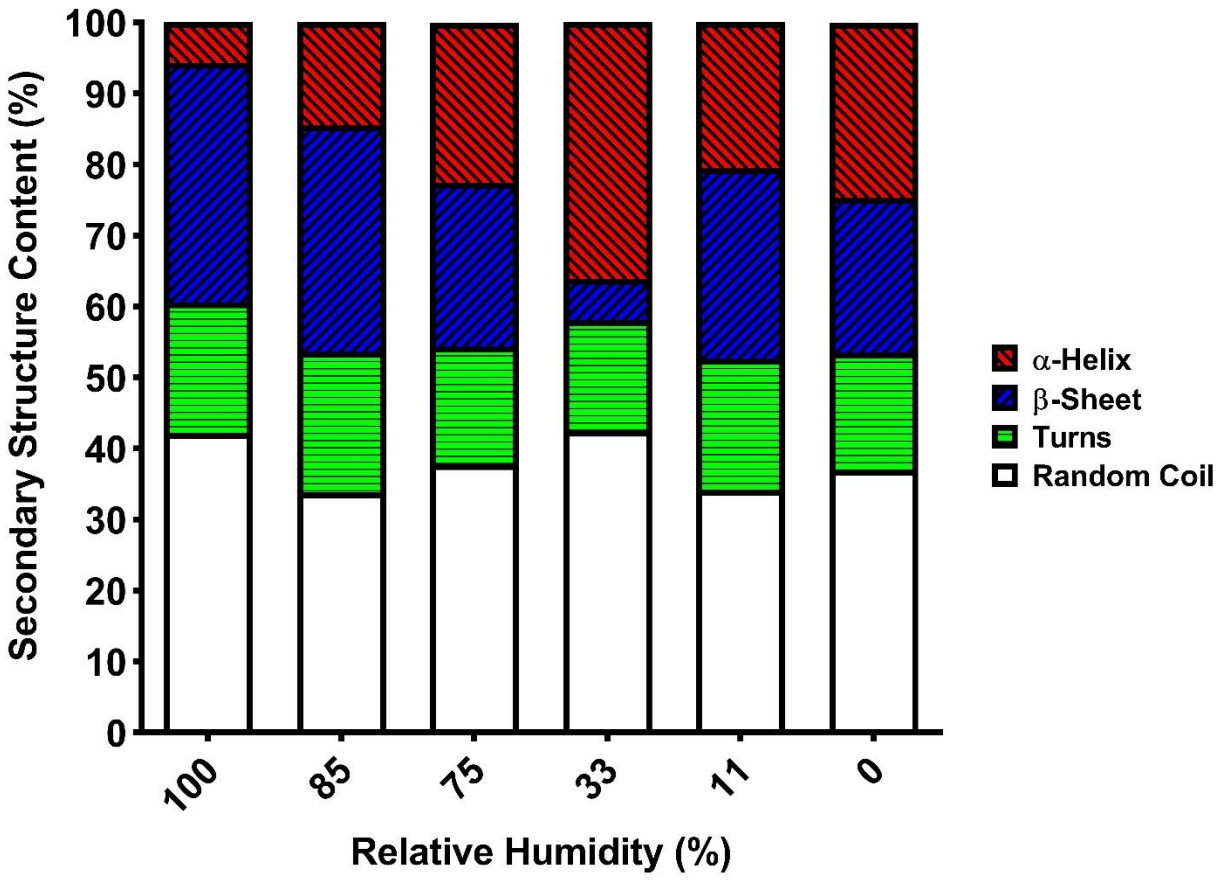

Figure 4.3. Comparison of secondary structure content for AfrLEA2 based on CD spectra recorded after equilibration for 48 h at specific relative humidities. AfrLEA2 was measured at a protein concentration of 0.55 mg/ml. Analysis of secondary structure was performed using the Dichroweb server with the Contin-LL method (see methods).

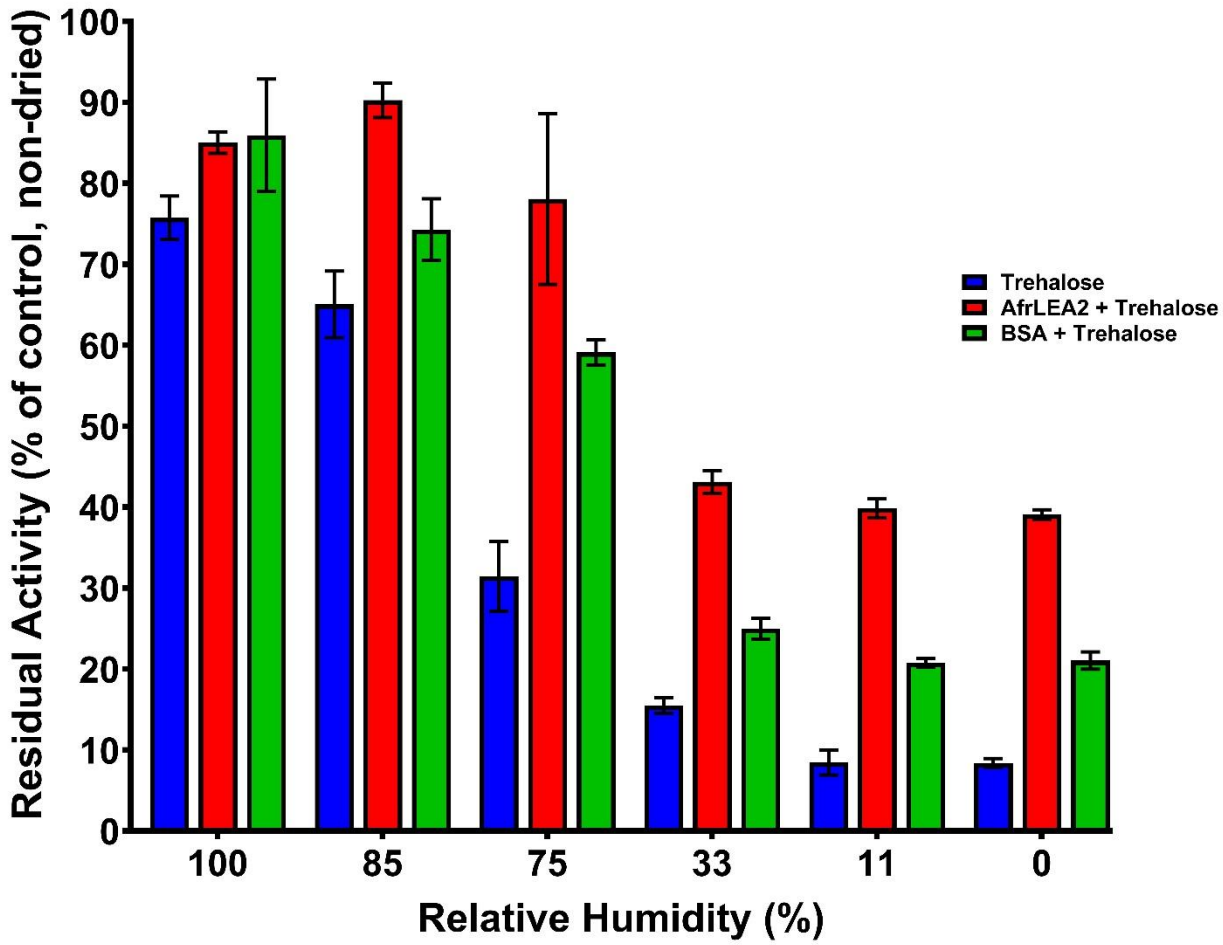

Figure 4.4. Residual phosphofructokinase (PFK) activity after incubation at specific relative humidities (RH) for 48 h at room temperature in the presence of protectants. Data are expressed as a percentage of control PFK activity without drying (mean ± SD; n=3). The concentrations of protectant proteins were 400 μg/ml. All samples contained 100 mM trehalose. Both AfrLEA2 and BSA offered significant protection over trehalose alone starting at 75% RH (Welch's ANOVA plus Dunnett's T3 test, $P<0.05$), but were not statistically different from one another ($P>0.05$). The protection offered by AfrLEA2 was significantly higher than BSA at RH at and below 33% (Welch's ANOVA plus Dunnett's T3 test, $P<0.05$). Symbols denoting statistical significance were omitted from the figure for clarity.

especially at the lower RH values. Protection of PFK by AfrLEA2 was detectable at 85% RH, where residual activity was 90.6 ± 2.1% (mean ± SD; n=3) compared to 65.0 ± 4.1% (mean ± SD; n=3) for trehalose alone. As relative humidity decreased, the protection offered by AfrLEA2 became more apparent. For example, at 0% RH, AfrLEA2 preserved 39.1 ± 0.6% (mean ± SD; n=3) activity, nearly a fivefold increase over samples with trehalose alone (Fig 4.4).

The protection offered by AfrLEA2 was compared to BSA, which is documented to possess significant stabilizing capacity (Chang and Mahoney 1995). Indeed, BSA protected PFK, evidenced by a residual activity of 59.1 ± 1.6% (mean ± SD; n=3) at 75% RH where the enzyme became heavily damaged in samples containing trehalose (Fig. 4.4). However, the effectiveness of BSA significantly decreased as RH decreased, where BSA preserved only 21.1 ± 1.1% (mean ± SD; n=3) of PFK activity. A comparison of residual activity between samples containing AfrLEA2 and BSA revealed that the protection offered by AfrLEA2 began to exceed that of BSA as RH decreased (Fig. 4.5). For example, residual activity in samples at 33% RH containing AfrLEA2 was 278.5% higher than samples containing trehalose alone, whereas residual activity in samples containing BSA was only 161.5% higher than trehalose alone (Welch's ANOVA plus Dunnett's T3 test, $P<0.05$; Fig. 4.5). At the lowest RH, AfrLEA2 offered nearly twice the protection to PFK than BSA. Furthermore, the ability of AfrLEA2 to protect PFK during drying coincided with the gain of α-helix secondary structure as the relative dryness of the sample increased (Fig 4.6). To statistically support this relationship, best fit lines (not shown) for the α-helix and residual activity data sets were evaluated and there was no significant difference between the slopes of these lines ($P=0.077$).

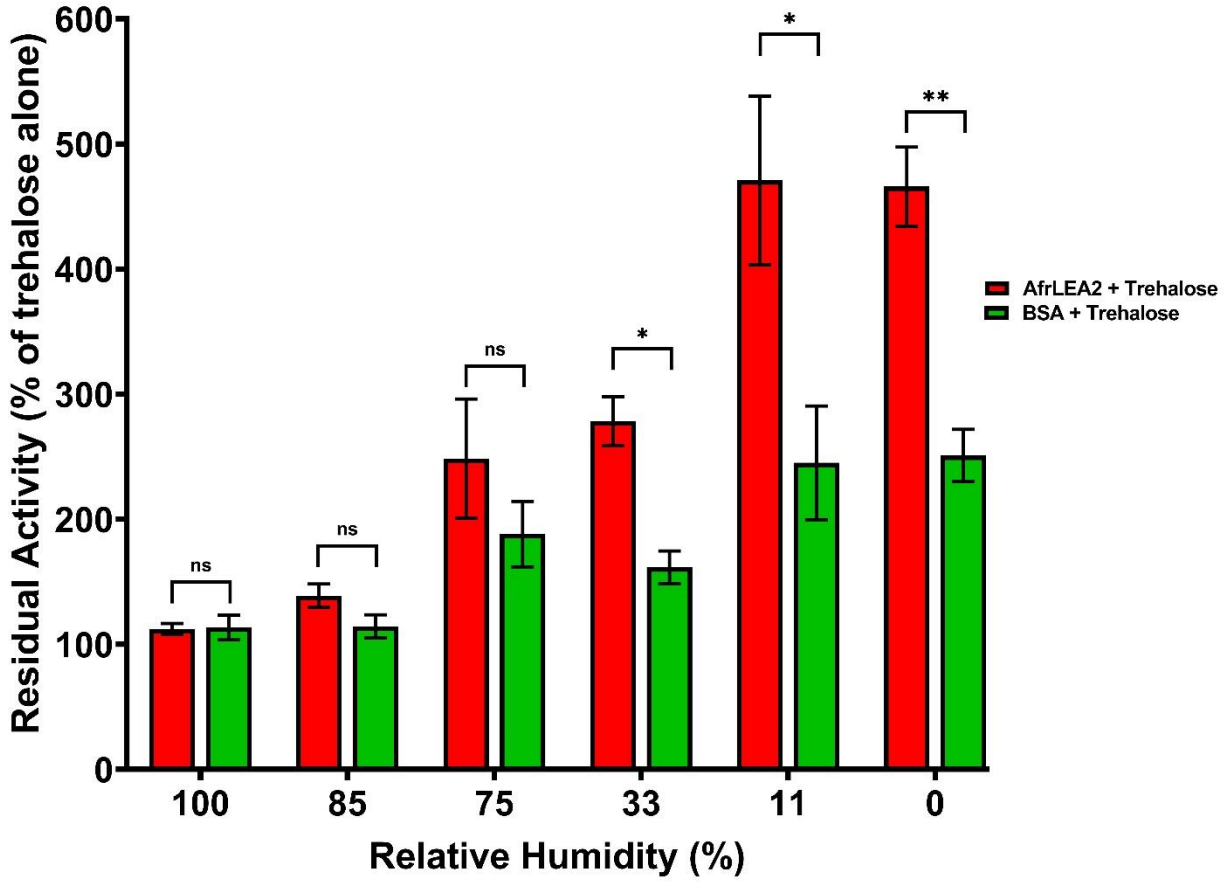

Figure 4.5. Residual phosphofructokinase (PFK) activity, expressed as a percentage of values for trehalose alone versus % relative humidity (RH). Samples were incubated at specific RH for 48 h at room temperature in the presence of protectants. Data are expressed as a percentage of the residual PFK activity for samples containing trehalose alone (mean ± SD; n=3) to clearly emphasize the added effects of proteins. The concentrations of protectant proteins were 400 µg/ml. All samples contained 100 mM trehalose. AfrLEA2 protected PFK significantly more than BSA did at 33% RH and below (Welch's ANOVA plus Dunnett's T3 test, $P<0.05$). Asterisks denote statistical significance among means (ns = not significant, * = $P<0.05$, ** = $P<0.01$).

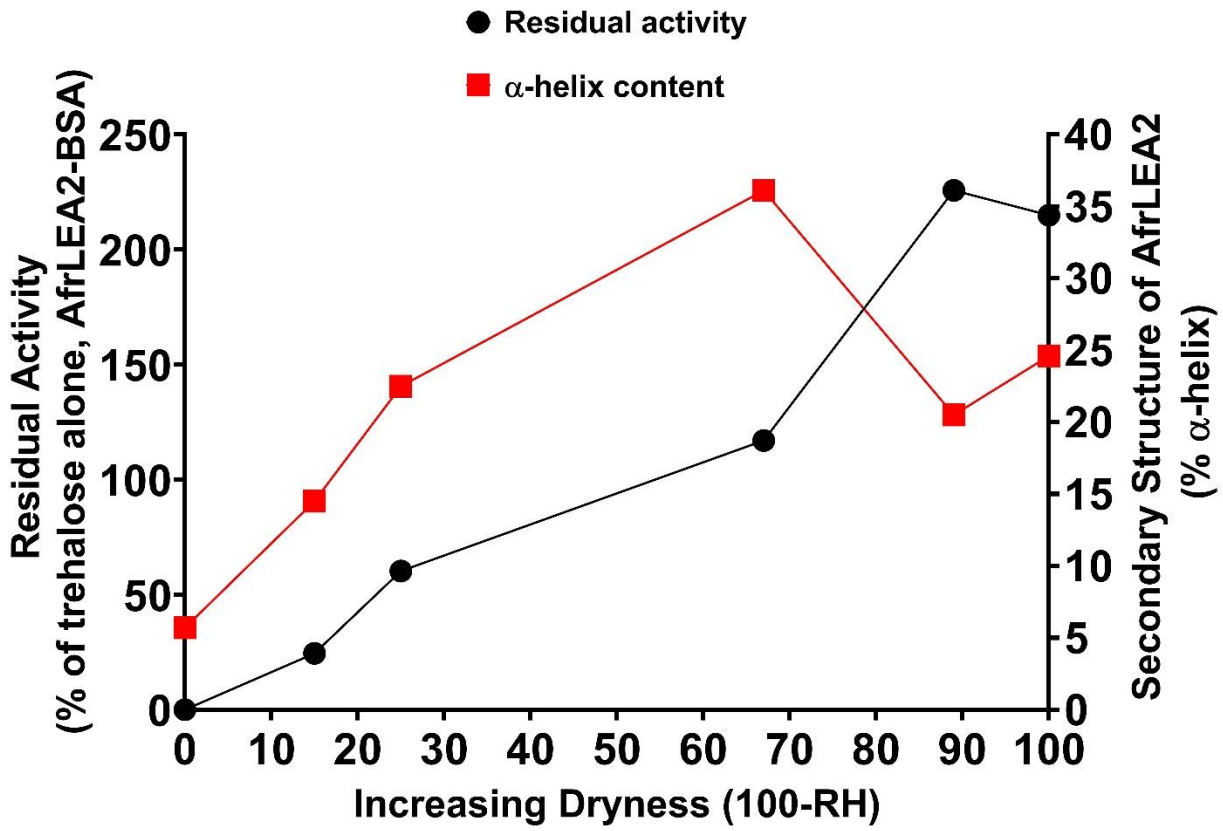

Figure 4.6. Progressive increase in protection of PFK by AfrLEA2 is paralleled by the gain of secondary structure in AfrLEA2 as a function of dryness. Data on the left axis represent the difference in residual activity of PFK between samples containing AfrLEA2 versus BSA (from Fig. 4.5). The specific stabilizing effect of AfrLEA2 is emphasized by subtracting the influence of BSA. Data on the right axis denote α-helix structure of AfrLEA2 expressed as a percentage of total secondary structure (from Fig. 4.3).

4.4. Discussion

Results presented in this chapter address important questions about LEA proteins that have remained unanswered thus far. I provide the first experimental evidence for the cytoplasmic localization of a Group 6 LEA protein within an anhydrobiotic animal, which supports the concept that multiple LEA proteins are needed across a range of subcellular compartments for desiccation tolerance. Additionally, I resolve a technical problem by removal of unwanted antibodies arising from protein linkers released during chitin-based affinity chromatography (IMPACT system) that may be generally applicable. Finally, drying to intermediate states of hydration revealed a progressive change in secondary structure for AfrLEA2 that correlated with its enhanced ability to protect phosphofructokinase. This finding provides the first evidence that protection of protein targets by LEA proteins is dependent on a gain of secondary structure.

AfrLEA6 localization

Results presented here demonstrate that AfrLEA6 is solely a cytoplasmically localized LEA protein and does not seem to be present in mitochondria like AfrLEA3m or in the nuclei like AfrLEA2 (Boswell and Hand 2014). The observation is supported by the targeting prediction of a cytoplasmic location from bioinformatics. The morphology of diapause embryos exhibits a high density of yolk platelets (Fig. 4.1 A) which are essential for meeting the biosynthetic and energetic demands upon embryo emergence and hatching (Clegg et al. 2000; Utterback and Hand 1987). However, these yolk platelets crowd and restrict the available free cytoplasm which can make the imaging of cytoplasmically localized proteins difficult (Boswell and Hand 2014). Despite this additional challenge, many areas were located where AfrLEA6 fluorescence was present without intrusion by mitochondria, nuclei, or yolk platelets (Fig. 4.1 and Fig. 4.2).

Unexpectedly strong fluorescence with anti-AfrLEA6 antibody was observed in the ECL, which is a tripartite structure composed of the outer cuticular membrane (OCM), a loose, fibrous middle layer, and an inner cuticular membrane (ICM) (Belk 1987; Morris and Afzelius 1967). Based on these observations, I predicted that AfrLEA6 antiserum possibly contained antibody against bacterial CBD that inadvertently released from the chitin moiety of the affinity column. Indeed, Ma et al. (2013) provide evidence that *Artemia* chitin-binding proteins (*Ar*-CBP) play a critical role in the development of the ECL. As seen in Fig. 4.2 A, I confirmed the co-migration of CBD-intein (28 kDa) and AfrLEA6 (27 kDa) with Western blots. Significant cross-reactivity of antiserum (which apparently contained antibody against bacterial CBD) with *Ar*-CBPs in the ECL generated fluorescence that was nonspecific for AfrLEA6. Adsorption of the AfrLEA6 antiserum by incubation with dechorionated embryos removed CBD antibodies in the serum. As a result, confocal images obtained with pre-adsorbed antiserum showed complete elimination of fluorescence in the ECL (Fig. 4.2 B, C). Although the IMPACT expression and purification system is advantageous for yielding recombinant AfrLEA6 free of exogenous tags (hexa-histidine), trace contaminants released from the chitin column are clearly antigenic in chickens. Pre-adsorption of antiserum with dechorionated embryos of *A. franciscana* is an effective way to remove unwanted CBD-intein antibodies found in antiserum raised against IMPACT-purified antigens.

Intermediate drying experiments

Results support a role of LEA proteins in desiccation tolerance across a range of hydration states. Clearly, PFK is highly sensitive to desiccation damage, as evidenced by <10% residual activity at 0% RH in samples containing trehalose alone. The protection offered by AfrLEA2 was equivalent to BSA down to 75% RH, but far surpassed BSA at lower relative

humidities (Fig. 4.4). Both proteins stabilized PFK much better than trehalose alone starting at 75% RH and below. Importantly, the stabilization by AfrLEA2 was accompanied overall by a gain of α-helical structure (Fig. 4.6), and slope analysis of best fit lines for these relationships (α-helix gain and residual activity versus dryness) confirmed there was no significant difference between the slopes. An unexplained observation was the decrease in α-helix content (and gain of β-sheet) at 11% and 0% RH. Perhaps trehalose enters a glassy state that impacts the structure of AfrLEA2 at lower water contents; certainly, in the absence of trehalose, AfrLEA2 exhibits a very high α-helix content of 45.6% in the fully dried state (Boswell et al. 2014a).

Previous work has shown that LEA proteins may display certain functions in the fully hydrated state when they are still intrinsically disordered. Prevention of aggregation has been documented at full hydration in human cell lines expressing LEA protein from the nematode *Aphelenchus avenae* (Chakrabortee et al. 2007; Chakrabortee et al. 2012a). Similarly, Marunde et al. (2013) provided evidence of a Group 1 LEA protein to protect respiration and proliferation of desiccation-sensitive cell lines from insects during moderate osmotic stress at hydration states where LEA proteins predictably were still disordered. It is possible that LEA proteins are functional across a broad range of hydration states, and that the nature of the protection conferred could differ depending on water availability (Hand and Menze 2015). An individual LEA protein could function as a molecular shield (Chakrabortee et al. 2012b) in solution, and the same LEA protein could gain structure as water is removed to further protect the cell in the dry state by interacting with membranes, stabilizing sugar glasses, and forming filamentous networks. Although it has been suggested that function of LEA proteins in the dry state might mirror an increase in α-helix structure (e.g., Li and He, 2009), this research provides the first direct

evidence linking gain of secondary structure with stabilization of a target protein across a graded series of drying.

CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

This dissertation research has provided further evidence for the role of LEA proteins in desiccation tolerance by examining various aspects of the novel Group 6 LEA protein, AfrLEA6 from embryos of *Artemia franciscana*. In chapter 2, AfrLEA6 was successfully expressed and purified; the recombinant protein was used for in-vitro studies as well as the creation of custom antibodies used in in-vivo studies. Purification of AfrLEA6 was accomplished using an IMPACT expression system (Intein-Mediated Purification with an Affinity Chitin-binding Tag), which allowed for an improved purification of native AfrLEA6 without N or C terminal affinity tags as previously used with Group 3 LEA proteins. Higher purity was achieved by anion exchange chromatography following the IMPACT purification. Estimates of AfrLEA6 secondary structure were accomplished with circular dichroism spectroscopy, and it was determined that AfrLEA6 is an intrinsically disordered protein that gains a majority of α-helix structure upon drying or treatment with 2% SDS or TFE. Compared to AfrLEA2, a Group 3 LEA protein, AfrLEA6 was found to have less β-sheet structure upon drying. Additionally, this study investigated whether trehalose, an important sugar implicated in desiccation tolerance in embryos of *A. franciscana*, could promote a gain of secondary structure for AfrLEA6, AfrLEA2, or AfrLEA3m at various physiological concentrations. These findings indicate that in the fully aqueous state, trehalose alone does not impact the structure of LEA proteins.

The AfrLEA6 polyclonal antibody was used to probe embryos of *A. franciscana* to quantify the expression of AfrLEA6 at various timepoints throughout pre-emergence development. Based on western blot analysis, the highest titer of AfrLEA6 was calculated during diapause where it existed at a concentration of 0.173 ± 0.016 mg per ml embryo water. Upon 0 h of pre-emergence development, there was a 35% reduction in AfrLEA6 which declined steadily

throughout 8 h of development and was completely undetectable in free-swimming nauplii, which parallels the loss of desiccation tolerance in adults. Unexpectedly, the titer of AfrLEA6 throughout development was determined to be roughly ten-fold lower than what has been reported for Group 3 LEA proteins AfrLEA2 and AfrLEA3m (Boswell et al. 2014b). The combined results of chapter 2 suggest that AfrLEA6 may function at a much lower concentration than other LEA proteins in *A. franciscana* to aid in desiccation tolerance of the embryo.

In chapter 3, the ability of AfrLEA6 to protect target enzymes and liposome models of biological membranes from damage due to desiccation was assessed. Target enzymes were chosen based on their sensitivity to desiccation as well as the subcellular compartment from which they originate. My results indicate that AfrLEA6 alone is effective at stabilizing cytoplasmic (LDH and PFK), but not a mitochondrial (CS) enzyme. In contrast, other LEA proteins from *A. franciscana* seem to offer unspecific protection. When combined with trehalose, the protection of LDH and CS by AfrLEA6 increased, but not with PFK. In any case, the protection offered by AfrLEA6 matched or exceeded the protection offered by BSA but did not display the magnitude of protection that was seen with Group 3 LEA proteins (Boswell et al. 2014a). Based on the low titer of AfrLEA6 in-vivo, it was expected that a protective role might be fulfilled at a much lower concentration than other LEA proteins. Instead, protection by AfrLEA6 was generally seen at the highest concentrations tested.

Liposomes were dried that simulated the inner and outer mitochondrial membranes as well as the inner leaflet of the plasma membrane in the presence of varying protein:lipid mass ratios of AfrLEA6. The results of membrane leakage assays suggest that AfrLEA6 does not play a major role in stabilizing liposomes regardless of their lipid composition. The lipid ratios used in this research were based on data for mammalian membranes, which has previously been used

successfully with LEA proteins from *A. franciscana* (Moore and Hand 2016; Moore et al. 2016). Clearly under these experimental conditions, AfrLEA6 is not as stabilizing as other LEA proteins studied in *A. franciscana*. One possible explanation for the limited protection of mammalian-based liposomes by AfrLEA6 may be that AfrLEA6 interacts with specific liposome compositions. Although this specificity was not shown for AfrLEA2 or AfrLEA3m, there have been cases where LEA proteins protected membranes of a specific composition and involved interactions with certain lipids (e.g. COR15; Thalhammer et al. 2010; Thalhammer et al. 2014). It may be revealing for future experiments to be repeated using liposomes with compositions simulating the membranes of *A. franciscana*, because recent literature has suggested differences in the lipid profile of *A. franciscana* and mammals. For example, concentrations of phosphatidylserines and phosphatidylglycerols were reportedly much higher in the inner mitochondrial membranes of *A. franciscana* compared to mammalian species (Chen et al. 2016). Nothing at all is known regarding the lipid profile for the outer mitochondrial membrane of *A. franciscana*, but work is in progress (J. Anderson and S. Hand, unpublished observations). Once defined, studies using liposomes modeled after membranes of *A. franciscana* could reveal specific protection by AfrLEA6 or even an improvement in the protective capabilities of Group 3 LEA proteins.

Chapter 4 examined the subcellular localization of AfrLEA6 to gain insight into its role in desiccation tolerance. Diapause embryos were imaged because they contain the highest titer of AfrLEA6 as outlined in chapter 2. Visualization of AfrLEA6 in diapause embryos was achieved using the AfrLEA6 polyclonal antibody raised in chicken eggs. Through confocal microscopy, it was determined that AfrLEA6 is located in the cytoplasm of diapause embryos and does not co-localize with either the mitochondria or nuclei – a finding that is supported by bioinformatic

targeting software. This location of AfrLEA6 may help explain why AfrLEA6 alone only protected cytoplasmically localized enzymes as outlined in chapter 3. Additionally, I described the resolution of a technical problem associated with chitin-based affinity chromatography (i.e., the IMPACT system) used for AfrLEA6 purification that should be generally applicable

Finally, the link between structure and function of LEA proteins in desiccation tolerance was investigated. These experiments were designed to test the ability of AfrLEA2 to protect PFK after drying the enzyme to various intermediate water levels. The use of PFK was critical to this study due to its extreme sensitivity to desiccation, and consequently, the protective effects of LEA proteins at intermediate water contents were able to be resolved. The use of saturated salt solutions allowed me to control the relative humidity within airtight chambers containing my samples. As a result, PFK was dehydrated to intermediate water levels while in the presence or absence of protein protectants. Results show increasing damage to PFK as a function of RH, and that AfrLEA2 was able to protect PFK at every RH tested. In parallel, the secondary structure of AfrLEA2 was analyzed with circular dichroism and provided the first evidence that links gain of secondary structure with stabilization of a target protein across a graded series of drying.

It is unclear why the magnitude of protection by AfrLEA6 and its titer during diapause are so low compared to Group 3 LEA proteins. Perhaps these differences may be due to unresolved mechanisms by which AfrLEA6 and Group 3 LEA proteins function in desiccation tolerance. As mentioned previously, the amino acid sequence of AfrLEA6 contains features associated with proteins known to undergo liquid-liquid phase separations in the hydrated state under certain conditions (Janis et al. 2018; Janis et al. 2017). LLPS is a phenomenon that has not been seen with other LEA proteins in *A. franciscana* (Janis et al. 2019). Also referred to as "membraneless organelles," the first described occurrence was the P granule in embryos of *C.*

elegans (Strome and Wood 1983; Wolf et al. 1983). In some cases, LLPS allows cells to compartmentalize important chemical reactions within the cytoplasm without the use of a membrane (for reviews, see Hyman et al. 2014 and Mitrea and Kriwacki 2016). However, LLPS has also been implicated in disease states such as Alzheimer's Disease whereby soluble tau proteins may form droplets that serve as precursors to harmful tau aggregates (Wegmann et al. 2018). *In vitro* conditions such as molecular crowding, changes in pH, and cooling can trigger AfrLEA6 to undergo LLPS (Janis et al. 2019). Many of these triggers are physiologically relevant to embryos of *A. franciscana*. For example, diapause embryos may experience severe desiccation, a reduction in cytoplasmic pH during hypoxia, or cold temperatures in the winter months. One hypothesis is that AfrLEA6 may be involved in the partitioning of signaling peptides or other biomolecules that regulate diapause in the cyst. Due to the increasing concentration of solutes in the drying cell, AfrLEA6 may precipitate from solution into another liquid phase that partitions such biomolecules at moderate to low water content and release them as it dissolves upon rehydration. Results presented here show that AfrLEA6 does not protect enzymes or liposomes from damage incurred during desiccation to the same degree as AfrLEA2 or AfrLEA3m. Therefore, future experiments should investigate the ability of AfrLEA6 to protect target enzymes or liposomes under other conditions which are thought to trigger LLPS, such as cold or acidic pH, which may shed light on potential functions.

Although the question remains as to why anhydrobiotic organisms commonly express multiple LEA proteins, their expression is clearly important to the survival of anhydrobiotes. For example, *Arabidopsis thaliana* is known to express at least 51 different LEA genes, with most LEA genes being expressed in seeds (Hundertmark and Hincha 2008). In embryos of *A. franciscana,* the expression of Groups 1, 3, and 6 expands this concept to animals (Hand and

Menze 2015; Tunnacliffe and Wise 2007; Warner et al. 2012; Warner et al. 2010). One possible explanation for the expression of multiple LEA proteins by a single organism is that LEA proteins work together to confer tolerance to extreme water stress. Results presented in this dissertation show that although AfrLEA6 is localized to the cytoplasm like AfrLEA2, these two proteins clearly differ in both their protective properties and expression. Perhaps AfrLEA2 and AfrLEA6 cooperate within the same subcellular compartment (i.e. the cytoplasm) but fulfill different roles during water stress, which accounts for differences in specificity of protection and titer.

Applications of this dissertation research may one day extend to the biomedical field with the ultimate goal of using LEA proteins to improve the long-term stabilization of mammalian cells in the dried state (Crowe et al. 2005; Hand and Hagedorn 2008). Storage of cells, tissues, and organs is vital to both research as well as patient care, and in some cases, the use of LEA proteins as protectants has led to exciting possibilities. For example, the expression of AfrLEA2 or AfrLEA3m in mammalian cell lines improved survivorship during acute desiccation (Li et al. 2012). More recently, AfrLEA3m along with two other LEA proteins were transfected into mammalian cells to determine whether a combination of LEA proteins targeted to different subcellular compartments could confer improved desiccation tolerance compared to expression of an individual LEA protein (Czernik et al. 2020). Interestingly, their results showed that survivorship and proliferation of cells after drying and rehydration was improved when cells expressed all three LEA proteins versus one LEA protein. Similar experiments using AfrLEA6 could provide further information about its role and expand upon the hypotheses presented in this chapter. In any case, the results presented in this dissertation underscore the concept that multiple

LEA proteins exist at different amounts and within different cellular compartments within a single organism and may act together to protect the organism during events of water stress.

APPENDIX
PUBLICATION AGREEMENT

Cell Stress Society International

Publication Agreement

Date: 6/3/19 **Ms. #** CSAC-D-19-00114

Authors: B. LeBlanc, M. Le, B. Janis, M. Menze, S. Hand

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VITA

Blase Matthew LeBlanc was born in Baton Rouge, LA as an only child. He took an early interest in math, science, and technology through the talented and gifted (TAG) program. In high school, Blase distinguished himself a member of multiple student organizations and was a student intern at a local hospital. In May 2011, Blase graduated from Dutchtown High School. As a first-generation college student, Blase chose to attend Louisiana State University to remain close to his family and friends. In May 2015, Blase earned his B.S. in biological sciences from LSU and chose to continue his undergraduate research on LEA proteins by applying to the Ph.D. program under the advice of Dr. Steven Hand. Through his tenure as a graduate student, Blase grew to love teaching and mentoring students. As such, Blase plans to pursue a career in academia at a professorial level to share his knowledge and love of biology with others.