Louisiana State University

LSU Scholarly Repository

Honors Theses

Ogden Honors College

4-2020

Protein Expression and Characterization of a Mitochondrial Group 3 LEA protein, AfrLEA3m29, from embryos of Artemia franciscana

Caretia JeLayne Washington

Follow this and additional works at: https://repository.lsu.edu/honors_etd

Part of the Biology Commons

Recommended Citation

Washington, Caretia JeLayne, "Protein Expression and Characterization of a Mitochondrial Group 3 LEA protein, AfrLEA3m29, from embryos of Artemia franciscana" (2020). *Honors Theses*. 1537.
https://repository.lsu.edu/honors_etd/1537

This Thesis is brought to you for free and open access by the Ogden Honors College at LSU Scholarly Repository. It has been accepted for inclusion in Honors Theses by an authorized administrator of LSU Scholarly Repository. For more information, please contact ir@lsu.edu.

Protein Expression and Characterization of a Mitochondrial Group 3 LEA protein, AfrLEA3m29, from embryos of *Artemia franciscana*

Caretia JeLayne Washington

Undergraduate honors thesis under the direction of

Dr. Steven C. Hand

Department of Biological Sciences

Submitted to the LSU Roger Hadfield Ogden Honors College in partial fulfilment of

The Upper Division Honors Program

April 2020

Louisiana State University & Agricultural and Mechanical College

Baton Rouge, Louisiana

TABLE OF CONTENTS

Acknowledgements……..................……………………………………………...3

Abstract………………….................……………………………………………4

Introduction………………................……………………………………...…5

Objective……………………...................…………………………………….11

Experimental Design………...................………………………….………..12

Anticipated Results……………………………………………………………...18

Discussion.…………………….................………………………….………...….…20

Literature Cited………………….................………………………………...22

ACKNOWLEDGEMENTS

With sincere gratitude, I thank Dr. Steven C. Hand, Ron and Mary Neal Distinguished Professor, for granting me the incredible opportunity to conduct research in a biochemistry/physiology laboratory setting. He expertly guided me through extensive research and the process of constructing and writing an honors thesis. Thank you to Dr. Steven Hand, Dr. Leaf Boswell, and Dr. Lisa Fultz, the members of the defense committee, for generously committing the time to evaluate my honors thesis and contributing valuable feedback that will greatly influence my future career. Additionally, I extend a special acknowledgement to Dr. Scott Herke for his assistance in DNA sequencing. I would also like to acknowledge the Roger Hadfield Ogden Honors College for supporting me during my enrollment in the honors program and encouraging me to pursue an honors thesis. Thank you to Dr. Hand's research students who extensively helped me with my thesis research project -- Elbert Hoang, Blasé LeBlanc, and Ainsley Rothschild. Lastly, I would like to extend my warmest thanks to Louisiana State University for ensuring students are afforded the opportunity to contribute to scientific research.

ABSTRACT

Embryos of *Artemia franciscana* tolerate extreme environments, including ones that promote desiccation. Group 3 LEA proteins are thought to contribute to the stability of macromolecules in the dried state. Three variants of AfrLEA3m, a LEA protein from embryos of *A. franciscana* that is targeted to the mitochondrion, have been identified and their DNA sequences determined -- *Afrlea3m_47*, *Afrlea3m_43*, and *Afrlea3m_29*. However, the functions of these new variants are unknown. Therefore, this study proposes an experimental design for the expression and characterization of the AfrLEA3m_29 protein. The *Afrlea3m_29* sequence will be ligated into a pET-30a expression vector and used for transforming Rosetta 2 (DE3) competent cells. After expression, the recombinant protein will be purified by His-Tag and anion exchange chromatography. SDS-PAGE and Western blotting will be used to characterize the AfrLEA3m_29 protein. Ultimately, the proposed experimental design should facilitate structural and functional studies with AfrLEA3m_29 in the future to reveal its role in the desiccation tolerance of *A. franciscana* embryos.

INTRODUCTION

Artemia franciscana

The brine shrimp, *Artemia franciscana,* is an excellent experimental animal with which to study physiological and biochemical mechanisms of tolerance to challenging environmental conditions. Specimens of *A. franciscana* used in my study originated from the Great Salt Lake, Utah. Depending on the specific stage in its life cycle, such as diapause (a period of arrested development), *A. franciscana* can survive many extreme conditions, such as subzero temperatures, desiccation to less than 2% tissue water, high salinity, and years of exposure to anoxia (Crowe et al., 1981; Eads and Hand, 2003; Hengherr et al., 2011; Lenz and Browne, 1991). The multiple tolerances are rare among macro-planktonic organisms (Gajardo and Beardmore, 2012). My thesis focuses specifically on mechanisms by which embryos of *A. franciscana* survive severe desiccation. Many of these encysted embryos wash up on the shoreline, dry out, and then are dehydrated during winter (Patil et al., 2013). One contributor to the remarkable desiccation tolerance of *A. franciscana* is the expression of multiple types of Late Embryogenesis Abundant (LEA) proteins. In this thesis, I provide an overview of the experimental design to transfer a previously-cloned gene for one specific LEA protein from *A. franciscana*, AfrLEA3m29 (Boswell et al., 2014b), into a bacterial expression system, to induce AfrLEA3m29 expression, to extract this protein, to purify it by His-Tag chromatography, and to characterize AfrLEA3m29 by SDS-PAGE and Western blotting.

Late Embryogenesis Abundant Proteins

Late Embryogenesis Abundant (LEA) proteins are intrinsically disordered proteins that have been reported in a variety of organisms such as bacteria, fungi, plants, and four animal phyla (Nematoda, Rotifera, Tardigrada, and Arthropoda) (Hand et al., 2011; Hundertmark and

Hincha, 2008; Tunnacliffe and Wise, 2007). Late Embryogenesis Abundant (LEA) Proteins were first discovered in mature cotton seeds (Dure et al., 1981). Most LEA proteins are heat soluble and belong to the “hydrophilin” family which consists of highly hydrophilic, unstructured proteins (Garay-Arroyo et al., 2000; Tanaka et al., 2015). Additionally, LEA proteins contain amphipathic alpha helices which are known to promote interaction of LEA proteins with cellular membranes and other proteins during desiccation (Moore and Hand, 2016; Olvera-Carrillo et al., 2011; Thalhammer et al., 2014).

There are six groups of LEA proteins that have been identified, sequenced, and expressed (Groups 1-6; Tunnacliffe and Wise, 2007). Apart from the LEA proteins found in *A. franciscana*, all LEA proteins that have been discovered in animals have been categorized as Group 3 LEA proteins (Wise, 2003). *A. franciscana* is unique in that it is the first animal species to be identified that can express more than one group of LEA proteins (Hand et al., 2007). *A. franciscana* express three different groups of LEA proteins that differ in their amino acid sequences (Groups 1, 3, and 6) (Hand and Menze, 2015). Group 1 LEA proteins are known to protect *A. franciscana* from water stress, such as desiccation and freezing (Toxopeus et al., 2014). Group 3 LEA proteins are characterized by a repeating motif of 11 amino acids (TAQAAKEKAGE) with an overrepresentation of charged and acidic amino acid residues. During desiccation, these proteins adopt an alpha-helical structure, which may contribute to their ability to protect biological structures during desiccation (Dure, 1993; Goyal et al., 2003). Additionally, Group 3 LEA proteins play a significant role in environmental stress tolerance in plant seeds (Liu, 2016). Group 6 LEA proteins have only been identified in one animal species, *A. franciscana*, and may play a role in desiccation tolerance as well (Hand and Menze, 2015; Janis et al., 2018; LeBlanc et al., 2019). Although LEA proteins have been noted for a multitude

of potential functions, such as ion sequestration and stabilization of sugar glasses (like trehalose), LEA proteins are most notable for their protective abilities during desiccation (Dure, 1993; Grelet et al., 2005; Hand et al, 2011; Shimizu et al., 2010; Tunnacliffe and Wise, 2007).

LEA proteins and desiccation tolerance in *A. franciscana*

Water is essential for life; however, some organisms can experience water loss and desiccation due to evaporation or freezing during periods of extremely low temperatures or winter (Hand and Menze, 2015). Desiccation can cause extensive damage to proteins resulting in protein unfolding and aggregation (Chakrabortee et al., 2007). Additionally, desiccation can damage cellular membranes, such as chloroplasts and mitochondrial membranes (Bohnert et al., 1995). There are various factors that may serve as protectants of cellular structures and macromolecules during desiccation, such as stabilizing solutes like trehalose, defenses against reactive oxygen species (ROS), and LEA proteins (Erkut et al., 2013; Hand et al., 2011; Yancey, 2005; Yancey et al., 1982).

LEA proteins are unstructured in aquatic solutions; however, in dry conditions, LEA proteins fold and form secondary structure (Goyal et al., 2003). Therefore, induction of secondary structure by desiccation suggests that LEA proteins may play a potential role in protecting macromolecules and membrane-bound organelles from damage when structurally ordered (Hand et al., 2011). Consequently, Group 3 LEA proteins may protect cellular membranes, such as the mitochondrial membranes (Tolleter et al., 2007). However, the exact mechanisms by which Group 3 LEA proteins protect mitochondrial membranes is unknown.

Characteristics of Mitochondrial-targeted LEA Proteins

LEA proteins are localized in different cellular organelles and compartments, such as the endoplasmic reticulum, cytosol, and mitochondria (Hundertmark and Hincha, 2008) and are

predicted to protect other proteins and macromolecules in the cellular compartments in which they reside. For instance, in desiccation-tolerant organisms, mitochondria are expected to be highly protected, since mitochondria are the primary energy-generating organelles (Tolleter et al., 2007). Mitochondrial LEA proteins may potentially contribute to membrane integrity and tolerance of water stress (Tunnacliffe and Wise, 2007). Additionally, mitochondrial LEA proteins fold into an alpha-helical structure during desiccation and stabilize mitochondria by protecting the inner mitochondrial membrane in plant seeds (Tolleter et al., 2007).

The first mitochondrial-targeted LEA protein in animals was reported for embryos of *A. franciscana* (Menze et al., 2009). Mitochondria of *A. franciscana* embryos contain Group 1 (AfrLEA1) and Group 3 proteins (AfrLEA3m plus variants) in desiccation-tolerant stages of development (Boswell and Hand, 2014; Boswell et al., 2014b; Warner et al., 2016). Mitochondrial AfrLEA1 was shown to protect cells during water stress (Marunde et al., 2013). Additionally, AfrLEA3m was identified as a protectant of desiccation-intolerant enzymes, such as phosphofructokinase, citrate synthase and lactate dehydrogenase (Boswell et al., 2014a). Therefore, these mitochondrial isoforms are predicted to play a role in desiccation tolerance in embryos of *A. franciscana*.

Variants of AfrLEA3m

Recent studies identified additional variants of AfrLEA3m that are also targeted to mitochondria (Boswell and Hand, 2014; Boswell et al., 2014b). Boswell et al. (2014b) cloned *Afrlea3m* and its variants from *A. franciscana* embryos. The *Afrlea3m* sequence was ligated into the Novagen pET-30a expression vector and transformed into Rosetta Single Competent cells. After the AfrLEA3m protein was purified by His-Tag column chromatography, a Western blot was conducted for protein expression. In addition to AfrLEA3m, antibody raised against

AfrLEA3m detected three additional protein variants in extracts of *A. franciscana* embryos: AfrLEA3m_47, AfrLEA3m_43, and AfrLEA3m_29. Consequently, total RNA was isolated from the embryos and cDNA for the three *Afrlea3m* variants was generated through reverse transcription. PCR products for each *Afrlea3m* variant was ligated into sequencing vectors and transfected into competent *Escherichia coli* cells. Cells were cultured, plasmids isolated, and the DNA for each *Afrlea3m* variant was sequenced. Deduced amino acid sequences and bioinformatic analyses predicted that the AfrLEA3m protein variants, like the original AfrLEA3m protein, were highly hydrophilic due to their amino acid residues. The suffixes for AfrLEA3m_47, AfrLEA3m_43, and AfrLEA3m_29 indicate the protein masses deduced from their DNA sequences. As discussed previously (Tompa, 2002), LEA proteins often display reduced binding of SDS and consequently migrate as if they are larger proteins on SDS PAGE. Accordingly, Boswell et al. (2014b) showed that AfrLEA3m_47, AfrLEA3m_43 and AfrLEA3m_29 displayed larger apparent molecular masses than predicted: 62.3 ± 0.4 kDa, 48.6 ± 1.4 kDa, and 32.4 ± 1.1 kDa, respectively. The DNA sequences for *Afrlea3m_47*, *Afrlea3m_43* and *Afrlea3m_29* show similarities with *Afrlea3m*, but each has a stretch of sequence that is absent in at least one of the others (Boswell et al., 2014b). The variants also possess several single nucleotide differences in their genetic sequences. For example, there are five single nucleotides differences in *Afrlea3m_43* that are not shared by *Afrlea3m*, *Afrlea3m_47* or *Afrlea3m_29*. Due to these differences, Boswell et al. (2014a) concluded that AfrLEA3m_47, AfrLEA3m_43, and AfrLEA3m_29 are encoded by different individual genes, as opposed to representing splice variants from the same gene.

Boswell et al. (2014b) documented that the protein expression levels of AfrLEA3m_47, AfrLEA3m_43, and AfrLEA3m_29 are the highest in diapause embryos and stages of

preemergence development (which are desiccation-tolerant) and are lowest in desiccation-intolerant nauplius larvae. Additional studies could explain more fully the roles played by AfrLEA3m variants in desiccation tolerance of *A. franciscana* embryos. Expression of each recombinant protein would be key in such future experiments. Therefore, in this study, an experimental design is proposed for the expression and partial characterization of AfrLEA3m_29. Experiments consist of the transfer of the *Afrlea3m_29* gene from the sequencing vector to the expression vector, the induced expression of AfrLEA3m_29 in transformed bacteria containing the plasmid, protein purification of AfrLEA3m_29, and partial characterization of the AfrLEA3m_29 protein.

GENERAL OBJECTIVE

The purpose of this research study is to present an experimental design that can be utilized to successfully express and characterize the AfrLEA3m_29 protein variant. The proposed experimental design intends to establish a foundation for future studies to determine the function and mechanism of AfrLEA3m_29 during desiccation in *A. franciscana* embryos.

SPECIFIC OBJECTIVES:

A. A method will be proposed to transfer the AfrLEA3m_29 plasmid from the sequencing vector to the expression vector.

B. Experimental techniques for the transfection of AfrLEA3m_29 plasmid into Rosetta 2 (DE3) cells and the induction and expression of AfrLEA3m_29 protein will be proposed.

C. The experimental design will describe the procedure of His-Tag and anion-exchange column chromatography for AfrLEA3m_29 protein purification. SDS_PAGE and Western blot procedures to characterize AfrLEA3m_29 will be outlined.

EXPERIMENTAL DESIGN

A. Transfer of *Afrlea3m_29* from Sequence Vector to Expression Vector

Experimental Rationale

The purpose for transferring the *Afrlea3m_29* from the sequencing vector to an expression vector is to allow the protein expression of AfrLEA3m_29 with a hexa-his tag attached to the N-terminus in Rosetta 2 (DE3) competent cells. The presence of *Afrlea3m_29* first must be verified in the sequencing vector. The proposed phosphatase treatment of the pET-30a expression vector (once linearized) should increase the ligation efficiency of the digested DNA insert (i.e., reduce the re-ligation of empty vector). Methods are similar to those described for the original *Afrlea3m* from *A. franciscana* embryos described by Boswell et al. (2014b).

Materials and Methods

Verification of the Size and Sequence of Afrlea3m_29

The *Afrlea3m_29* sequence was originally inserted into a sequencing vector (pENTR/D-TOPO) (Boswell et al., 2014b). To isolate this *Afrlea3m_29* vector, the One Shot Top10 Chemically competent *Escherichia coli* cells were grown in autoclaved Luria-Bertani (LB) broth, containing 51.5 µM kanamycin. The cultures were shaken at 300 rpm at 37°C overnight in the Innova 44 Incubator Shaker Series. The AfrLEA3m_29 plasmid then was purified from the overnight culture using a QIAprep96 Turbo Miniprep Kit (Qiagen). The concentration of the DNA was measured on a Thermo Fisher Scientific NanoDrop 2000 Spectrophotometer.

In order to determine the size of the insert, a vector sample was amplified with PCR. The PCR reaction containing 2 µL of miniprep product, 2 µL of the LEA3m specific primers, and 44 µL of master mix was amplified using the GeneAmp PCR system 9700, and the product was purified using a QIAquick PCR purification kit (Qiagen). The purified PCR product and a

biotinylated protein ladder (Cell Signaling Technology) were loaded onto 2% agarose gels containing 0.5 µg/ml ethidium bromide. The gel then was electrophoresed for 90 min at 120 volts in Tris-acetate-EDTA buffer. The gel was imaged using the Bio-Rad ChemiDoc XRS Image Lab software. To verify that the isolated vector contained *bona fide Afrlea3m_29*, DNA sequencing was performed using M13 forward and M13 reverse primers with BigDye terminator chemistry and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Insert Preparation, Ligation, and Transformation

To prepare the purified PCR product and the pET-30a expression vector (EMD Millipore, Novagen) for ligation, 60 µL of vector and 20 µL insert were first independently digested with XhoI and NdeI restriction enzymes (New England Biolabs) at 37°C for 2 h. To prevent re-ligation of the empty vector, the pET-30a vector was treated with phosphatase by adding 5 µL of Calf Intestinal Alkaline Phosphatase (CIAP; 0.01 units/µL) in CIAP buffer to 40 µL of digested vector. The phosphatase solution was incubated at 37°C for 1 h, and then incubated for 15 min at 75°C to inactivate the CIAP. After agarose gel electrophoresis, the AfrLEA3m_29 insert and pET-30a vector were extracted from the agarose with a QIAquick Gel Extraction Kit (Qiagen).

To ligate the insert into the vector, a ligation reaction composed of 4 µL DNA insert, 2 µL phosphatase-treated vector, 100 mM dithiothreitol, 10 mM ATP, 1 µL T4 DNA ligase (New England Biolabs) and 10 µL ligase buffer was incubated at 12°C for 16 h. After the overnight incubation, 1 µL of ligation reaction and 20 µL of Rosetta 2 (DE3) competent cells will be incubated on ice for 5 min, heat-shocked at 42°C for 30 s, and then placed on ice. A positive transfection control for the Rosetta 2 (DE3) cells provided by the manufacturer will be used in a separate test to ensure that the cells are competent. Next, 80 µL of Super Optimal broth with

Catabolite repression (SOC broth) will be added to the transformed cells, and the mixture will be incubated at 37 C for 1 h with shaking (300 rpm) to improve recovery. Then 25 µL of the diluted preparation will be spread on a culture plate (30 ml of 1.5% agarose in LB broth with 51.5 µM kanamycin and 105 µM chloramphenicol). The plate will be incubated at 37°C for up to 36 h if needed to allow adequate colony growth. Colonies will be picked for culture.

B. Verification of Transformed Bacteria and Protein Expression

Experimental Rationale

The aims are to verify that the ligated AfrLEA3m_29 plasmid is properly transfected into the Rosetta Single Competent cells, and next, to express the recombinant protein by induction with isopropyl β-d-1-thiogalactopyranoside (IPTG).

Materials and Methods

Bacterial colonies will be cultured overnight at 37°C at 300 rpm in LB broth containing 51.5 µM kanamycin and 105 µM chloramphenicol. The ArfLEA3m_29 plasmid then will be isolated with the QIAprep 96 Turbo Miniprep kit. The remainder of the bacterial culture will be frozen at -80°C until successful transformation can be verified. The miniprep products will be sequenced as previously described.

After verification, a starter culture of transformed cells will be shaken overnight at 300 rpm at 37°C overnight. The starter culture then will be used to inoculate 1 L of LB broth, which will also contain 51.5 µM kanamycin and 105 µM chloramphenicol. After 3 h of growth at 37°C and 150 rpm, cells will be induced with 1 mM IPTG. Cells then will be cultured for an additional 2 h. Afterwards, the transformed bacterial cells will be centrifuged (15 min, 5000 x g, 4°C) and resuspended in HisTrap binding buffer, composed of 20 mM imidazol, 20 mM sodium

phosphate, and 0.5 M NaCl. The HisTrap binding buffer will contain EDTA-free protease inhibitor tablets (Roche, EMD Millipore; Burlington, Massachusetts).

C. Purification of Recombinant AfrLEA3m_29

Experimental Rationale

The purpose of the experiment is to purify AfrLEA3m_29 protein from the transformed Rosetta Singles Competent Cells. The transformed cells must be lysed so that the extract can be applied to a HisTag affinity column. A second chromatography step of anion exchange will likely be needed based on previous work with AfrLEA3m (Boswell et al., 2014b).

Materials and Methods

Bacterial cells will be lysed with sonication, and the cellular debris will be removed by centrifugation (20,000 g, 30 min) at 4°C. Affinity chromatography will be performed on the cell lysate using the HisTrap™ FF crude column (GE Healthcare, Waukesha, WI; 5 ml column size) with FPLC (AKTA Prime Plus; GE Healthcare Life Sciences). A step elution will be used with an elution buffer, composed of 0.5 M imidazole, 20 mM sodium phosphate, and 0.5 M NaCl, and the flow rate will be set to 5 ml/min. The fractions that contain the AfrLEA3m_29 protein will be dialyzed at 4°C overnight in the anion exchange buffer (20 mM triethanolamine, 10 mM NaCl, pH 7.0). The dialyzed AfrLEA3m_29 protein sample then will be applied to an anion exchange column (HisTrap™ Q FF; GE Healthcare, 5 ml column size) and eluted with elution buffer containing 20 mM triethanolamine and 1 M NaCl, pH 7.0. The Primeview Evaluation program (GE Healthcare) will be utilized to confirm the elution of protein based on absorbance at 280 nm. The fractions that contain the AfrLEA3m_29 protein will be pooled and exchanged into LEA storage buffer (20 mM HEPES, 50 mM NaCl, pH 7.5). The pooled fractions then will be

concentrated using Amicon® Ultra Centrifugal filters (Ultracel®-10K; Millipore, Billencia, MA, USA).

D. Characterization of AfrLEA3m_29 Protein

Experimental Rationale

Electrophoretic characterization is an important first step with the purified recombinant AfrLEA3m_29. Western blot analysis will also be used to verify that the purified protein cross-reacts with the polyclonal antibody raised against AfrLEA3m.

Materials and Methods

To determine the purity of the protein, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) will be used. Ten µg of purified AfrLEA3m_29 protein will be loaded onto each lane of acrylamide gels (4% stacking gel and 11% resolving gel) and electrophoresed at 125 Volts for 80 min in a Bio-Rad mini-Protean 3 Cell. Proteins will be stained with Coomassie Blue.

For Western blotting, the AfrLEA3m_29 protein then will be transferred from the gel to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 80 V for 60 min in a transfer buffer containing 192 mM glycine, 0.025% SDS, 20% methanol, and 25 mM Tris. Panceau S then will be used to stain and visualize the protein bands on the nitrocellulose membrane. The nitrocellulose membrane then will be blocked for 1 h in a 5% fat-free dry milk prepared in TBS-T (0.1% Tween 20, 500 mM NaCl, 20 mM Tris-HCl, pH 7.6) at room temperature with rocking. Primary antibody raised against the AfrLEA3m_29 (diluted 1:100,000 in 5% dry milk in TBS-T; Aves Lab Inc.) will be incubated with the membrane at 4°C overnight. Afterwards, the membrane blots will be washed four times for 15 min over the course of 1 h with TBS-T. The

membranes will be incubated with the horse radish peroxidase-linked secondary antibody (1:10,000 dilution; goat anti-chicken; Aves Lab Inc.) for 1 h at room temperature. After washing with TBS-T, the protein bands will be visualized using LumiGLO chemiluminescent substrate (Cell Signaling Technology) and quantified using Quantity One Basic 4.6.9 software (Bio-Rad Laboratories).

ANTICIPATED RESULTS

It is expected that the sequencing vector will contain the *bone fide* AfrLEA3m_29 DNA and that the size of the PCR product will approximate that reported for *Afrlea3m_29.* Thus, AfrLEA3m_29 will migrate on agarose gels between 0.7-0.8 kb compared to the biotinylated DNA ladder. AfrLEA3m_29 should be composed of 777 bases, which I anticipate will be confirmed by sequencing and that the sequence will yield greater than 99% identity with that previously reported (Boswell et al., 2014b).

Acceptable ligation efficiency and transformation are expected to be confirmed by the successful growth of colonies for the Rosetta 2 (DE3) cells on the antibiotic-containing culture plates. The plasmid miniprep of the transformed Rosetta cells should yield sequence data that matches bona fide *Afrlea3m_29*.

Culture of bacterial cells induced with IPTG should give good expression of the AfrLEA3m_29 protein based on Western blotting. The protein purification methods that were proposed in the experimental design hopefully will yield a protein with a purity of greater than 95% based on SDS-PAGE. I expect that the primary antibody raised against AfrLEA3m will bind successfully to the AfrLEA3m_29 protein during Western blotting. Westerns blots could indicate the presence of breakdown products, analogous to those previously seen for other AfrLEA proteins (Boswell et al., 2014b; Leblanc et al., 2019).

The SDS-PAGE performed on the purified protein sample should yield a molecular mass for AfrLEA3m_29 of approximately 32.4 kDa, as compared to the molecular weight ladder. As previously discussed, the larger than anticipated size often obtained by SDS_PAGE is a typical behavior for LEA proteins. It is hoped that the purified AfrLEA3m_29 will facilitate further

studies to discover the role AfrLEA3m_29 may play in desiccation tolerance of *A. franciscana* embryos.

Current Progress

At this point, I have successfully identified Afrlea3m_29 in the sequencing vector. I have carried out the procedures for amplifying the AfrLEA3m_29 gene by PCR, digesting and treating the vector, and ligating the PCR product into the pET-30a expression vector. I have been unsuccessful at transforming the Rosetta 2 (DE3) cells in order to obtain bacterial colonies that will grow on agar plates containing kanamycin and chloramphenicol. Factors that could have contributed to the unsuccessful transformation include noncompetent Rosetta 2 (DE3) cells and low ligation efficiency due to inactive ligase and/or suboptimal insert to vector ratio. Further research is presently on hold due to the university closure as a result of the COVID-19 outbreak.

DISCUSSION

The experimental design proposed will hopefully provide an accurate and reliable method to express and characterize the AfrLEA3m_29 protein. AfrLEA3m_29 is a newly discovered mitochondrial LEA protein, the expression of which has been quantified throughout the development of *A. franciscana* (Boswell et al., 2014b). There has not been any research conducted on the characteristics of AfrLEA3m_29. The characterization of AfrLEA3m_29 is essential to understanding its structure and potential function in desiccation tolerance in *A. franciscana*. Included here would be experiments to test the ability of AfrLEA3m_29 to stabilize target macromolecules and to understand whether it behaves like other intrinsically disordered proteins.

Certainly, it is well documented that the original AfrLEA3m protects proteins (Boswell et al., 2014a) and lipid bilayers (Moore and Hand, 2016; Moore et al., 2016) from damage during water stress. During drying and freezing, AfrLEA3m protects liposomes of various compositions that simulate the plasma membrane, outer mitochondrial membrane, and inner mitochondrial membrane. Dehydration of the lipid bilayers, like the mitochondrial membrane, can increase the likelihood of nonbilayer configuration and bilayer fusion, thus decreasing the stability of the mitochondrial membrane (Disalvo and Simon, 1995). Mitochondrial LEA proteins interact with the negatively charged phosphate groups in phospholipids in the dry state (Tolleter et al., 2010). Additionally, AfrLEA3m shows synergistic interactions with trehalose to protect various metabolic enzymes against drying damage. AfrLEA3m, along with trehalose, provides protection against acute, short-term drying damage in human HepG2 cells (a human cell line from liver) (Li et al., 2012). Trehalose has the ability to force protein assembly/folding through preferential

exclusion from the protein backbone (Street et al., 2006). By comparison, AfrLEA3m_29 could show similar capabilities or even alternative roles during drying.

The experimental design developed here could be applied to other AfrLEA3m variants, including AfrLEA3m_43 and AfrLEA3m_47. Hopefully, future studies with the mitochondrial-targeted LEA proteins will help explain why so many different isoforms are apparently necessary to protect a single subcellular compartment. The ultimate goal is to gain a clearer picture of the roles of AfrLEA3m variants in the desiccation tolerance of *A. franciscana* embryos.

LITERATURE CITED

Boswell L.C. and S.C. Hand. 2014. Intracellular localization of group 3 LEA proteins in embryos of *Artemia franciscana*. Tissue and Cell 46: 514–519.

Boswell L.C, M.A. Menze, and S.C. Hand. 2014a. Group 3 LEA proteins from embryos of *Artemia franciscana*: Structural properties and protective abilities during desiccation. Physiol Biochem Zool 87: 640–651.

Boswell L.C., D.S. Moore, and S.C. Hand. 2014b. Quantification of cellular protein expression and molecular features of group 3 LEA proteins from embryos of *Artemia franciscana*. Cell stress & chaperones 19: 329–341.

Bohnert H.J., D.E. Nelson, and R.G. Jensen. 1995. Adaptations to environmental stresses. Plant Cell 7: 1099–1111.

Chakrabortee S., C. Boschetti, L.J. Walton, S. Sarkar, D.C. Rubinsztein, and A. Tunnacliffe. 2007. Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. PNAS 104: 18073–18078.

Crowe J.H., L.M. Crowe, and S.J. O'Dell. 1981. Ice formation during freezing of *Artemia* cysts of variable water contents. Mol Physiol 1: 145–152.

Disalvo E.A. and S.A. Simon. 1995. Freeze-Induced Destabilization of Cellular Membranes and Lipid Bilayers. In: Permeability and Stability of Lipid Bilayers. Boca Raton FL: CRC Press Pp. 75-103.

Dure L. 1993. A repeating 11-mer amino acid motif and plant desiccation. Plant J 3: 363–369.

Dure L., S.C. Greenway, and G.A. Galau. 1981. Developmental biochemistry of cottonseed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by in vitro and in vivo protein synthesis. Biochemistry 20: 4162–4168.

Eads B.D. and S.C. Hand. 2003. Transcriptional initiation under conditions of anoxia-induced quiescence in mitochondria from *Artemia franciscana* embryos. Journal of Experimental Biology 206: 577–589.

Erkut C., A. Vasilj, S. Boland, B. Habermann, A. Shevchenko, and T.V. Kurzchalia. 2013. Molecular strategies of the *Caenorhabditis elegans* dauer larva to survive extreme desiccation. PLoS ONE 8: 82473.

Gajardo G.M. and J.A. Beardmore. 2012. The brine shrimp Artemia: adapted to critical life conditions. Frontiers in physiology 3: 185.

Garay-Arroyo A., J.M. Colmenero-Flores, A. Garciarrubio, and A.A. Covarrubias. 2000. Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. J Biol Chem 275: 5668–5674.

Goyal K., L. Tisi, A. Basran, J. Browne, A. Burnell, J. Zurdo, and A. Tunnacliffe. 2003. Transition from natively unfolded to folded state induced by desiccation in an anhydrobiotic nematode protein. J Biol Chem 278: 12977–12984.

Grelet J., A. Benamar, E. Teyssier, M.H. Avelange-Macherel, D. Grunwald, and D. Macherel. 2005. Identification in pea seed mitochondria of a late-embryogenesis abundant protein able to protect enzymes from drying. Plant Physiol 137: 157–167.

Hand S.C., D. Jones, M.A. Menze, and T.L. Witt. 2007. Life without water: expression of plant LEA genes by an anhydrobiotic arthropod. J Exp Zool 307: 62–66.

Hand S.C. and M.A. Menze. 2015. Molecular approaches for improving desiccation tolerance: insights from the brine shrimp *Artemia franciscana*. Planta 242: 379–388.

Hand S.C., M.A. Menze, M. Toner, L. Boswell, and D. Moore. 2011. LEA Proteins During Water Stress: Not Just for Plants Anymore. Annual Review of Physiology 73: 115–134.

Hengherr S., R.O. Schill, and J.S. Clegg. 2011. Mechanisms associated with cellular desiccation tolerance in the animal extremophile *Artemia*. Physiol Biochem Zool 84: 249–257.

Hundertmark M. and D.K. Hincha. 2008. LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. BMC Genomics 9:118.

Janis B., V.N. Uversky, and M.A. Menze. 2018. Potential functions of LEA proteins from the brine shrimp *Artemia franciscana*—anhydrobiosis meets bioinformatics. J Biomol Struct Dyn 36: 3291–3309.

LeBlanc B.M., M.T. Le, B. Janis, M.A. Menze, and S.C. Hand. 2019. Structural properties and cellular expression of AfrLEA6, a group 6 late embryogenesis abundant protein from embryos of *Artemia franciscana*. Cell Stress and Chaperones 24: 979-990.

Lenz P. and R. Browne. 1991. Artemia *Ecology*. Artemia Biology 1: 237–254.

Li S., N. Chakraborty, A. Borcar, M.A. Menze, M. Toner, and S.C. Hand. 2012. LEA proteins protect cells during desiccation. Proceedings of the National Academy of Sciences 109: 20859–20864.

Liu Y., J. Liang, L. Sun, X. Yang, and D. Li. 2016. Group 3 LEA Protein, ZmLEA3, Is Involved in Protection from Low Temperature Stress. Frontiers in plant science 7:1011.

Marunde M.R., D.A. Samarajeewa, J. Anderson, S. Li, S.C. Hand, and M.A. Menze. 2013. Improved tolerance to salt and waters stress in *Drosophila melanogaster* cells conferred by late embryogenesis abundant protein. J Insect Physiol 59: 377-386.

Menze M.A., L. Boswell, M. Toner, and S.C. Hand. 2009. Occurrence of mitochondria-targeted Late Embryogenesis Abundant (LEA) gene in animals increases organelle resistance to water stress. J Biol Chem 284: 10714–10719.

Moore D.S. and S.C. Hand. 2016. Cryopreservation of lipid bilayers by LEA proteins from *Artemia franciscana* and trehalose. Cryobiology 73: 240–247.

Moore D.S., R. Hansen, and S.C. Hand. 2016. Liposomes with diverse compositions are protected during desiccation by LEA proteins from *Artemia franciscana* and trehalose. Biochem. Biophys. Acta (Biomembranes) 1858: 104-115.

Olvera-Carrillo Y., J. Luis Reyes, and A.A. Covarrubias. 2011. Late embryogenesis abundant proteins: versatile players in the plant adaptation to water limiting environments. Plant Signal Behav 6: 586–589.

Patil Y., B. Marden, M.D. Brand, and S.C. Hand. 2013. Metabolic downregulation and inhibition of carbohydrate catabolism during diapause in embryos of *Artemia franciscana*. Physiol Biochem Zool 86: 106-118.

Shimizu T., Y. Kanamori, T. Furuki, T. Kikawada, T. Okuda, T. Takahashi, H. Mihara, and M. Sakurai. 2010. Desiccation-induced structuralization and glass formation of group 3 late embryogenesis abundant protein model peptides. Biochemistry 49: 1093–1104.

Street T.O., D.W. Bolen, and G.D. Rose. 2006. A molecular mechanism for osmolyte-induced protein stability. Proc Natl Acad Sci USA 103: 13997–14002.

Tanaka S., J. Tanaka, Y. Miwa, D.D. Horikawa, T. Katayama, K. Arakawa, A. Toyoda, T. Kubo, and T. Kuineda. 2015. Novel mitochondria-targeted heat-soluble proteins identified in the anhydrobiotic Tardigrade improve osmotic tolerance of human cells. PLoS One 10: 118272.

Thalhammer A., G. Bryant, R. Sulpice, and D.K. Hincha. 2014. Disordered Cold Regulated15 Proteins Protect Chloroplast Membranes during Freezing through Binding and Folding, But Do Not Stabilize Chloroplast Enzymes. Plant Physiology 166: 190–201.

Tolleter D., D.K. Hincha, and D. Macherel. 2010. A mitochondrial late embryogenesis abundant protein stabilizes model membranes in the dry state. Biochem Biophys Acta-Biomembranes 1798: 1926–1933.

Tolleter D., M. Jaquinod, C. Mangavel, C. Passirani, P. Saulnier, S. Manon, E. Teyssier, N. Payet, M.H. Avelange-Macherel, and D. Macherel. 2007. Structure and function of a mitochondrial late embryogenesis abundant protein are revealed by desiccation. Plant Cell 19: 1580–1589.

Tompa P. 2002. Intrinsically unstructured proteins. Trends Biochem Sci 27: 527-533.

Toxopeus J., A.H. Warner, and T.H. MacRae. 2014. Group 1 LEA proteins contribute to the desiccation and freeze tolerance of *Artemia franciscana* embryos during diapause. Cell stress & chaperones 19: 939–48.

Tunnacliffe A. and M.J. Wise. 2007. The continuing conundrum of the LEA proteins. Naturwissenschaften. 94: 791–812.

Warner A.H., Z.H. Guo, S. Moshi, J.W. Hudson, and A. Kozarova. 2016. Study of model systems to test the potential function of Artemia group 1 late embryogenesis abundant (LEA) proteins. Cell Stress Chaperones 21: 139–154.

Warner A.H., O. Miroshnychenko, A. Kozarova, P.O. Vacratsis, T.H. MacRae, J. Kim, and J.S. Clegg. 2010. Evidence for multiple group 1 late embryogenesis abundant proteins in encysted embryos of *Artemia* and their organelles. J Biochem 148: 581-592.

Wise M.J. 2003. LEAping to conclusions: A computational reanalysis of late embryogenesis abundant proteins and their possible roles. BMC Bioinform 4: 52.

Yancey P., M. Clark, S.C. Hand, R. Bowlus, and G. Somero. 1982. Living with water stress: The evolution of osmolyte systems. Science 217: 1214-1222.

Yancey P.H. 2005. Organic osmolytes as compatible, metabolic, and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 208: 2819–2830.